Walter K.H. Krause
Rajesh K. Naz
Editors

Immune Infertility

Impact of Immune Reactions on Human Fertility

Second Edition

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Preface

Infertility is defined as an inability to conceive after unprotected intercourse for a year. Infertility is increasing worldwide and has various causes, which occur in the male and/or female partner of an infertile couple. Immune reaction(s) to sperm is an important cause of infertility as described in the first edition of the book published in 2009. Our knowledge in this field has considerably increased since publication of the first edition. The immune reaction to sperm is recognized as a unique condition, and it is crucial to update the knowledge in this field. A lot of new exciting scientific findings has come during the last 6 years, especially as related to sperm proteomics, gene knockouts, and vaccinology. Due to huge success of our first edition and emerging knowledge in the field, it became imperative to publish the second edition of the "Immune infertility" to update the information for our readers. The second edition covers the basic information on the immunological factors and sperm antigens and their role in clinical infertility and immunocontraception. All the authors of the first edition have incorporated emerging recent information in their chapters for the second edition.

Sperm cell has both autoantigenic and isoantigenic potential and is thus capable of producing antisperm antibodies (ASA) and sperm-reactive T cells in both infertile men and women. Also, over 75 % vasectomized men produce autoantibodies to sperm that can cause a problem in regaining fertility even after successful reanastomosis in vasovasostomy. Early claims regarding the incidence and involvement of ASA in involuntary human infertility were probably overemphasized because of unreliable techniques and naivety concerning the complexity of the immune response and antigenic nature of the sperm cell. These factors, the lack of well-designed and controlled experimental studies and the dearth of effective therapeutic modalities, resulted in confusion of the occurrence and importance of ASA in human infertility. Consequently, evaluation of infertile couples for ASA and their possible role in infertility was not considered a significant proposition. The development of more accurate assays and the discovery of mucosal immunity capable of responses independent of systemic immunity have caused inclusion of sperm cells and genital tract secretions in the analysis of ASA. Furthermore, with progress in assisted reproductive and hybridoma technologies, recent development in

proteomics and genomics has tremendously increased our understanding regarding the induction and role of ASA in infertility. It is becoming clearer now that any immunoglobulin that binds to sperm cannot be called an “antisperm antibody” unless it is directed against an antigen that is relevant to fertilization and fertility.

Although there are numerous reports on ASA and their role in immunoinfertility, there is no book comprising various aspects of immunoinfertility under a single comprehensive treatise. This book is unique and the first of its kind in bringing together our current knowledge on immune mechanisms, proteomics and genomics of sperm structure and function, and diagnosis and treatment of ASA-mediated infertility. Also included are chapters on the application of these immune reactions in the development of novel nonsteroidal immunocontraceptives.

It has 19 chapters, arranged into four sections, written by the well-renowned experts in the field of immune infertility from all over the world. In the first section, various sperm antigens involved in immune infertility are enumerated. The first chapter describes the protein structure of spermatozoa, the proteome, followed by a chapter dealing with the methods of analysis. In the next chapter, the proteins inducing immune reactions which cause an impairment of sperm function are summarized. The second section is dedicated to the different aspects of the nature of ASA. First, the status of immune privilege of the testis is discussed. The following chapters describe the immune chemistry of ASA, involvement of sperm-specific T cells, the site and risk factors of ASA production, and the prevalence of ASA in the different compartments of the body. Two other chapters on the occurrence of ASA in women and the significance of sperm immobilizing ASA are included. The next section addresses the clinical impact of ASA. The chapters in this section discuss autoimmune infertility, tests for detection of sperm antibodies, impact of ASA on male fertility, and the role of assisted reproductive technologies and other methodologies to treat immunoinfertility. The fourth section includes three chapters discussing the application of immune reactions to gametes and hormones in the development of novel immunocontraceptives for wildlife and humans.

In conclusion, the second edition is a unique and novel treatise in offering up-to-date information on ASA-mediated infertility. The authors of this book are expert investigators who are pioneers in their fields. In the second edition, its contributors provide profound insights into the complex field of immune infertility in a timely manner. It will provide a model source of authentic, vital, and viable information on the latest scientific developments in the field of immune infertility and immunocontraception to clinicians, scientists, students, residents, and fellows working in the field of reproductive biology, obstetrics and gynecology, and urology.

Marburg, Germany — Walter K.H. Krause
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Part I

Sperm Antigens

Proteomic Analysis of Human Spermatozoa

1

Brett Nixon, Matthew D. Dun, and R. John Aitken

Abstract

Novel technological innovations in high-resolution mass spectrometry have ushered in a new era in proteomic analyses. Coupled with enhanced methods for cellular and protein pre-fractionation, such developments have enabled the detailed characterization of proteomes from various cell types, including the spermatozoa of a number of species. Collectively, these studies have generated complex inventories consisting of thousands of sperm proteins and served as an important platform for advancing our understanding of sperm biology. In this context, exciting advances have been made into comparative and quantitative approaches that enable sophisticated analysis of the proteomic signature of spermatozoa in different functional states (immature vs. mature, non-capacitated vs. capacitated, fertile vs. infertile). These techniques have helped to define which specific elements of the proteome are of functional significance and improved our understanding of the cascade of post-translational modifications

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(e.g. phosphorylation, glycosylation, acetylation, proteolytic cleavage) involved in generating a fertilization competent spermatozoon. Such fundamental information holds considerable promise for identifying key biomarkers of male fertility in addition to elements of sperm maturation that might be targeted for fertility regulation both in the context of contraceptive development and therapeutic intervention. In this review, we have sought to present an overview of the use of contemporary proteomics to address many of the long-standing challenges in the field of human sperm biology as well as to speculate on the future clinical applications of these technologies.

1.1 Introduction

Unraveling the complexities of human sperm biology is an area of research that continues to attract considerable attention owing to the prevalence of male infertility. Nevertheless, despite their pivotal role in reproduction, we still have much to learn about the overall molecular composition of this unique and highly specialized cell. The production of spermatozoa represents the culmination of an extraordinary process of cytodifferentiation that occurs within the testes [1]. This process, known as spermatogenesis, produces a highly differentiated and compartmentalized spermatozoon with a number of defined intracellular domains and a mosaic surface architecture [2, 3]. The balance of evidence suggest that the extensive chromatin remodeling that accompanies the latter phases of spermatogenesis also results in the silencing of the nuclear genome such that on leaving the testis, spermatozoa are both transcriptionally and translationally inactive. In the absence of de novo protein synthesis, the functionality of these cells is largely, if not solely, dependent on post-translational modifications to their protein complement. This applies equally to the maturation of these cells in the male reproductive tract (epididymis) and to their post-ejaculatory capacitation in the female reproductive tract [4]. These features, combined with the relative ease of obtaining large numbers of purified spermatozoa that can be driven into different functional states, make this cell type particularly amenable to proteomic analyses. Indeed, while modern genetic profiling methods including differential display [5, 6], serial analysis of gene expression [7], microarray [8–11], next-generation sequencing [12], genome-wide association study [13] and proteogenomic technologies [14] have proved highly effective for analyzing differentiating germ cells within the testes [15], recent studies have revealed limited semblance in the transcriptomic and proteomic signatures of mature human spermatozoa [16], thus indicating that these techniques are of little value in characterizing the changes that confer functionality on the male gamete.

Focus has instead rested on resolving the proteomic composition of human spermatozoa, a field that is evolving at a rapid pace as advances are made in protein and peptide separation, detection and identification [17, 18]. This expanding resource has recently been consolidated into a reference library comprising 6,198 unique proteins, a comprehensive list that represents ~80 % of the estimated 7,500 total

proteins that constitute a human spermatozoon [19]. Among the key challenges that remain in harnessing the full potential of this dataset, is to characterize the targets impacted by post-translational modifications, investigate the protein interactome, and define anomalies in protein expression associated with specific lesions in sperm function. Herein, we review literature pertaining to contemporary proteomic analysis of human spermatozoa, assess the relative merits of the different methods that have been employed and discuss future directions that may help realize the full transformative potential of this field of research. Where relevant, we have also sought to direct the reader to a number of excellent reviews which critically appraise our current progress with proteomic characterization of the human spermatozoon [4, 15, 17, 19–30].

1.2 Assessment of the Complete Human Sperm Proteome

The past two decades have witnessed unprecedented technological improvements in the tools available for characterization of complex cellular proteomes [31–34]. The application of such technology in large-scale shotgun (or 'bottom up') sequencing initiatives has radically changed the landscape of cell biology research. This is particularly evident in the field of human sperm biology where substantial progress has been made in cataloguing the estimated 7,500 unique proteins that constitute this highly differentiated cell [19]. What is more, an ever-expanding repertoire of comparative strategies have begun to elucidate anomalous protein signatures correlated with some of the most common infertility phenotypes [35–40]. In the following section we discuss the contemporary analytical techniques that have been employed to dissect the global human sperm proteome. As indicated, such studies have been dominated by the application of two complementary proteomic strategies focused on gel-based and gel-free separation platforms.

1.2.1 Technologies Employed for Studying the Human Sperm Proteome

1.2.1.1 Gel-Based Separation Platforms

With its origins dating back to the 1970s, two-dimensional electrophoresis (2DE) was among the first large-scale approaches employed to separate human sperm proteins for the purpose of downstream identification by mass spectrometry. The application of this preparative platform, which facilitates the resolution of protein mixtures on the basis of both isoelectric point and apparent molecular mass, to identify human sperm proteins was pioneered by Herr and colleagues who were extremely prolific in the sequencing and characterization of novel sperm proteins [41–47]. The mining of this resource to identify immunodominant sperm antigens for contraceptive purposes eventually led to the establishment of the Human Sperm Protein Encyclopedia, a database mapping some 1397 protein spots [46]. Among this original data set, at least 98 protein spots were accessible to both ^{125}I vectorial

labeling and biotinylation, suggesting an association with the sperm surface. Furthermore, 22 protein spots were immunologically reactive to a phosphotyrosine antibody, thus emphasizing the contribution of post-translational modifications to the overall complexity of the sperm proteome [41, 43]. In subsequent detailed analyses, Oliva and colleagues resolved >1,000 spots by 2DE, with identifications being secured for 131 different proteins, almost a quarter of which had not previously been recorded in human spermatozoa [48, 49]. Interestingly, in addition to the anticipated abundance of cytoskeletal, mitochondrial, flagellar and membrane proteins, these data also provided some surprising findings such as the presence of a large proportion of proteins involved transcription, protein synthesis and turnover; functions that are not typically ascribed to mature human spermatozoa [48, 49]. Higher resolution 2DE maps of normozoospermic human sperm proteins have since been generated using a series of overlapping, narrow pH ranges for the initial isoelectrofocusing step [50]. Although this approach resolved a total of 3,872 different protein spots, only 16 novel protein identities were reported.

Such studies serve to highlight both the utility of 2DE-based approaches in cataloguing the overall nature and complexity of the sperm proteome, as well as some of its inherent limitations in terms of securing definitive protein identifications. The latter has generally relied on immunoblotting procedures employing antibodies against defined antigens or the use of matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), a technique that has now been largely superseded. Proteomic strategies employing 2DE also suffer from a number of additional limitations including: the laborious nature of the technique, its limited dynamic range, the difficulty of resolving hydrophobic (membrane) proteins, an inherent variability that often confounds inter-gel comparisons, and it is not strictly quantitative [51]. This latter problem, is a reflection of the nature of many of the widely used staining techniques, such as silver staining, which themselves suffer from a limited dynamic range, so that the intensity of less abundant spots is not linearly correlated to that of more abundant spots. Moreover, some types of proteins, especially those bearing post-translationally modifications, can give quantitatively and qualitatively different staining in comparison to similar amounts of other proteins.

Difference In Gel Electrophoresis (DIGE) Several of these limitations have been addressed through the advent of DIGE (Difference In Gel Electrophoresis) technology that permits the simultaneous separation of up to three samples in a single 2D gel [52]. In most DIGE applications, two samples and one internal standard are covalently labeled with size and charge-matched, spectrally resolvable dyes (CyDyes). The labeled protein mixtures are then combined and resolved on the same gel, thus eliminating inter-gel variability in electrophoretic migration patterns. Following separation, the migration of individual protein populations can be resolved by scanning the gel with lasers tuned to the excitation wavelengths of the corresponding CyDyes [53, 54]. Statistical analyses are then performed using powerful software packages designed to compare and match protein spots of gel replicates in order to detect both qualitative (presence/absence) and quantitative (spot

intensities) changes in the proteome. Importantly, the incorporation of an internal standard facilitates accurate inter-gel normalization thus improving the measurement of even subtle changes in protein abundance. Indeed, with this technology, statistically significant differences in the migration of individual protein spots can be ascertained with just six replicates [53–55], whereas conventional proteomic techniques require several times this number of replicates to be mapped before any differences can be identified with confidence. Labeling with DIGE fluorophores is also extremely sensitive and displays a linear response in protein concentration over five orders of magnitude.

On the basis of these properties, DIGE technology has proven to be readily amendable for the assessment of global proteomic changes associated with sperm function [55]. Examples of where DIGE has been used to directly visualize physiologically relevant proteins include comparisons of the post-translational changes that occur in mammalian spermatozoa as they engage the process of epididymal maturation [55]. This analysis revealed significant decreases in protein spots identified as α-enolase, heat shock protein (HSP) 90B1, lactate dehydrogenase 3, testis lipid binding protein and cytokeratin, while spots associated with the β-subunit of the F1 ATPase, HSP70 and phosphatidylethanolamine binding protein (PEBP1) all increased during epididymal transit. The rise in PEBP1 was particularly dramatic, amounting to a 4.8 fold increase during transition from the caput to cauda epididymis. This protein is especially interesting because independent studies have identified PEBP1 as a key component of a decapacitation system that regulates the rate at which capacitation occurs in mature mouse spermatozoa [56, 57]. In more recent applications, DIGE has also been applied to: assess protein concentrations in different germ cell types to identify those proteins specifically or preferentially expressed at each stage of spermatogenesis [58]; determine the temporal expression pattern of spermatogenesis-associated proteins in newborn, young adult, and aged men [59]; screen human seminal plasma as a potential source of biomarkers for disorders of the male reproductive system associated with male infertility [60]; compare the proteome of normozoospermic donors with that of infertile patients afflicted with either globozoospermia (a severe form of teratozoospermia in which all spermatozoa are round-headed) [61] or an idiopathic failure of sperm-zona pellucida binding [62]; and correlate perturbations in the sperm proteome with physiological insults such as diabetes and obesity [36] and oxidative stress [63]. A striking theme that has emerged from the latter studies is that, despite bearing pronounced anomalies in sperm morphology and/or function, most cases of infertility appear to be associated with only a limited number of proteomic changes. Thus, in the case of globozoospermia, a total of only 35 protein spots were identified that exhibited significant changes in expression between normal and round-headed spermatozoa [61].

In a similar context to the improvements afforded by DIGE, 2D electrophoresis analyses of the cellular proteomes have also benefited from the recent development of a suite of fluorescent stains that facilitate multiplexing approaches in which post-translational modifications such as phosphorylation (Pro-Q Diamond),

glycosylation (Pro-Q Emerald) as well as total protein (Sypro) expression patterns can be determined within a single gel [64]. Notwithstanding these exciting developments and the instrumental role that traditional 2D electrophoresis techniques will continue to hold in helping to define the elementary aspects of the sperm proteome, such gel-based approaches are rapidly being superseded for large-scale proteomic analyses in favor of higher throughput techniques based on chromatographic separation platforms.

1.2.1.2 Chromatographic Separation Platforms

While the analysis of intact proteins with 2D electrophoresis is likely to continue to play an important role in comparative studies of the sperm proteome [24], recent technical developments have heralded a new era in proteomics where the emphasis is placed not on whole proteins but on peptides. By virtue of their smaller size, peptides are much more homogenous structures than proteins, which can exhibit significant variation in physiochemical properties such as size, charge, and hydrophobicity as a consequence of post-translational modifications such as glycosylation or proteolytic cleavage. As an average sized protein of around 30–50 kDa will produce approximately 50 tryptic peptides, the number of entities that have to be analyzed increases dramatically when attention shifts from proteins to peptides. However, the continued maturation of nanoscale chromatographic strategies to purify individual peptides combined with improved MS systems has made the rapid detailed analysis of large numbers of tryptic peptides a realistic possibility [51].

The same purification techniques available for peptide purification are used for whole protein purification and include size-exclusion chromatography, ion-exchange chromatography, and reversed-phase high performance liquid chromatography (RP-HPLC). It is the latter of these techniques that is most commonly used for peptide purification in proteomics. In RP-HPLC, the peptides are generally retained due to hydrophobic interactions with the stationary silica phase. Polar mobile phases, such as water mixed with methanol or acetonitrile, are subsequently used to elute the bound peptides in order of decreasing polarity (increasing hydrophobicity). While RP-HPLC can be used as the sole separation procedure for moderately complex peptide mixtures prior to tandem mass spectrometric analysis, it is generally considered to have insufficient resolution for the analysis of more complex mixtures. This reflects the fact that although an MS instrument can perform mass measurements on several co-eluting peptides, if many peptides co-elute the instrument cannot fragment them all and therefore valuable information is likely to be irretrievably lost.

The first comprehensive analysis of the human sperm proteome utilizing an LC-MS/MS approach recorded the identification of greater than 1,760 proteins [65]. In this study, spermatozoa from a single fertile individual were fractionated into detergent-soluble and detergent-insoluble fractions and resolved by SDS-PAGE. The gel was then separated into 35 slices and digested with trypsin. Of the 1760 proteins identified within these gel sections, 1,350 proteins were uniquely present in the soluble fraction, 719 in the insoluble fraction, and 309 in both fractions. However, the individual proteins identified were not reported [65]. Using a similar approach,

Baker and colleagues reported the identification of 1,056 unique gene products in human spermatozoa, approximately 8 % of which had not previously been characterized [66]. Similar experimental strategies in which the sperm samples were first enzymatically digested and focused in immobilized pH gradient (IPG) strips before being run through a nanoflow reversed-phase column coupled to a linear ion trap, provided identifications of 858 and 829 unique gene products in mature spermatozoa of the mouse and rat, respectively [67, 68]. In the latter species, bioinformatics demonstrated that at least 60 of these proteins were specifically expressed in the genitourinary tract, including: pyruvate dehydrogenase 1, ropporin, testis-specific serine kinase 4, testis-specific transporter, and retinol dehydrogenase 14.

Among the most recent developments in this field, Wang and colleagues employed an advanced mass spectrometry platform to reveal that the human sperm proteome was in fact far more complex than previously anticipated [16]. Indeed, by focusing on populations of high quality spermatozoa purified by density gradient centrifugation, the authors reported the successful resolution of some 30,903 peptides corresponding to 4,675 unique proteins. Importantly, among these proteins, 4,401 (94 %) were identified in two independent experiments and a total of 3,777 represented new additions to the human sperm proteome [16]. Using an elegant suite of bioinformatics tools, Amaral et al. subsequently collated the information from this and an additional 29 proteomic studies into a catalogue of 6,198 different proteins [19]. Although this list represents the entire complement of human sperm proteins that have been identified to date, the authors predict that our current coverage represents only ~78 % of the complete proteome of these cells [19]. Thus, defining the additional 22 % of proteins that constitute a mature human spermatozoon remains a key challenge for future proteomic studies. Nevertheless, these resources have already begun to yield important insights with confirmation that little overlap (only ~29 %) exists between the sperm proteome and that of its transcriptome [16]. Curiously however, it has also revealed an unexpected overrepresentation of several biochemical pathways including those involved in nucleic acid metabolism and protein synthesis. Since neither of these functions are typically ascribed to mature human spermatozoa, confirmation of their activity and biological significance remains to be fully investigated. However, such data highlight the enormous potential of proteomic-based studies to fuel paradigm shifts in our understanding of sperm biology. Indeed, this approach has become increasingly attractive as a means of not only extracting as much information as possible from the sperm proteome but also generating insights into the proteins that are functionally important. One of the ways in which such information can be generated is through comparative proteomics.

Isobaric Labeling In addition to their utility for rapidly building an in-depth understanding of the sperm proteome, gel-free strategies have also proven to be particularly amenable for use in comparative profiling applications. Indeed, since peptides are inherently less variable than their parent proteins, it has been argued that they constitute a more reliable basis for quantitative comparisons. This property has been exploited for the development of a suite of isobaric labeling strategies to compare

the complex proteomic mixtures in different cell populations. The most common of these approaches seek to introduce stable isotope tags (e.g. ^{2}H, ^{13}C, ^{15}N, ^{18}O) into peptides via either in vivo metabolic labeling [stable isotope labeling by amino acids in cell culture (SILAC)] or in vitro chemical reactions [e.g. isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), tandem mass tags (TMT)] or enzymatic incorporation during proteolysis (e.g. stable isotope dimethyl labeling [69]). In each technique, the stable isotopes possess identical chemical properties that ensure similar behavior during chromatographic peptide purification and MS applications. Thereafter they present an easily distinguishable mass difference that enables relative quantification based on the intensities of the reporter ion produced by precursor ion fragmentation in the low mass/charge (m/z) region of spectra. As such, chromatographic separation platforms have become viable alternatives to 2D electrophoresis for the differential analysis of complex protein mixtures [70, 71].

While neither SILAC (owing to a lack of compatibility with translationally inert spermatozoa) nor ICAT technologies have gained favor for the analysis of human spermatozoa, a number of recent studies have featured quantitative analyses based on either iTRAQ [72] or TMT [35, 40, 73] modification chemistries. Both labeling strategies rely on derivatization of peptides with an amine-reactive tagging reagent and their subsequent quantification on the MS/MS level. In addition to enhanced sensitivity, the availability of several isotope-coded variants, each of which possess an identical molar mass (isobaric), means that both strategies are readily amenable for multiplex profiling applications of up to eight (iTRAQ) to ten (TMT) different samples. As with DIGE, the application of these isobaric labeling technologies has predominantly focused on quantitative alterations in the sperm proteome of normozoospermic individuals compared with that of males afflicted with either motility (asthenozoospermic) [35] or idiopathic [40, 72, 73] infertility lesions. The former of these studies identified 80 differentially expressed proteins that were subsequently mapped to core cellular pathways associated with sperm motility dysfunction [35]. An unanticipated finding was the prevalence of post-glycolytic enzymes with altered expression levels in low-motility sperm populations. Such findings offer tantalizing evidence that several bioenergetic pathways (including those associated with the mitochondria: tricarboxylic acid cycle, oxidative phosphorylation, beta-oxidation of fatty acids) contribute to human sperm motility, and thus challenge the long-held view that glycolysis is the sole player in this process [74]. The degree of proteome dysregulation is also somewhat unique to this study as a majority of others have tended to identify relatively few overall changes, irrespective of the functional lesion. For instance, despite the imposition of a very modest threshold (>1.2-fold change), Zhu et al. identified only 21 (out of a total of 2,045 proteins detected) that were differentially expressed in the spermatozoa of fertile men compared to those from IVF patients who failed to produce a clinical pregnancy [40]. Such findings accord with those previously reported for DIGE-based analyses [61] and thus raise the prospect that even relatively minor changes to the sperm proteome can have profound

influences on the functioning of the mature male gamete. This notion is supported by the work of our own laboratory focusing on the molecular chaperone HSPA2 [37, 75–78], a protein originally identified in our attempt to dissect the molecular basis of gamete interactions through the application of a label-free MS-based quantification approach [37].

Label-Free Quantification Label-free quantitative mass spectrometry has recently emerged as an important tool for both relative and absolute quantification of proteins in biological specimens. In the complete absence of chemical modifications, this rapid, low-cost technology relies on a workflow in which individual samples are analyzed separately (e.g. by LC-MS or LC-MS/MS) prior to protein quantitation via either ion profiling or spectral counting. The former is typically applied to high precision mass spectra and facilitates the extraction of ion peak intensity on the MS1 level, thereby uncoupling the quantification and identification processes. The m/z ratios for all ions are detected and their signal intensities at a particular chromatographic retention time recorded. Owing to the tight correlation between signal intensity and ion concentration, relative peptide levels between samples can be determined directly from these peak intensities. Similarly, spectral counting exploits the strong correlation between protein abundance and the number of MS/MS spectra. This approach involves counting the number of peptide-specific spectra identified in different biological samples and the subsequent integration of these data for all measured peptides of the protein(s) that are quantified. Although such approaches have been used sparingly in the field of human sperm proteomics to date, they have nevertheless provided key molecular insight into important processes such as capacitation [37, 79–81].

In one such application, our laboratory employed label-free MS technology to map defects in human sperm-zona pellucida (ZP) adhesion resulting in the detection of significant alterations in the expression of ten proteins [37]. Chief among these was the molecular chaperone, HSPA2 which displayed a significant, >10 fold reduction in infertile spermatozoa (Fig. 1.1). Such findings accord with independent evidence that the overall levels of HSPA2 present in mature human spermatozoa provide a robust discriminative index of the success of cumulus-oocyte interactions and fertilizing potential [82, 83]. At least two models have been proposed to account for the role of HSPA2 in promoting ZP recognition. Thus, Huszar and colleagues postulate that the chaperoning activity of HSPA2 facilitates major cycles of protein transport that drive cytoplasmic extrusion and plasma membrane remodeling during spermiogenesis [82–85]. Alternatively, our own evidence suggests that HSPA2 may play an important functional role in mature spermatozoa following their morphological differentiation within the testes [76]. This model draws on evidence that HSPA2 is retained in mature spermatozoa and appears to facilitate the assembly and/or presentation of zona recognition complexes on the surface of these cells [76, 86, 87]. Indeed, we have shown that HSPA2 stably interacts with a number of high molecular weight multimeric complexes, which include proteins involved in mediating cumulus-oocyte complex recognition as well as regulating the stability of the chaperone itself [37, 77, 88, 89].

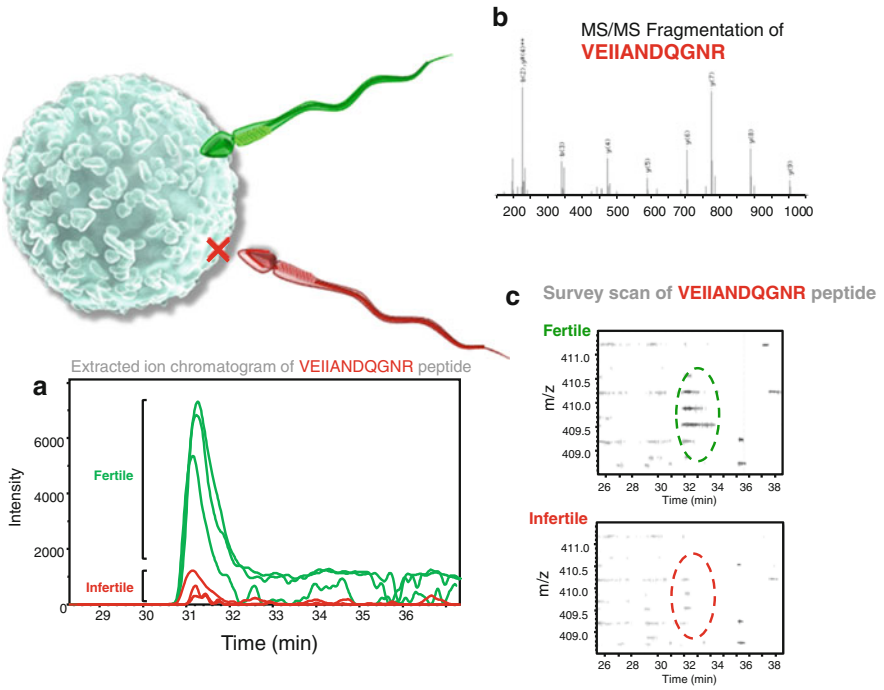

Fig. 1.1 The power of proteomics. A proteomic comparison of tryptic peptides isolated from the spermatozoa of patients whose gametes lack a capacity to bind to the zona pellucida demonstrated the central importance of a heat shock protein, HSPA2, in orchestrating this physiological process [76–78]. (**a**) Extracted ion chromatogram demonstrating the difference in expression of a specific peptide which was traced to the HSP70 family. (**b**) MS/MS spectrum of the peptide. (**c**) A small window from the MS survey scan demonstrating the difference in peptide expression between the patient's spermatozoa and a normal fertile control

While such work highlights the utility of comparative proteomic strategies as a powerful starting point for dissecting the functional lesions associated with male infertility, the main disadvantages of this approach are concerned with post-experimental data processing. Indeed, the extremely large volume of data collected in such experiments presents a significant problem in terms of both the time required to collate and assemble the data into a useable format and the computing power needed to complete database searching. This problem may eventually be alleviated as mass spectrometric instrumentation, sequencing algorithms, and the performance of computing resources continue to improve and become more affordable. These improvements, coupled with the increasing stringency of data requirements of journals are helping to make results more transparent and address the burden of erroneous identification of proteins that appear in many published proteomes [90].

SRM/SWATH-MS Researchers in the field of human sperm biology have traditionally been quick to embrace innovative developments in proteomic analyses.

However, to the best of our knowledge there are presently few reports capitalizing on recent technological breakthroughs in label-free quantitative proteomics, such as Selected Reaction Monitoring [(SRM), also known as Multiple Reaction Monitoring (MRM)], Parallel Reaction Monitoring (PRM), and Sequential Windowed Acquisition of all Theoretical fragment ion spectra-mass spectrometry (SWATH-MS) analyses. Driven by recent advances in the speed and sensitivity of the new generation of high resolution mass spectrometry instrumentation, these technologies afford the ability to not only determine which proteins are present in the sperm proteome, but also to accurately quantitate them in a variety of biological contexts at a resolution that far exceeds that obtained using traditional quantitative approaches. As such, these techniques are being heralded as among the most important recent developments in proteomics research.

SRM is an absolute quantitation method that exploits the unique capabilities of a triple quadrupole mass spectrometer. This analysis is performed by the acquisition of selected events across the LC retention time domain of predefined pairs of precursor and product ion masses. The technique becomes an absolute quantitation tool by spiking isotope-labeled synthetic peptide(s) into the complex sample of interest, which acts as an internal standard for any peptide(s) of interest. The labeled peptide standards are designed to mimic those generated by tryptic sample digestion, thus enabling them to co-elute and be subjected to MS/MS analysis along with the target peptides. A calibration-response curve based on the labeled peptides is subsequently used to accurately determine the absolute concentration of targeted peptides, a procedure that is repeated for each target within the sample. It follows that assay development and optimization are key elements of the SRM proteomic strategy. Indeed, the labeled peptides must be synthesized based on *a priori* knowledge for each target, taking into account those tryptic fragments that possess optimal electrochemical characteristics. While sophisticated software packages are available to help predict the most suitable peptide sequences, an element of trial-and-error and the necessity for instrument optimization renders the process of quantitation via SRM a time-consuming and costly endeavor. Notwithstanding such limitations, SRM is now firmly established as a method of choice for quantitative clinical applications. This reflects its unparalleled ability to characterize and quantify a set of proteins reproducibly, selectivity and with high sensitivity. Indeed, SRM readily extends analytical capability to low-abundance proteins without bias from abundant analytes, with recent reports suggesting the technique can detect proteins with as few as 50 copies per cell from among complex unfractionated lysates [91].

In one of the first applications to illustrate the potential of SRM in the context of male infertility research, Drabovich et al. employed the technique to assess a cohort of prospective seminal plasma biomarkers for their ability to discriminate between fertile, post-vasectomy and non-obstructive azoospermia patients [92]. From an initial investigation of 31 proteins identified in a multiplex SRM assay, the authors synthesized heavy isotope-labeled internal standards to reanalyze the concentration of 20 of the most promising candidates [92]. This approach was subsequently extended through investigation of diagnostic biomarkers to differentiate between

obstructive and non-obstructive azoospermia [93]. In both instances, key biomarkers were identified that performed with either absolute, or nearly absolute, specificities and sensitivities in these assays. Such results offer the promise of developing viable alternatives to alleviate the current need for invasive testicular biopsy as the only definitive diagnostic method to distinguish between obstructive and non-obstructive azoospermia.

The recent advent of faster acquisition MS equipment has fueled the development of a new proteomic approach referred to as SWATH-MS. In essence, SWATH-MS allows the generation of a complete and permanent spectral library constituting a record of all fragment ions of the peptide precursors present in a biological sample. In combining the unique and material advantages of traditional shotgun (high throughput) and SRM (high reproducibility and consistency) technologies, SWATH-MS can be deployed for both discovery and quantitation of all detectable peptides present in complex biological samples. It also affords the added advantage that it does not rely on prior knowledge of the precursor peptide ions, instead acquiring information in a data-independent manner and thus avoiding laborious assay development. The SWATH-MS workflow involves two key steps beginning with the generation of a spectral library (e.g. via conventional LC-MS/MS) through which acquired peptides are identified. During this acquisition mode, the mass spectrometer is programed to step within 2–4 s cycles through a set of precursor acquisition windows covering the mass range accessible by a quadrupole mass analyzer and also that in which most tryptic peptide precursors should fall (400–1200 m/z). During each cycle, the mass spectrometer fragments peptide precursors and records a complete, high accuracy fragment ion spectrum for all precursors that elute on the chromatograph. This is then followed by acquisition of SWATH-MS data for each sample under analysis, interrogating and matching against the spectral library to identify peptides, and finally extraction of specific peptide ions to enable area-under-the-curve quantitation between samples.

1.3 Sub-cellular/Proteomic Fractionation Strategies

Notwithstanding the significant advances this next generation of proteomic technologies is likely to afford in terms of defining the complete human sperm proteome, a considerable challenge that lies ahead rests with our ability to convert such a vast body of data into meaningful biological function [21]. In an effort to realize the transformative potential of this resource, there is an increasing interest in coupling comparative proteomics with methods of subcellular fractionation and protein/peptide enrichment techniques for investigation of the key functional domains and post-translational modifications that are required to achieve successful fertilization.

In this context, several groups have begun to characterize important sub-proteomes associated with human sperm capacitation. A focus for these studies has been analysis of the phosphoproteome of capacitated human spermatozoa utilizing pre-fractionation strategies in which phosphopeptide enrichment is coupled with MS/MS [94] or label-free quantitative phosphoproteomics [81]. In the former

study, more than 60 phosphorylated sequences were mapped leading to the identification of novel targets for capacitation-associated tyrosine phosphorylation including: valosin-containing protein, a homolog of the SNARE-interacting protein NSF, and A-kinase anchoring protein types 3 and 4 [94]. In the latter study, an expanded cohort of some 3,303 phosphorylation sites, corresponding to 986 proteins, were identified following immobilized metal affinity chromatography (IMAC)-TiO_2 phosphopeptide enrichment. Among these candidates, the phosphorylation levels of 231 sites were increased significantly, including that of insulin growth factor 1 receptor, a tyrosine receptor kinase implicated in the regulation of hyperactivated motility [81]. Such studies have also recently been extended to provide valuable insight into the functional impact of alternative forms of post-translational modification including: S-nitrosylation [95], lysine acetylation [96, 97], lipid aldehyde (4-hydroxynonenal) adduction [98], N-glycosylation [99], and sumoylation [100] on human spermatozoa.

Among the common themes emerging from these studies is the striking increase in proteome complexity generated by the dynamic post-translational modification that accompany sperm development and post-testicular maturation processes. Indeed, with in excess of 400 different forms of post-translational modification, it is likely that we are only beginning to scratch the surface of the crucial role such modifications play in sperm physiology and pathology. This notion is reinforced by the relatively poor correspondence between the capacitation-associated phosphoproteomes identified in the spermatozoa of humans as opposed to those of model species such as the mouse [101], rat [102], and hamster [103]. While it is difficult to refute the contribution of methodological differences, these data also speak to the possibility that a large portion of the phosphoproteome remains unexamined.

One of the most promising approaches to reduce this overall complexity is the use of subcellular fractionation strategies that seek to break the cell down into its constituent parts prior to MS analysis. Indeed, as one of the most highly differentiated cell types in the human body, spermatozoa are uniquely amenable to this form of analysis. Accordingly, several studies have begun to emerge in which the protein signature of discrete functional domains has been reported. These proteomic catalogues now include membrane microdomains involved in mediation of oocyte interactions (plasma membranes [47, 104, 105], detergent resistant membranes [106]), the sperm nucleus [107], chromatin [108, 109], head [110], and flagellum [110–112]). Through the reduction of the dynamic range and enrichment of less abundant proteins, these combined approaches have helped increase our coverage of the human sperm proteome and apportion protein function to specific subcellular domains [110]. A central tenet of this work has been the marked division of labor that exists among sperm proteins/domains. Thus, among a total of 1,429 proteins successfully identified in their comparative proteomic analysis of the human sperm head and flagellum, Baker et al. [110] reported only 179 (~12 %) proteins that were detected in both cellular domains. In addition to the anticipated partitioning of metabolic enzymes within the flagellum, the sperm head featured an abundance of proteasomal and signaling machinery. These findings mirror those reported in a more comprehensive analysis of the sperm

flagellum proteome in which Amaral et al. identified a total of 1,049 proteins [111]. Interestingly, however, less than half of these flagellum proteins were identified in both datasets [110, 111], illustrating that we still have some considerable ground to cover before achieving the ultimate goal of documenting the complete sperm proteome.

Conclusions

Simultaneous advances in mass spectrometry design, computing power, and the availability of genomic sequence data for a variety of organisms have fueled rapid growth in the field of proteomic analysis and served to enhance the utility of this approach for the study of human sperm function. Ambitious, large-scale, mass-spectrometry-based proteomic analyses have identified complex inventories comprising thousands of sperm proteins with a dynamic range of abundance of several orders of magnitude. In fact, obtaining mass-spectrometry data has already ceased to be the limiting step in sperm proteomics. Instead, the main challenge that lies ahead is to exploit this valuable resource in order to define which specific elements of the proteome are of functional significance and understand the cascade of post-translational modifications involved in generating a functional spermatozoon. Ultimately the success of such studies will be measured by our progress in understanding the molecular mechanisms that may be targeted for contraceptive purposes or implicated in the etiology of defective sperm function.

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Methods of Analysis of Sperm Antigens Related to Fertility

2

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Abstract

Progress in the identification of novel sperm antigens related to fertility, particularly, immune infertility, has been made in recent years employing advanced proteomic analyses, mass spectrometry microsequencing, and recombinant DNA technology. This chapter summarizes several approaches employed to identify and characterize previously unidentified sperm antigens and their encoding genes that are relevant to fertility and/or infertility, focusing on strategies that have the potential to yield candidate protein targets for immunocontraceptive or drug development programs, including novel sperm proteins that may fall in the cancer-testis antigen category. Methods aimed to enrich, isolate, and identify the relevant antigens include vectorial labeling, probing with antisperm antibodies (ASA) or monoclonal antibodies, phage display, subcellular fractionation, differential extraction, treatment of sperm to isolate and identify glycosyl phosphatidylinositol (GPI)-anchored proteins, identification of glycoproteins by lectin blotting and fluorescent dye staining, cDNA library screening, and PCR amplification. Several techniques employ 2-D gel electrophoresis to first separate and visualize immunoreactive protein spots followed by mass spectrometry to sequence and identify the protein to obtain sequence information which can then be used to clone the corresponding gene. The recombinant proteins can be expressed in a variety of expression vectors and host systems, specific

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immunoreagents may be developed to the novel proteins and subsequent functional characterizations can lead to definition of the sub-cellular localization, definition of biological roles in various sperm functions, fertility, and validation of contraceptive potential.

2.1 Introduction

The discovery of sperm antigens involved in fertilization requires a comprehensive understanding of the composition of the sperm plasma membrane as well as the membranes involved in sperm-egg plasma membrane interaction. The plasma membrane of the sperm contacts the outer investments of the oocyte including the corona radiata and zona pellucida and subsequently undergoes the acrosome reaction. Following the acrosome reaction, the anterior aspect of the sperm head is remodeled and the inner acrosomal membrane becomes the limiting membrane on the anterior two thirds of the sperm head. The membrane overlying the equatorial segment of the acrosome is believed to be the initial site of fusion with the egg plasma membrane [1]. Knowledge of the molecular composition of the sperm plasma membrane and the structural domains involved in various sperm functions leading to fertilization may be employed to design contraceptive vaccines, "intelligent spermicides" that target surface receptors, or contraceptive antagonists. Toward these applied endpoints a target discovery strategy should be employed that identifies those sperm antigens that are unique, germ cell specific, immunogenic and accessible to antibodies, or antagonists at the cell surface. Several conventional gene profiling methods, including differential display [2, 3], serial analysis of gene expression [4], RNA sequencing (RNA-seq) [5], and microarray [6–9] technologies are suitable for profiling relative levels of messenger RNAs in precursor germ cells such as spermatogonia, spermatocytes, or spermatids. These "transcriptomic" techniques can only be complimentary to understanding the comprehensive proteomic composition of fully differentiated spermatozoa. Recently, the combination of RNA-seq and mass spectrometry (MS)-based proteomic has led to the development of the so-called proteomics informed by transcriptomics (PIT) approach [10]. Proteomics can verify the presence of a target protein, provide an estimate of its relative abundance, and provide evidence of its immunogenicity. In this context, spermatozoa are in some respects ideal cells for proteomic analyses since they can be easily purified in large numbers and they can be pharmacologically manipulated to drive them into different physiological states such as capacitation and the acrosome reaction. Several recent reviews on sperm proteomics elaborate on the progress in this field [11–15]. With the recent developments in protein microsequencing by mass spectrometry, along with the wealth of information now available from the genome projects, the technologies are at hand to readily determine amino acid sequences of a protein spot on a 2-D gel or from a complex mixture of enriched proteins. The peptide sequence can be used to deduce and synthesize a set of gene-specific oligonucleotides which

can then be used to PCR amplify or hybridization clone and sequence a corresponding cDNA, leading to characterization of genes and proteins of hitherto unknown sperm components, particularly those associated with the acrsosome, plasma membrane, and acrosomal membrane domains. This chapter reviews several proteomic methods of analysis that are currently being employed to identify and characterize the sperm antigens that are relevant to sperm function and fertility.

2.2 Two-Dimensional Gel Electrophoresis

The functionality of spermatozoa is significantly influenced by post-translational modifications that may take place during spermiogenesis, during the maturation of spermatozoa in the epididymis, during the post-ejaculatory capacitation processes in the female reproductive tract, or following the acrosome reaction. Two-dimensional (2-D) gel electrophoresis offers a classical proteomic tool to dissect the molecular transformations that spermatozoa undergo in various functional states through a comparison of 2-D gel images from various experimental conditions. The pioneering innovation of O'Farrell [16] led to the development of 2-D gel electrophoresis. Proteins are resolved in two dimensions: the first dimension separates proteins in a pH gradient according to their isoelectric point (pI) by isoelectric focusing (IEF), while in the second dimension the proteins are separated according to their molecular weight by SDS-PAGE. Since its introduction, IEF has undergone several advances. The first dimension may be carried out in polyacrylamide gel rods that are formed in glass tubes and contain ampholytes that form a pH gradient in an electric field. The introduction of immobilized pH gradients (IPGs) by Bjellqvist et al. [17] had a significant impact on the use of IEF to separate complex mixtures over a wide pH range. The IPGs enable the formation of stable and reproducible pH gradients capable of focusing acidic and basic proteins on a single gel prepared with broad pH gradients. Once a broad pH gradient gel is stained with a reagent that allows the repertoire [encyclopedia] of proteins to be resolved, a narrow pH gradient gel may then be employed to expand particular pI regions of the protein encyclopedia. Visualization of low abundant proteins separated on 2-D electrophoresis gels is also governed by staining sensitivity. For example, classic Coomassie has a narrow dynamic range with detection limit of only about 100 ng. Colloidal Coomassie is relatively higher in sensitivity with detection limit of 10 ng. A detection level of below 1 ng can be achieved with a highly sensitive silver-staining method [18] and a variety of fluorescence dyes such as SYPRO-Ruby and Deep Purple flurophore dye. Use of sensitive stain increases protein sample dynamic range and leads to successful mass spectrometric identification and immunological validation. Two-dimensional electrophoresis followed by Western blotting is widely applied as a tool to study sperm-antibody interactions or other protein-protein interactions [as in Far-Western analysis of receptor-ligand interactions] and to analyze proteins from different domains of the sperm. A critical requirement of proteomic research is high quality separation of cellular constituents, usually referred to as

protein resolution. 2-D gel electrophoresis may be preceded by various methods of subcellular fractionation, isolation, and enrichment of proteins to generate a proteome of particular subcellular compartments.

2.2.1 Vectorial Labeling of Surface Proteins

Molecules exposed on the cell surface represent key targets for both understanding the complex processes of differentiation and function of the spermatozoa and offer candidate contraceptive vaccinogens or receptors for drug agonists or antagonists. Surface labeling of the proteins exposed on the spermatozoa may be achieved by labeling with radioactive ^{125}I (radioiodination) or biotin (biotinylation). Radioiodination involves the introduction of radioactive iodine into certain amino acids, usually tyrosines in proteins and peptides. The iodo-bead idodination method [19] is a convenient, gentle, and efficient method for vectorial iodination of membrane bound proteins. Identification of the iodinated cell surface proteins is achieved by performing an autoradiograph of the proteins after resolution by 2-D gel electrophoresis. Surface labeling with biotin may be accomplished using the reagent sulfo-NHS-LC-biotin which reacts with primary amines exposed at the sperm surface. The biotinylated proteins along with non-biotinylated proteins may be subsequently fractionated by 2-D gel electrophoresis and the biotinylated proteins can be identified by avidin blotting, using peroxidase-conjugated avidin followed by enhanced chemiluminescence [20].

Naaby Hansen et al. [19] identified a repertoire of proteins exposed on the surface of ejaculated human spermatozoa by utilizing high resolution 2-D gel system using both isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis (NEPHGE). A total of 181 surface proteins spots were identified by radiolabeling the intact sperm with ^{125}I, while 228 protein spots were biotinylated out of 1397 total protein spots that were digitized and catalogued from silver stained gels. The protein spots labeled with ^{125}I and biotin constituted a "sperm surface protein index". In a later study, Shetty et al. [20] reported surface labeling the sperm with sulfo-NHS-LC-biotin, which has an additional sulfur group which restricts the penetration of the biotin reagent through the cell membrane during surface labeling. Use of this improved biotinylating reagent limited the number of biotinylated proteins detected to 68 and enabled the identification of eight novel sperm molecules. D'Cruz et al. [21] identified target antigens recognized by clinically important complement-fixing antisperm antibodies (ASA) by indirect immunoprecipitation after surface biotinylation of motile sperm with ASA-positive sera from autoimmune, isoimmune, and vasectomized patients. Paradowska et al. [22] applied the same technique to identify human ASA reactive mouse sperm surface proteins. Stein et al. [23] used sulfo-NHS-LC-biotin to biotinylate mouse sperm surface proteins. Biotinylated proteins were further isolated using monomeric avidin beads and microsequenced after fractionating the proteins by SDS-PAGE. Pasten et al. [24] made use of sperm surface biotinylation experiments to determine the presence of proteasomes on the

sperm surface and their possible role in fertilization. In an experiment on the rearrangement of macaque hyluronidase PH-20, Yudin et al. [25] demonstrated this protein's localization on the surface by surface–labeling with biotin. Naaby-Hansen and Herr [26] demonstrated the presence of several heat shock proteins on the human sperm surface utilizing biotinylation and 2-D gel–based proteomic study. Recently Kasvandik et al. [27] have combined cell-surface biotinylation technique with the differential centrifugation and achieved a significant degree of enrichment for transmembrane and PM-targeted proteins in bovine sperm.

In our experience, even though biotinylation with the sulfo-NHS-LC-biotin improved the quality of surface labeling, and verification of labeling of known surface molecules such as angiotensin-converting enzyme (ACE), PH-20, and SAGA1 served as useful positive controls, the subsequent cloning and characterization of two unknown biotinylated protein spots yielded novel acrosomal membrane proteins namely SAMP14 [28] and SAMP32 [29]. This suggests that acrosomal membrane proteins may also be biotinylated by this procedure, possibly because of their exposure following spontaneous acrosome reaction among a subset of sperm. Another possible drawback of the procedure was the inability to visualize several of the biotinylated proteins on a silver-stained gel, probably due to their low abundance. The avidin-step, which amplifies reaction products and may provide higher sensitivity, resulted in spots on the western blot but not on the silver-stained gel. The current commercial availability of another biotinylating reagent Sulfo-NHS-SS-biotin (Thermoscientific) adds a few advantages to the isolation of sperm surface targets. The disulfide bond in the water soluble reagent allows the biotin label to be removed using reducing agents such as DTT. The cleavable spacer arm enables initially biotinylated proteins to be released from streptavidin affinity columns. The eluted proteins can be directly processed for microsequencing.

2.2.2 Identification of Fertility Related Sperm Antigens by Naturally Occurring Anti-sperm Antibodies

The occurrence of antisperm antibodies (ASA) in association with many cases of unexplained infertility has shown that fertilization can be blocked at various stages by ASA, causing sperm agglutination and/or immobilization, or interfering in the process of sperm-oocyte interaction [30, 31]. The presence of ASA does not exert in most cases any harmful effect on patients, except for their infertility. For this reason, identification of the cognate sperm antigens in these cases has been seen as a route to candidate contraceptive vaccinogens. Antisperm antibodies are found in 9–12.8 % of infertile couples. However, these antibodies are also present in approximately 1–2.5 % of fertile men [32, 33] and in 4 % of fertile women. The presence of ASA in the fertile population suggests that not all ASA cause infertility [34–36]. This observation requires consideration in the design of a discriminating method to identify the cognate antigens relevant to infertility utilizing sera or seminal plasma containing antisperm antibodies. Another aspect to be considered is the

accessibility of an antibody to its cognate sperm antigen. Sperm interact with their surroundings through their surfaces within the male and female tracts and it is through the plasma membrane that the sperm contacts the egg investments. Hence a strategy that identifies sperm surface antigens that elicit immune responses in humans might offer a particularly attractive subset of sperm proteins for contraceptive targeting. An extensive literature has emerged on the subject of identification of sperm antigens recognized by systemic and/or local auto and iso-antibodies from infertile individuals using immunoblotting techniques (see reviews from Chamley and Clarke [37] and Lombardo et al. [38]). Most of the cited studies used uni-dimensional gel electrophoresis for the separation of sperm proteins and as a result the molecular weights of relevant sperm antigens were conflicting, probably due to differences in methodology or in the immunodominant repertoires of ASA-positive individuals. Shetty et al. [39] employed a 2-D proteomic approach coupled with immunoblotting, vectorial labeling, and computer aided 2-D gel analysis to target sperm surface proteins relevant to fertility while screening the sperm proteins with sera from infertile men and women. A subset of six auto- and iso-antigens was identified as possibly relevant to antibody-mediated infertility. The serum samples were initially screened for the presence of ASA by the immunobead binding test (IBT) [30] and only those sera showing significant reactivity for the presence of ASA were utilized to identify immunodominant antigens. By monitoring bead binding to the sperm surface, the IBT test offers an opportunity to identify and pre-screen patients with auto-antibodies to surface exposed epitopes. In order to have a comprehensive overview of the repertoire of immunoreactive sperm proteins recognized by serum samples from fertile and infertile subjects of both sexes, the blots were serially incubated with five serum samples from each group of subjects. Figure 2.1 shows one of such experiments [39]. Serum samples from the infertile subjects were selected based on their high immunoreactivity, heterogeneity in the immunoreactivity, and unique recognition of certain protein spots following individual Western blot analysis of the sera. Comparison of Western blots probed with sera from fertile (Fig. 2.1a, b) and infertile subjects (Fig. 2.1c, d) demonstrated that several discrete sperm antigens were recognized by sera from infertile patients (arrows). Sperm antigens unique to infertile patients were identified by excluding those antigens recognized by serum samples from clinically fertile subjects using the software Bio Image "2D Analyzer" to compare 2-D immunoblots. A database of 2-D gel images of silver-stained proteins and a database of vectorially labeled sperm surface proteins (sperm surface index) [19] allowed the definition of a subset of sperm surface antigens relevant to antibody-mediated infertility. These immunogens were microsequenced, cloned, and characterized. Examples are ESP [40, 41], SAMP32 [29], SAMP14 [28] and CABYR [42]. Among these antigens ESP, SAMP32 and SAMP14 are intra-acrosomal proteins and become exposed on the sperm surface following the acrosome reaction. CABYR, in contrast, is primarily localized to the fibrous sheath of the principal piece of the sperm tail [42]. Vectorial labeling of CABYR with ^{125}I suggests this protein may be released and exposed even with gentle handling of sperm, or perhaps a subset of CABYR molecules are surface exposed since the single copy

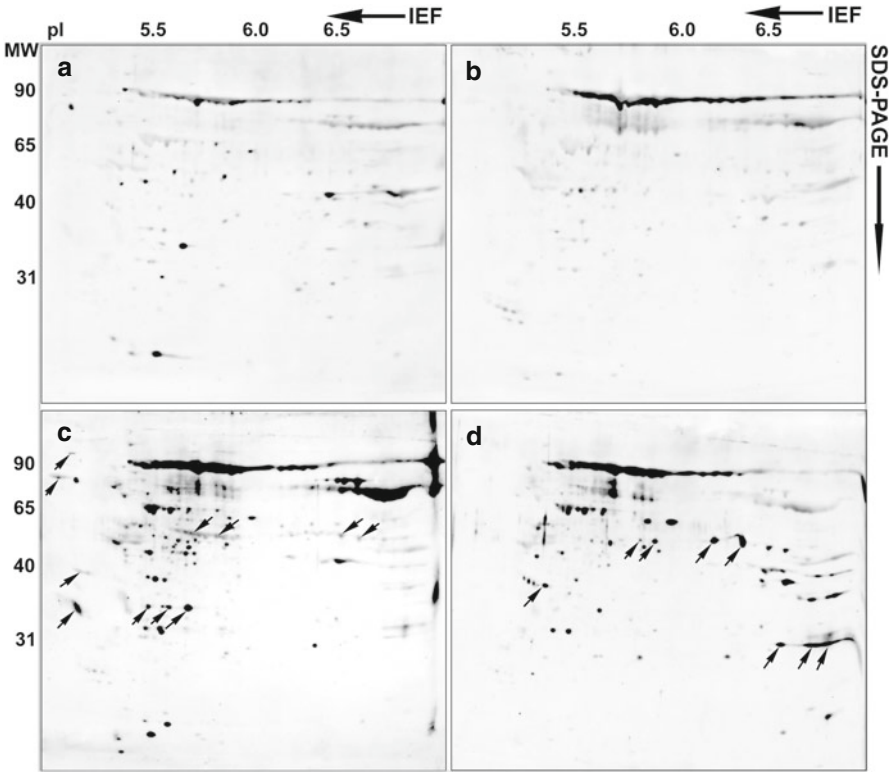

Fig. 2.1 2-D Western analysis by serial incubation of a single 2-D blot with serum from five fertile males (**a**) and fertile females (**b**) compared with those from five infertile males (**c**) and infertile females (**d**) (dilution: 1:2000). Note major auto- and isoantigens (*arrows*) that are uniquely recognized by the infertile subjects (This figure was originally published in Shetty et al. [39])

CABYR gene undergoes remarkable alternative splicing resulting in protein microheterogeneity.

Other studies employing 2-D immunoblotting led to the identification of discrete specific sperm proteins recognized by sperm immobilizing sera [43], immunoinfertile male and female sera containing ASA [44, 45], and seminal plasma containing ASA [46]. A well-known risk factor for the development of antisperm antibodies (ASA) in the male is disruption of the vas deferens, which is achieved during vasectomy for sterilization. Failure to restore the fertility in several cases of vasectomy reversal is attributed to the development of antisperm antibodies following vasectomy [47]. Sera from vasectomized male subjects can be useful reagents to identify fertility related sperm antigens [48, 49]. Employing a battery of such sera, Shetty et al. [50] identified potential fertility related antigens unique to sera collected following vasectomy by comparing the immunoreactivity of serum samples from pre- and post-vasectomy with a 2-D Western blot approach. Domagala et al. [51] employed a similar approach in identifying six novel antigens; four of which were

recognized by the ASA positive infertile males, one recognized by a vasectomized man and one recognized by ASA positive seminal plasma.

Another approach to identify antigens involved in fertility is by probing 2-D Western blots with antibodies that were recovered from the surface of sperm obtained from the ejaculate of infertile men (as opposed to using circulating anti-sperm antibody). Such studies have the advantage of potentially detecting antigens identified by antibodies that transudate from the serum or are produced locally within the reproductive mucosae, including iso- and auto-antigens recognized by secretory IgA, if the appropriate secondary anti-IgA reagents are employed. Auer et al. [52] employed enhanced chemiluminescence and immumoblotting techniques to analyze sperm antigens recognized by antibodies eluted from the surface of spermatozoa obtained from infertile men with unsuccessful in vitro fertilization. The study identified immuno-reactive proteins from 37/36 and 19/18 k Da zones.

Naz [53] used the powerful phage display technology to identify peptide sequences that were specifically recognized by immunoinfertile sera with a long-term goal of identifying sperm peptide sequences that might find applications in the specific diagnosis and treatment of immunoinfertility in humans, and in the development of a contraceptive vaccine.

The study led to the identification of seven dodecamer peptide sequences that were specifically recognized by the immuno-infertile sera. This technical approach aided in the discovery of a novel peptide sequence that was designated YLP12 [54, 55]. Vaccination with YLP12 or its cDNA is reported to cause long-term, reversible contraception, without side effects, in female mice. Further, infertile, but not fertile, men and women were found to have antibodies to YLP12 peptide [56]. Naz's laboratory has successfully isolated, cloned, and sequenced cDNA encoding human single chain variable fragment (scFv) antibody from infertile men which reacts with YLP12 peptide [56].

2.2.3 Differential Extraction

Differential extraction of sperm proteins involves various solubilization methods to preferentially enrich for peripheral membrane proteins, hydrophobic membrane associated proteins, or hydrophilic proteins. One well known and powerful technique to enrich the membrane associated hydrophobic proteins is temperature-induced phase partitioning in TX-114 [57], which allows the separation of hydrophobic integral membrane proteins from the hydrophilic proteins. The technique is based on the ability of the nonionic detergent TX-114 to partition into two distinct phases above 23 °C: a detergent-rich phase and a detergent-depleted or aqueous phase. Amphipathic membrane proteins, whether anchored to a lipid by a GPI moiety or a hydrophobic polypeptide, partition predominantly into the detergent-rich phase, whereas hydrophilic proteins partition predominantly into the aqueous phase [58]. Shetty et al. [20] exploited this technique to identify sperm membrane associated proteins. The sperm surface proteins were labeled before extraction using sulfo-NHS-LC-biotin and the proteins were resolved on large

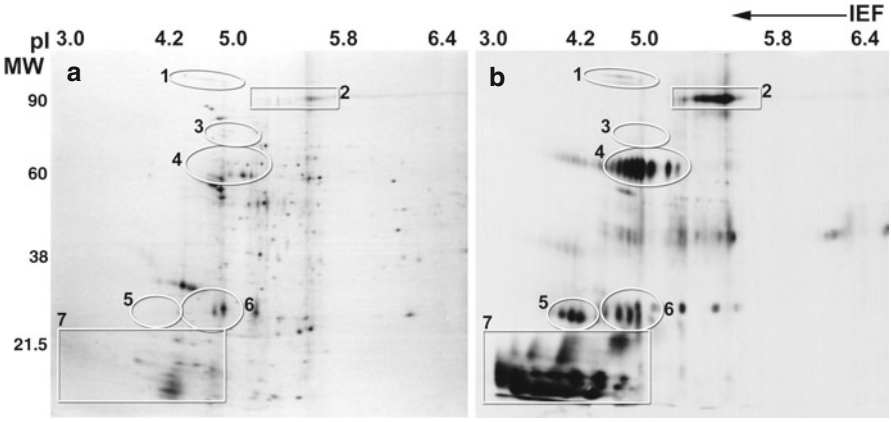

Fig. 2.2 Analysis of surface biotinylated, TX-114 phase partitioned sperm proteins. A silver-stained 2-D gel from the detergent phase extract enriched for membrane associated proteins (**a**) is matched with an avidin-ECL blot from the same phase (**b**). Clusters of biotinylated putative surface protein spots are circled or boxed in the avidin blot and their corresponding location on the silver stained gel is indicated [20]

format 2-D gels capable of high resolution. Surface localized proteins were identified by avidin blotting of the biotinylated proteins transferred to nitrocellulose membranes. Figure 2.2 demonstrates the identification of several membrane associated sperm surface localized proteins by 2-D gel analysis and avidin blotting [20]. The method facilitated the identification of eight novel sperm proteins in addition to several known membrane proteins. For example, two acrosomal membrane proteins: SAMP14 [28], a GPI-anchored Ly6/uPAR superfamily protein, and SAMP32 [29], an autoantigenic protein, emerged from this approach. Antibodies against recombinant human SAMP14 and SAMP32 inhibited both the binding and the fusion of human sperm to zona free hamster eggs, suggesting that these molecules may have a role in sperm-egg interaction. Triton X-114 phase partitioning is routinely applied to characterize a protein or to check the presence or absence of a known hydrophobic protein under various experimental conditions [59–61].

Salt Extraction Peripheral membrane proteins are known to be extracted by relatively mild treatments. One of the ways to isolate a soluble form of an extrinsic membrane protein is by treatment with high ionic strength solution (1 M NaCl or 1 M KCl). The procedure aims at the disruption of weak electrostatic interactions and hydrogen bonds, and, occasionally, weak hydrophobic interactions in order to break the interactions between the extrinsic proteins and the membrane [62]. Johnson et al. [63] were able to extract an adenylate cyclase-activating factor from bovine sperm by treating the sperm with various salts such as NH4HCO3, NaCl, and Na acetate. Combining vectorial labeling and 2-D gel electrophoresis, Shetty et al. [20] identified several surface labeled, peripheral membrane proteins and obtained a novel peptide sequence by mass spectrometry using mild treatment with NaCl (1 M).

2.2.4 Subcelluar Fractionation of the Sperm

Detergent Resistant Membranes Domains (Lipid Rafts) Lipid rafts are plasma membrane microdomains which are defined as small, heterogeneous, highly dynamic regions that serve to compartmentalize cellular processes [64]. A multiplicity of cellular functions has been associated with these lipid microdomains, such as membrane trafficking, cellular signal transduction, viral entry, and fertilization [65]. The lipid content contributes to the hydrophobic nature of raft domains and leads to two inherent biochemical properties: insolubility at 4 °C in Triton X-100 (TX100) detergent, and light buoyant density after centrifugation in a sucrose density gradient. These properties are used to isolate detergent-resistant membrane (DRM) as a biochemical correlate of lipid rafts [66].

Initial evidence for raft formation in male germ cells came from identification of the raft protein, caveolin-1, in the head and flagellum of mouse and guinea pig sperm, implicating these structures in the regulation of both motility and sperm–zona interaction [67]. In order to determine alteration of the protein composition in DRMs following capacitation, Sleight et al. [68] performed a proteomic analysis of mouse sperm proteins isolated in the light buoyant-density fraction. The immunoglobulin superfamily protein Izumo, a well-known sperm-egg fusion protein [69], was also one of the predominant protein enriched in the preparation. Thaler et al. [70] made a similar kind of study and reported that several individual proteins became enriched or depleted in DRM fractions following capacitation. Studies done on the pig [71] and boar [72] support the hypothesis that capacitation induced increased levels of sperm DRMs, with an enhanced zona pellucida affinity. Nixon et al. [73] have shown that DRMs isolated from spermatozoa possessed the ability to selectively bind to the zona pellucida of unfertilized, but not fertilized, mouse oocytes. Watanabe and Kondoh [74] recently demonstrated that mouse sperm undergo GPI-anchored protein release associated with lipid raft reorganization and acrosome reaction to acquire fertility. Collectively, these data provide compelling evidence that mouse spermatozoa possess membrane microdomains that provide a platform for the assembly of key recognition molecules on the sperm surface.

An alternative method for the enrichment and isolation of sperm membrane proteins involves preparation of membranes through hypoosmotic swelling, homogenization, and sonication [75]. Membranes are further isolated by differential centrifugation steps. Purified human sperm membrane proteins can be separated by 2-D gel electrophoresis and further analysis of the sperm antigens can be achieved. Oko's group [76] has devised a methodology to obtain a sperm head fraction consisting solely of the inner acrosomal membrane (IAM) bound to the detergent-resistant perinuclear theca. On the exposed IAM surface of this fraction, they define an electron dense protein layer that was termed IAM extracellular coat (IAMC). Their approach has led to the identification of a novel inner acrosomal protein IAM38 from bovine sperm with a demonstrated role in sperm egg interaction along with the matrix metallo-proteinase2 (MMP2) and acrosin in the inner acrosomal membrane [76, 77]. Amaral et al. [12] focused on the sperm tail proteins, as the role of the sperm flagellum is specific and very distinct from the role of the sperm head.

Isolation of the sperm tails was performed by sonication followed by ultracentrifugation in a sucrose gradient; the proteins were then solubilized in lysis buffer that was compatible with 2DE.

2.2.5 Use of Polyclonal and Monoclonal Antibodies

Polyclonal or monoclonal antibodies may also be used for the identification, isolation, and characterization of sperm antigens that are relevant to fertility. A number of sperm antigens have been identified by means of monoclonal antibodies [59, 69, 78–86]. Among the most significant findings was the discovery of the immunoglobulin superfamily protein Izumo1, by using monoclonal antibody OBF13, which interfered with sperm-egg interaction [69]. Izumo1 was shown to localize within the acrosome and to have a definitive role in sperm-egg fusion during fertilization. Izumo1 was identified by separation of the crude extracts from mouse sperm by 2-D gel electrophoresis and subsequent immunoblotting with the monoclonal antibody. Recent studies by Clark and Naz [87] have shown that significant percentage of immunoinfertile female sera have circulating isoantibodies against this protein with none of the fertile women's sera showing any reactivity, suggesting a possible application of Izumo1 in diagnosis and treatment of infertility, and human contraceptive vaccine development.

2.2.6 Identification of GPI Anchored Proteins

In mammals, more than 200 cell surface proteins with various functions, such as hydroxylation, cellular adhesion, and receptor activity, are anchored to the membrane by a covalently attached GPI moiety [88–90]. GPI deficiency causes developmental abnormalities, failure of skin barrier formation, and female infertility in mice indicating that GPI anchor is essential for cell integrity [91, 92].

A few GPI anchored proteins discovered from sperm are CD59, CD52, TESP5, TEX101, Ly6K, PH20 [hyaluronidase], and SAMP14. PH-20 is proposed to have multiple functions involved in cell signaling and serve as a receptor for the zona pellucida, in addition to its hyaluronidase activity [93]. The discovery that a testicular isoform of angiotensin converting enzyme (ACE) is a GPI-anchored protein-releasing factor which is crucial for fertilization [94] has shown the importance of GPI-anchored proteins in the fertilization process. It is known that TEX101 has to be shed and to disappear from testicular germ cells by the GPI-anchored protein-releasing activity of ACE for the correct localization of ADAM3 on the mature sperm surface. LY6K a recently discovered GPI-anchored protein interacted with TEX101 and ADAM3 in the testicular germ cells but disappeared from mature spermatozoa and is believed to be a new factor involved in sperm fertilizing ability [95].

A standard method to isolate GPI-anchored molecule from the cell surface is treatment of the cells with GPI-specific phospholipase C (PIPLC) that cleaves GPI anchors specifically, leaving the lipid moiety in the membrane and releasing the protein with a terminal cyclic phosphoinositol [96]. Even though there is no single

report on the identification of GPI anchored molecules from the sperm surface on a global scale, an experiment carried out on oocytes by Coonrod et al. [97] investigated human sperm-hamster oocyte interactions and determined that PI-PLC cleavable glycosylphosphatidylinositol (GPI)-anchored proteins are involved in sperm-egg binding and fusion. Two-dimensional electrophoresis was then utilized to visualize proteins released from hamster oocytes following PI-PLC treatment. The authors demonstrated that treatment of hamster oocytes with PI-PLC inhibits sperm-egg interaction and releases a 25–40 kDa protein cluster (pI 5–6) from the oolemma suggesting that this released protein cluster represents an oolemmal GPI-linked surface protein(s) which is involved in human sperm-hamster egg interaction. A comprehensive search for all the GPI-anchored molecules in spermatozoa and oocyte by proteomic analysis may be needed to identify the full repertoire of molecules that are directly involved in fertilization.

2.2.7 Two-Dimensional Differential In-Gel Electrophoresis (2D-DIGE)

The recent development of 2D-DIGE [98] is beginning to have an impact on the field of reproductive immunology assisting in the identification and characterization of potential fertility related proteins. This technology is based on the creation of a family of size and charge-matched spectrally resolvable dyes that are used to label different protein preparations prior to 2-D gel electrophoresis, allowing up to three distinct protein mixtures to be separated within a single 2-D PAGE gel. By running such differentially labeled protein mixtures on the same gel, between-gel differences in electrophoretic migration patterns can be entirely eliminated. Baker et al. [99] used this technique to isolate and characterize those proteins that undergo processing in rat spermatozoa as they transit the epididymal tract. The technique can be effectively applied to determine the post-translational modifications of the sperm at various functional states (non-capacitated, capacitated and acrosome reacted) and also to identify fertility related proteins by using sperm proteins from healthy fertile specimens versus infertile specimens. Hamada et al. [100] utilized the technique to identify the relative abundance of proteins in pooled reactive oxygen species (ROS)–positive (ROS+) and ROS-negative (ROS−) semen samples and found significantly different expression of protective proteins against oxidative stress in ROS-compared with ROS+ samples. Since so few genes related to human infertility are now known, this method may open up the field of male infertility genetics by “back-tracking” from the proteomes of affected individuals compared to fertile controls.

2.2.8 Identification of Phosphoproteins and Glycoproteins

In addition to the proteomic methods described above, several other strategies being employed to identify interesting sperm proteins include the identification of phosphoproteins and glycoproteins. It is well established that capacitation, a prerequisite event for fertilization requires a cyclic AMP-dependent increase in tyrosine phosphorylation.

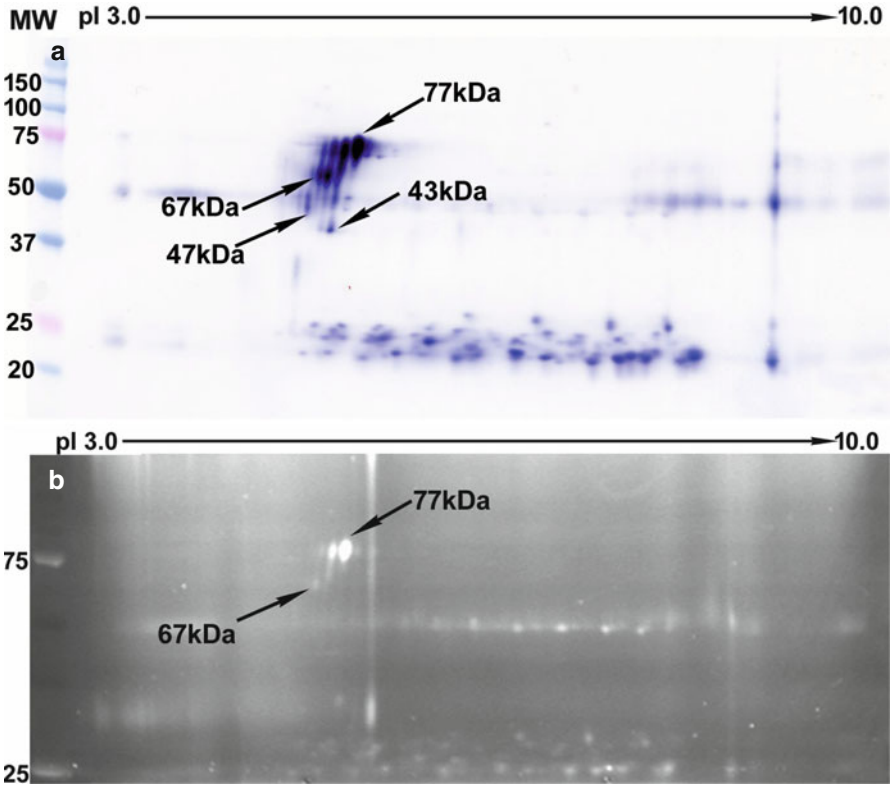

Fig. 2.3 Glycoprofile staining of immunoprecipitated mouse testicular SPESP1. (**a**) SPESP1 isoforms immunoprecipitated from mouse testis were analyzed by 2D SDS-PAGE Western blot using SPESP1 antibody revealing trains of SPESP1 charge variants at 77 and 67 kDa. (**b**) Several 77 kDa SPESP1 charge variants and one 67 kDa variant stained with glycoprofile stain showing that these forms of SPESP1 are glycosylated. Nonimmune immunoprecipitates probed with SPESP1 immune serum showed only heavy and light chains (Data not shown, see Ref. [104]) (Modified with permission from Suryavathi et al. [104])

One of the approaches employed to target phosphoproteins involved in the capacitation event is two-dimensional gel analysis coupled to anti-phosphotyrosine immunoblots and tandem mass spectrometry [101]. The molecular probe, Pro-Q® diamond phosphoprotein gel staining, is an advanced method that provides a precise strategy for selectively staining phosphoproteins in polyacrylamide gels [102]. The glycoproteins, on the other hand, may be identified by lectin blotting coupled to 2-D gel electrophoresis [41]. A commercially available fluorescent dye Lissamine rhodamine B sulfonyl hydrazine (LRSH) has been introduced recently to specifically stain the glycoproteins [103]. Suryavathi et al. [104] utilized this technique successfully to analyze the glycosylation sites on the mouse Equatorial Segment Protein 1 (SPESP1) using a glycoprotein specific fluorescent stain with the trade name Glycoprofile (Sigma). Mouse testis SPESP1 was immunoprecipitated with the SPESP1 antibody and analyzed by 2D-SDS-PAGE Western blotting and glycoprofile stain (Fig. 2.3).

2.2.9 Identification and Mining of Low Abundant Proteins

An important challenge in most of the proteomic methods described above is the identification and mining of antibody reactive, low abundant proteins. Pre-fractionation of the proteins by continuous elution preparative electrophoresis (using PrepCell from Bio-Rad) and preparative isoelectric focusing (using Rotofor from Bio-rad) are useful methods of choice for enriching these low abundant proteins.

2.2.10 Coring of Protein Spots for Microsequencing and Cloning of Novel Genes

In excising proteins from a 2-D gel, it is essential to precisely define the boundaries of individual protein spots to obtain pure proteins for microsequencing. Figure 2.4 demonstrates exceptionally useful tools for coring spots of interest from a silver-stained 2-D gel. These custom-made coring tools consists of hollow cylindrical steel tubes with specialized tips milled to a razor sharp edge. Different bore sizes range from a diameter of 0.5 to 5 mm and each coring tool holds a solid piston within the tube for expulsing gel plugs. A tube of appropriate diameter is chosen to just encompass an individual protein spot and the acrylamide plug is precisely drawn into the tube by placing the tube vertically on the center of the spot with the coring tip down and applying gentle pressure. The piston is used to extrude the gel piece into a sample tube. Use of these precision coring tools has resulted in a high incidence of unique and novel peptide sequence resulting from mass spectrometry. Such sequences may match an Expressed Sequence Tag (EST) sequence which may then be used to clone the corresponding gene by PCR. In the absence of any known sequence in the data base, the gene can be cloned by using a completely degenerate deoxyinosine-containing sense primer and adaptor primer, and performing a 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR [42].

2.3 Recent Advances in Mass Spectrometry Towards the Identification of Novel Sperm Antigens

Mass spectrometry proteomic-based technologies have proven to be powerful tools in the determination of sperm antigens and their associated post-translational modifications (PTMs). In a typical experiment, proteins are digested with enzymes and the resulting peptides separated by high performance liquid chromatography (HPLC) into a mass spectrometer over a period of 0.5–4 h depending on the complexity of the sample. This type of experiment is often referred to as LC-MS(MS). Modern mass spectrometers take high resolution spectra ($R = 15–250$ K) in both MS (molecular weight determination – MW) and MS/MS (fragmentation for identification) modes. In an experiment to identify as many proteins as possible (shotgun proteomics), the mass spectrometer is programmed to take 1 MS scan followed by 5–20 MS/MS scans and repeat this loop for the length of the HPLC gradient. These

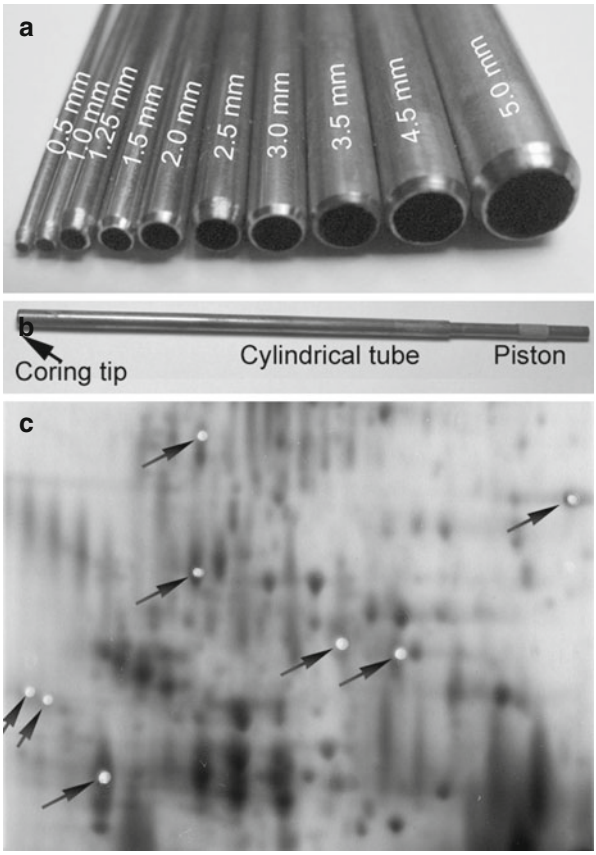

Fig. 2.4 Technique for protein spot excision from 2-D gels using finely milled coring tools of various bores. (**a**) The sharpened end of several coring tools. (**b**) A lateral view of a single coring tool showing the cylindrical tube with a solid piston used to extrude the cored gel plug from the tube. (**c**) Portion of a silver-stained 2-D gel showing various spots cored for protein digestion and microsequencing using a 1.5 mm bore coring tool

types of runs produce MW/fragment information for 5000–100,000 species in a given sample. The resulting fragment spectra (MS/MS) are searched by algorithms such as Sequest, Mascot, etc. against databases comprised of proteins predicted from the organism's genome/transcriptome. The end result is a list of peptides identified and mapped to parent proteins. Often shotgun experiments yield identifications for thousands of proteins in a sample [105]. The large amounts of data collected during a mass spectrometry run can be searched looking for differential mass addition to determine the presence and locations of PTMs. In some cases, a particular protein involved in a cellular event may be isolated to study its PTMs [104] while in others a shotgun type experiment can be performed to look at thousands of PTMs in a specific cell type [106]. Identification of proteins and PTMs gives a catalog listing of species present (qualitative analysis) but is often just the first step in determining biological activity. In most conditions or diseases, the amount of a certain protein and/or PTM and how that amount changes under different treatments (quantative analysis) is equally important. Quantitative changes can be determined relative to a standard condition using label free (ion current peak areas, spectral counts) or labeled (Stable Isotopes – SILAC, iTRAQ) approaches [107, 108]. In these cases,

the actual absolute amount is not known, only how it changes between conditions. In some cases, the absolute amount of the protein or PTMs needs to be determined between conditions. This type of quantitation is most often done by MRM (multiple reaction monitoring) with either an internal isotopically labeled standard or external standard curve [109, 110]. Using these techniques, proteins, peptides, and PTMs can be identified and quantified for any given set of samples providing fundamental data necessary for further experiments.

2.4 cDNA Library Screening

This strategy combines recombinant DNA technology with the experimental approach as described by Hjort and Griffin [111]. Typically a testis lambda expression library is used in this strategy and is plated with a chosen strain of E.coli as host bacterium. After growth at 42 °C and induction with isopropyl-b-thiogalactoside (IPTG), the nitrocellulose filters are screened with the sperm antibody of interest. Bound recombinant protein is detected by use of an isotype specific secondary antibody coupled to horse-radish peroxidase. The cDNA insert of the positive clone is utilized again to re-probe the testis lambda cDNA library to confirm the sequence of the identified clone and also to identify any additional clones.

Wright et al. [112] identified the SP-10 cDNA (ARCV1 gene) using the MHS-10 monoclonal antibody with this method. Diekman and Goldberg [113] used sera from ASA-positive infertile patients and vasectomized men to identify an antigen, designated AgX, expressed by recombinant bacteriophage in a human testis library. Later studies used similar methods to identify additional sperm/testis antigens [114, 115]. In another study, the FA-1 mAb was used for a screening of murine testis lambda gt11 cDNA expression library [116] and the novel sperm protein discovered was named FA-1 for its potential role in fertilization. A similar strategy was employed by the same group to identify two more sperm antigens NZ-1 [117] and NZ-2 [118] from mouse and human origin, respectively. Two monoclonal antibodies, designated S71 and S72 (by World Health Organization workshop on antisperm antibodies), were used to isolate their corresponding cDNA clones from a human testis λZAP cDNA library by Westbrook et al. [85]. The cloned gene was named SPAN-X for sperm protein associated with the nucleus on the X chromosome.

cDNA library screening techniques can also be used as a strategy to isolate the complete cDNA for an unknown candidate molecule when a small region of the cDNA corresponding to the gene is known (e.g. through EST data base). Shetty et al. [28] applied the technique to clone the full-lenth cDNA for SAMP14 using a PCR amplified partial cDNA (derived from EST) clone from human testis as a probe to screen a λDR2 cDNA library. A similar strategy was used earlier by Wolkowicz et al. [119] to clone the full-length cDNA for a human sperm flagellar protein tectin-B1. PCR-based cloning strategy and cDNA library screening utilizing the known sequences of potential drug targets and primary mediators of signaling network has also led to the discovery of novel testis specific targets. Testis specific serine threonine kinases (TSSK 1–6) are one such example. TSSK1 and 2, the first

two members of the family, were initially discovered using degenerate oligos corresponding to highly conserved motifs within the protein kinase catalytic domain and by cDNA library screening [120, 121]. Recent studies have validated these testis specific, post-meiotically expressed kinases as candidate male contraceptive targets [122–124].

In an attempt to identify peptide sequences that might be involved in zona pellucida (ZP) binding, Naz et al. [55] screened FliTrx random phage display library with solubilized human ZP. A novel dodecamer sequence, designated as YLP_{12}, was identified that is involved in sperm-ZP recognition/binding. A subtractive cDNA hybridization technology was employed by Naz et al. [125] to obtain sperm specific antigens that could be targeted as potential contraceptive vaccines.

Chen et al. [126] constructed phage-display peptide libraries to select epitope peptides derived from human posterior head 20 (*hPH20*) and homo sapiens sperm acrosome associated 1 (*hSPACA1*) using sera collected from infertile women harboring antisperm antibodies.

The studies led to the identification of four epitope peptides. The BSA-coupled synthetic peptides generated a sperm-specific antibody response with a contraceptive effect in both male and female mice. Samoylova et al. [127] used a modified phage display by using intact oocytes surrounded by ZP proteins in native conformation. The procedure led to the identification of an antigneic 12 aa peptide that produced antisperm antibody recognizing acrosomal region of the sperm surface.

The identification of a candidate molecule is followed by experiments to determine its localization, tissue specificity, and functional assays to elucidate its possible role in fertilization. This generally requires expression of a recombinant protein in bacteria or in a host of choice to produce sufficient protein to generate antibodies and for functional assays.

2.5 Summary

An integration of several methods of protein isolation, enrichment, and identification provide powerful tools to dissect the molecular architecture of spermatozoa. For example, combining vectorial labeling with phase partitioning and immunoblotting has the potential to enrich for and readily identify a subset of hydrophobic membrane associated auto-antigens. Figure 2.5 depicts an overview of the different methods that can be employed to identify and characterize sperm antigens that are functionally relevant for fertilization and events leading to fertilization. Even though 2-D electrophoresis technology has its limitations, it still remains a stalwart approach when coupled with mass spectrometry for the methodical characterization of proteomes. Currently one of the major challenges in identifying the functionally relevant sperm antigens is to devise methodologies to isolate and enrich the molecules from subdomains of the sperm involved in the fertilization event, so that a careful analysis of all the molecules including the low abundant ones can be made with multidimensional separation methods. Advances in bioinformatics are likely to assist researchers in understanding the vast amount of data collected in proteomic studies.

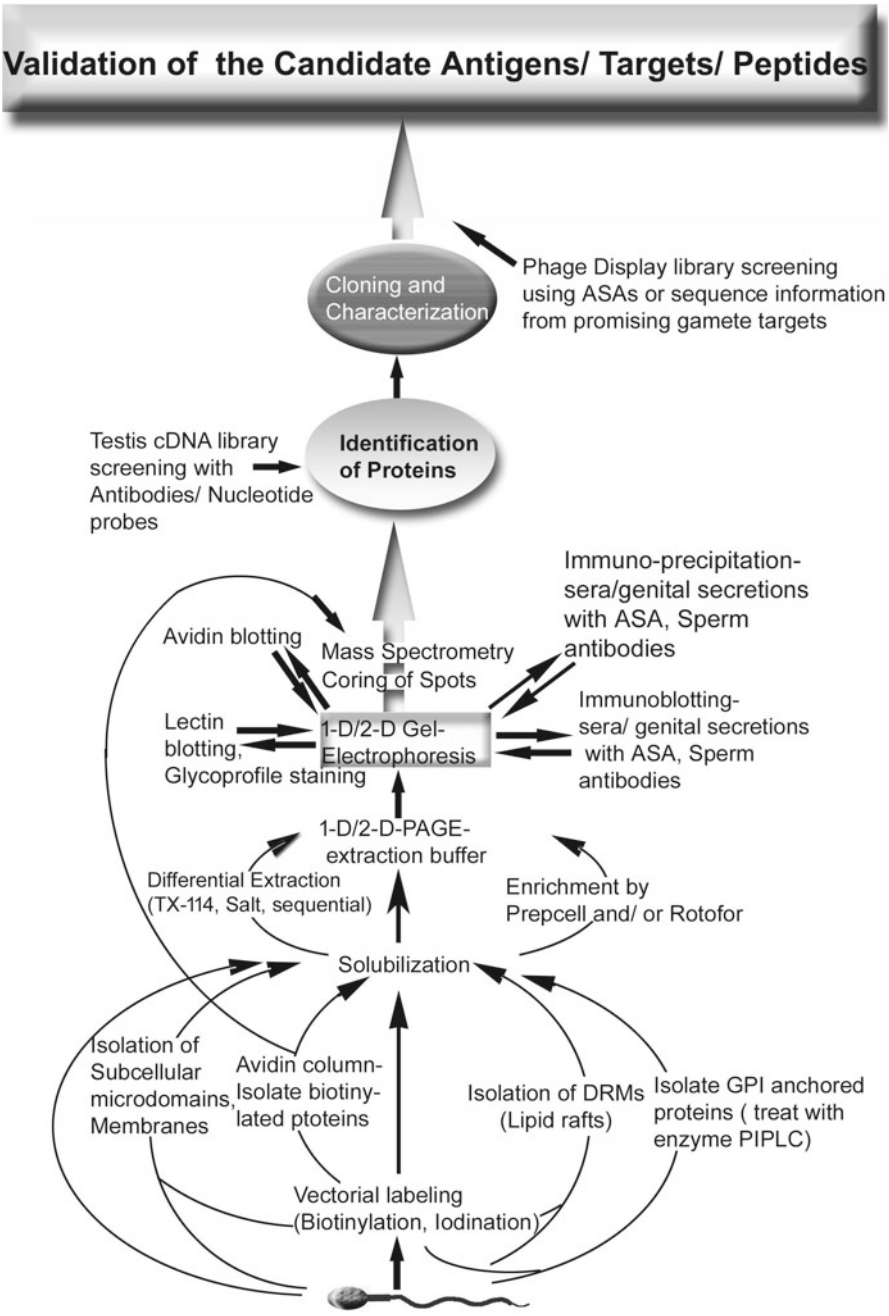

Fig. 2.5 An outline of molecular approaches applied to the sperm cell that are useful in the identification of sperm antigens involved in fertilization

The use of naturally occurring antisperm antibodies remains an attractive approach for the identification of immunoreactive antigens for immunocontraceptive purposes and for defining the auto-antigens associated with immuno-infertility. We predict that more sperm autoantigens will be defined by the methods discussed above as the full protein repertoire of mankind's immune diversity is probed. We envision a future time when 2-D immunoblots and databases of 2-D gels may become a standard clinical assay for the differential diagnosis of immuno-infertility. Protein arrays containing the major immunodominant proteins recognized by the human immune response may in the future offer simple diagnostic tests for determining patients with antibodies to sperm surface antigens essential for fertilization.

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Sperm Surface Proteomics

3

Ian A. Brewis and Barend M. Gadella

Abstract

This contribution will focus exclusively on the total (global) protein composition (the proteome) of the sperm surface. Immune responses directed towards sperm surface proteins may cause infertility since functionally intact sperm are under immune attack. The immune attack can be achieved directly by deteriorating sperm or by antibody blocking of a sperm surface protein with a specific function in the fertilization process. Antibodies that bind to the sperm surface proteins could also impair the fertilization potential of sperm more indirectly by causing lateral redistribution of the sperm surface proteins and/or by hindrance of the assemblage of functional membrane protein complexes involved in fertilization. Currently the information about the sperm plasma membrane proteome is increasing but has only led to limited understanding of the functionality that is related to the complex ordering and processing of this specific cell surface. New proteomic data and new strategies designed for complete coverage of the surface proteome of mammalian sperm will significantly increase our understanding of how fertilization is accomplished but also how immune responses may frustrate this process. This information will become highly relevant for studying immune infertility. An overview is provided about the current knowledge of the sperm

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surface and how this structure should experimentally be approached for proteomic studies. Comparative analysis of different mammalian species is covered as this will provide better understanding for the possibilities and limitations of analyzing the surface proteome of human sperm.

3.1 Introduction

3.1.1 The Sperm Surface

The sperm is a highly polarized cell with a minimum of cytosol and organelles [54, 157]. The sperm head has two organelles namely the nucleus that houses the male haploid genome which is highly condensed to protamines, and a large secretory granule called the acrosome which is oriented over the anterior area of the sperm nucleus. At the distal part of the sperm head, the flagellum sprouts. In the mid-piece of this flagellum, mitochondria are spiraled around the microtubules of the flagellum. In the tail part, specific cytoskeletal elements are surrounding the microtubules of the flagellum. The surface of the sperm head, mid-piece, and the tail parts of the sperm is heterogeneous [66, 118] and reflects the polar distributed organelles that lie under the surface. The sperm head surface is particularly heterogeneous, and at least four different regions can be distinguished with separate functions in the fertilization process. In general the sperm has lost many somatic cell features and does not house an endoplasmic reticulum, Golgi, lysosomes, or peroxisomes and has lost ribosomes. Primarily due to this, the sperm has lost the potential for gene expression (both transcription as well as translation processes are completely silenced [27]). In fact due to this the sperm has lost the de novo protein synthesis capacity as well as vesicle endocytosis/exocytosis-mediated cycling of the sperm surface. Related to this, the endogenous sperm surface proteins (that is, those not added postspermiation) are prone to very complex posttranslational modifications, which normally would be eliminated and are a challenge for proteomic detection [10]. The sperm has also lost almost the entire cytoplasm. The cell has a typical ordering of the remaining organelles and cytoskeletal elements, and probably this polar ordering is reflected into the lateral domained ordering of the sperm surface [67].

3.1.2 Function of Sperm Membrane Domains at Fertilization

The subdomains of the sperm head area have particularly diversified functions in the series of processes that are involved in fertilization. The apical ridge area of the sperm head specifically recognizes and binds to the zona pellucida (the extracellular matrix of the oocyte) [151], and a larger area of the sperm head surface (the preequatorial domain) is involved in the acrosome reaction, which results in the release of acrosome components required for zona penetration [58, 157]. The equatorial segment of the sperm head remains intact after the acrosome reaction and is the

specific area that recognizes and fuses with the oolemma (the egg plasma membrane) in order to fertilize the oocyte [154]. Although the mid-piece and tail surface of the sperm cell are also heterogeneous, the function of these surface specializations is not yet understood [82]. It is quite possible that they are involved in organization of optimal sperm motility characteristics. When studying sperm surface proteins, the researcher has to keep in mind that a rather complex surface is under study, and especially for studying the processes of fertilization in which the sperm head surface is involved (zona binding, acrosome reaction, and fertilization), this specific surface area needs first to be separated and purified.

3.1.3 Sperm Surface Dynamics Before Fertilization

The domained surface of sperm is already apparent in spermatids before spermiation in the seminiferous tubules of the testis [54]. The molecular dynamics involved in the establishment of surface specialization upon spermatogenesis is largely unknown. Moreover, once liberated in the lumen of the seminiferous tubule, the sperm will start its travel through the male and female genital tract and will meet a sequence of different environments. During this voyage, surface remodeling takes place most likely at any site of the two genital tracts: (1) upon somatic maturation in the epididymis major changes in the sperm proteome are reported [2, 8, 11, 69, 92, 132], (2) by re- and decoating events induced by the accessory fluids combined at ejaculation probably stabilizing the sperm for its further journey in the female genital tract [74, 75, 77, 95], (3) after their deposition in the female genital tract which is followed with the removal of extracellular glycoprotein coating (release of decapacitation factors) and further remodeling by (cervical) uterine and oviduct secretions are activating the sperm to meet the oocyte (in vivo capacitation) [72, 87, 104, 123, 138, 161, 162], and (4) sperm also interacts with cumulus cells and remaining follicular fluid components surrounding and impregnating the zona pellucida [71, 73] and even in the perivitelline space (that is, the fluid filled space between the zona pellucida and the oolemma) with components [21, 22]. All these changing environments may cause surface remodeling to the sperm and thus may influence its potential to fertilize the oocyte [43].

The possible mechanisms of altering the sperm surface are reviewed earlier [62] and are schematically drawn in Fig. 3.1. Note that recently proteomic studies have elucidated the protein composition of extracellular vesicles/exosomes from diverse origin such as in the male genital tract secreted by the epididymis and prostate [53, 70, 139] and female genital tract secreted by the uterus, the oviduct, or even the oocyte perivitelline space [3, 21, 22, 37, 106]. It has been demonstrated that at least epididymosomes, but probably also the other extracellular vesicles/exosomes can deliver certain proteins to the sperm surface. Therefore, the exact role of extracellular vesicles/exosomes in sperm surface physiology will become more and more relevant. Beyond this it is likely that the redox balance at both the extracellular and the intracellular side of the sperm surface is also subjected to changes which will cause thiol changes, which may relate to altered folding and even complexing of proteins [10, 11, 34].

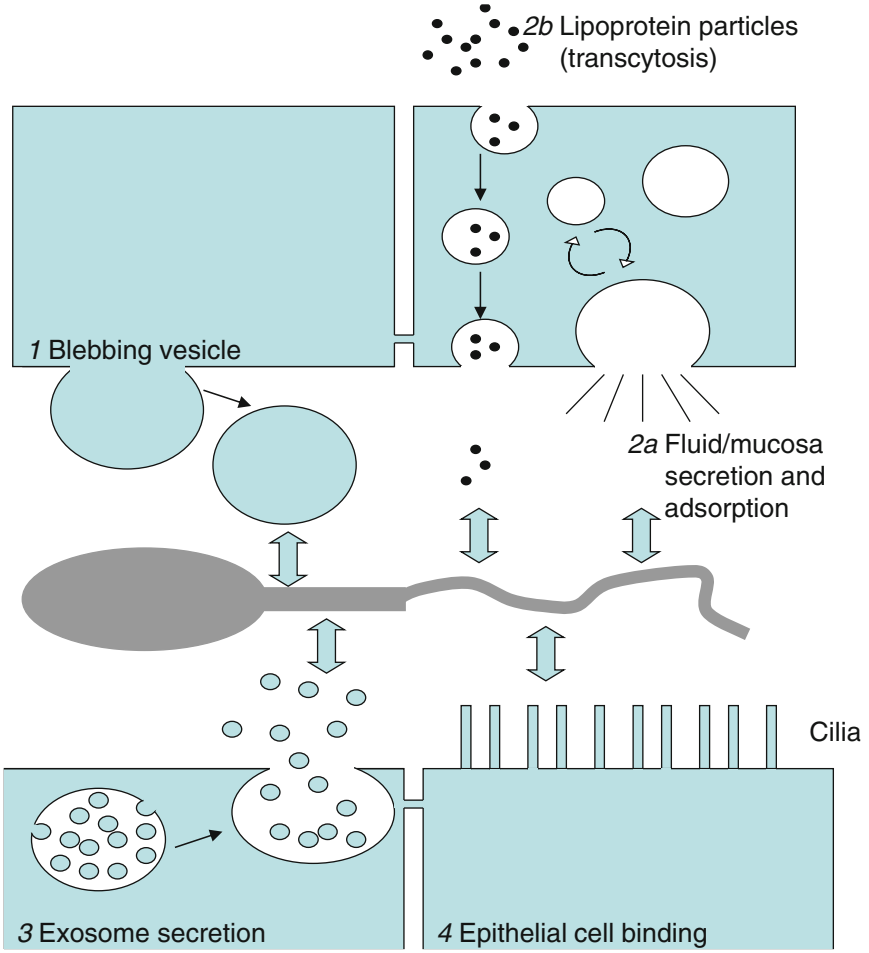


Fig. 3.1 Possible alterations at the surface of sperm due to somatic modifications in the lumen of the male and female genital tract. Possible interactions of male and female genital tract components with the sperm surface. (*1*) From the diverse epithelia of the male and female genital tract blebbing vesicles containing cytosol may be released into the genital fluids. Such vesicles may interact and exchange surface components with sperm. It is highly unlikely that such vesicles fuse with the sperm as this would dramatically increase the volume of sperm (which has been reduced to a minimum in order to obtain an ergonomically designed cell optimally suited for fertilization). (*2a*) Serum components can be released into the genital fluids by transcytosis [45]. Interestingly lipoprotein particles may invade the surrounding of sperm and may facilitate exchange of larger particles and the sperm surface. (*2b*) Fluid phase secretion and adsorption of either fluid or mucosa may directly alter the ECM of sperm. (*3*) Apocrine secretion of exosomes has been suggested to alter the sperm surface and sperm functioning. Exosomes have been demonstrated to be secreted by the epididymis (epididymosomes) and by the prostate (prostasomes) [72, 144] but likely are also secreted throughout the female genital tract. Interestingly exosomes may provide sperm with tetraspanins which are a group of membrane proteins involved in tethering of proteins into protein complexes. Recently the addition of CD9 onto the sperm surface by membrane particles has been described to occur even when sperm reaches the perivitelline space [21, 22]. (*4*) Sperm interacts with ciliated epithelial cells, which has been observed in the oviduct [135] and probably has a physiological role during in situ capacitation. Sperm interactions with other ciliated epithelial cells of the female and male genital tract have not been studied extensively. It is possible that such interactions are important for sperm surface remodeling and for sperm physiology (Adapted from Gadella [62])

It is very difficult to study the above-described sperm surface alterations in situ. However, for many mammalian species, including human, specific sperm handling and incubation media have been optimized for efficient in vitro fertilization purposes. In general mammalian sperm are activated in a medium that compares with the oviduct in that it contains the capacitation factors such as high concentrations of bicarbonate, free calcium ions, and lipoproteins such as albumin [58]. In some species specific glycoconjugates [105] or phosphodiesterase inhibitors are added for extra sperm activation [19]. All strategies are designed to evoke capacitation in vitro. This implies that the researcher can observe the relevant sperm surface reorganization primed under in vitro conditions for fertilization. The membrane composition as well as ordering of membrane components can be compared with control conditions (media without the capacitation factors) or with the membrane ordering of sperm at collection time. Sperm can be collected at ejaculation for human, boar, stallion, bull sperm but needs to be aspirated from the cauda epididymis for murine species (rat, mouse, guinea pig), which can also be the case under certain clinical conditions from male subfertile patients in the IVF clinic. In particular the surface reordering of membrane proteins and lipids in sperm head has been studied extensively under in vitro capacitation conditions (for reviews see [58, 64, 68, 95, 96]). It is important to stress the importance of the sperm surface reordering and changes in composition of membrane components by diverse extracellular factors. The induced lateral redistribution of membrane components appears to also be instrumental for the assembly of a functional sperm protein complex involved in sperm-zona binding as well as for the zona-induction of the acrosome reaction [1, 146–148, 151]. Therefore, the researcher interested in the surface proteome of sperm needs, beyond the composition of sperm surface proteins, to consider how these proteins are organized and whether they are functionally complexed for their physiological role in fertilization. In this light, it is also important to stress that sperm surface protein reordering can be imposed by processing semen for instance during density gradient washing, cryopreservation, or sex-sorting in a flow cytometer [63, 95, 153].

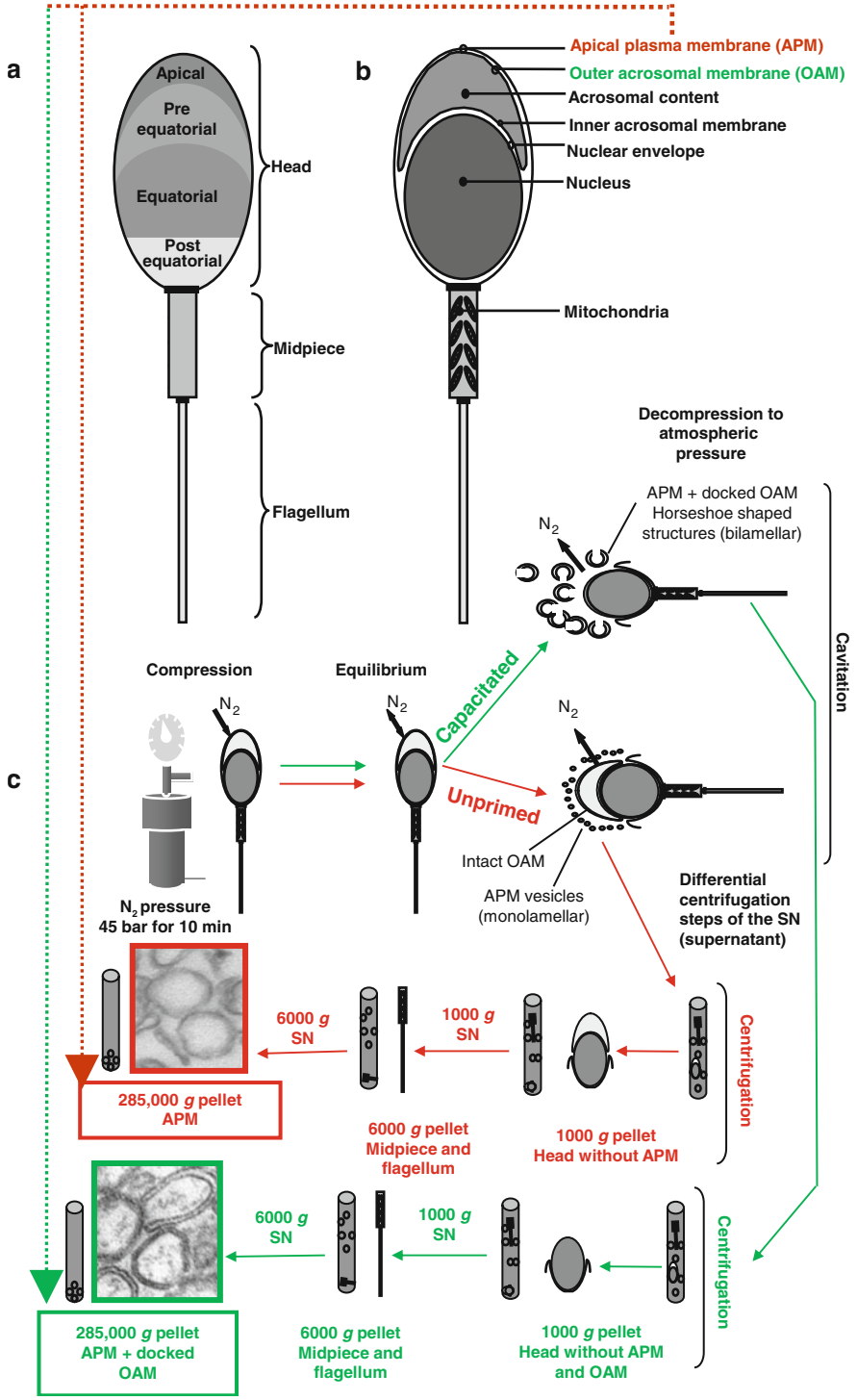
3.2 Isolation of Sperm Surface Proteins

Membrane proteins can be classified as integral membrane proteins and peripheral proteins. Most integral membrane proteins have an extracellular domain and a transmembrane domain (often an alpha helix region with the hydrophobic part exposed to the fatty acid moieties of the phospholipid bilayer). However, other integral membrane proteins interact by covalent lipid anchors such as glycosylphosphatidylinositol (GPI), acylation, and other modifications [52]. Peripheral proteins have electrostatic interactions with the integral membrane proteins or with the lipids of the membrane. Discrimination between these two types of membrane proteins can be done by treating membrane preparations with high salt which destabilizes the electrostatic interaction and results in the release of peripheral membrane proteins, while the integral membrane proteins remain in the insoluble membrane fraction. In general, to study the sperm surface proteins, one has to isolate the sperm membrane

from soluble proteins and insoluble nonmembrane material (such as cytoskeletal components and the condensed nuclear chromatin). Furthermore, researchers need to give particular attention to the indirect interactions of nonsurface material to the membrane extract.

To this end, specific sperm disruption methods such as ultrasonication and nitrogen cavitation (see Fig. 3.2) have been designed [59]. Sonication gives lower purification and less defined membrane fraction [17] although good results were obtained on bovine sperm [38]. After sperm disruption, differential centrifugation techniques need to be employed to isolate sperm membranes from insoluble cellular debris and soluble components. The researcher needs to consider that the disruption method as well as the isolation protocol is really delivering sperm plasma membrane or also intracellular membranes. This is especially relevant for studying proteins involved in zona recognition. When the plasma membrane preparation also contains acrosomal contamination, one can be sure that secondary (intraacrosomal) zona binding proteins will be identified and will possibly overwhelm the amount of primary (plasma membrane) zona binding proteins [60, 151]. To this end, the specific abundance of marker proteins or specific activities of marker enzymes of plasma membrane and intracellular membranes need to be quantified. The relative purification is indicative for the purity of the membrane fraction for surface proteins. In our hands, an optimized nitrogen cavitation method turned out to yield a 200 times enriched plasma membrane fraction over possible contaminating membranes with a yield of approximately 30 % of the sperm surface [59]. Moreover, ultrastructural analysis of this membrane fraction and of disrupted sperm showed that the isolated plasma membrane fraction contained resealed plasma membrane vesicles. The vesicles were so-called right-side outside unilamellar vesicles (see Fig. 3.2) implicating that the outer and inner side of the vesicle membranes had the same protein topology as in the intact plasma membrane of sperm and that the resealed plasma membrane vesicles have not encapsulated intracellular membranes.

Fig. 3.2 Subcellular fractionation of apical plasma membranes from boar sperm cells (Adapted from Brewis and Gadella [24]). (**a**) A schematic of the surface of the sperm cell showing the main subdomains in the head. The apical ridge specifically recognizes and binds to the zona pellucida, and a larger area of the preequatorial region is involved in the acrosome reaction. The equatorial segment of the sperm head remains intact after the acrosome reaction and is the specific area that recognizes and fuses with the oolemma in order to fertilize the oocyte. (**b**) A sectional view of the sperm cell. Note that all solid lines represent membrane bilayers. (**c**) Procedure to isolate apical plasma membranes (*APM*) from unprimed boar sperm cells (*red arrows*) using nitrogen cavitation and differential centrifugation. This results in a 200 times enriched apical plasma membrane fraction with the outer acrosomal membrane (*OAM*) remaining intact and represents an exceptional resource for further understanding zona binding and the acrosome reaction. Note that boar sperm capacitation (see *green arrows*) leads to acrosome docking which as a result leads to the isolation of APM and the outer acrosomal membrane (*OAM*) (see also [147]). As mentioned in the text, this phenomenon should be carefully considered as an artifact when interpreting proteomics data on sperm surface proteins of capacitated versus noncapacitated sperm samples)


a
Apical
Pre equatorial
Equatorial
Post equatorial
Head
Midpiece
Flagellum
b
Apical plasma membrane (APM)
Outer acrosomal membrane (OAM)
Acrosomal content
Inner acrosomal membrane
Nuclear envelope
Nucleus
Mitochondria
c
Decompression to atmospheric pressure
APM + docked OAM
Horseshoe shaped structures (bilamellar)
N2
Cavitation
Compression
Equilibrium
Capacitated
Unprimed
Intact OAM
APM vesicles (monolamellar)
N2 pressure
45 bar for 10 min
Differential centrifugation steps of the SN (supernatant)
6000 g SN
1000 g SN
Centrifugation
285,000 g pellet APM
6000 g pellet Midpiece and flagellum
1000 g pellet Head without APM
285,000 g pellet APM + docked OAM
6000 g pellet Midpiece and flagellum
1000 g pellet Head without APM and OAM

This membrane preparation turned out to be instrumental to study protein-protein interactions relevant for sperm-zona binding [151] and for the redistribution of membrane microdomains believed to represent lipid rafts [152]. Interestingly, multiple proteins known from the literature as being zona binding protein candidates were identified by proteomics on the isolated boar apical plasma membrane preparations [151] which were extremely enriched in the DRM fraction of sperm. Most notable were fertilin beta, P47, carbonyl reductase, and the sperm adhesion AQN3. Interestingly, proteomics revealed a possible role of chaperone proteins in formation of functional protein complexes involved in zona binding [35, 36, 122], which will be discussed later. The folding and grouping of sperm surface proteins is relevant for the observed capacitation-induced redistribution of sperm surface proteins. This phenomenon also allows the plasma membrane to firmly dock to the outer acrosomal membrane exactly at the area where the characteristic lipid ordered microdomains were clustering. A trans trimeric SNARE complex (containing VAMP, syntaxin, and SNAP proteins) was formed at multiple sites of the apical sperm surface and was stabilized in the trans configuration by complexin [148]. In fact the isolation of the vesicles that were formed after a calcium-ionophore-induced acrosome reaction showed the declipping of complexin and the trans- to cis-configuration of the trimeric SNARE protein complex which coincided with the hybird vesicles formed after multipoint fusions between the apical plasma membrane and the docked outer acrosomal membrane [148].

Note that the isolated hybrid vesicles are an interesting source for proteomic analysis with a number of interesting proteins identified such as synaptotagmin-4 involved in cis-configuration of the trimeric SNARE complex [148] but also of a number of acrosome-specific and surface-specific proteins involved in zona binding zona penetration. The identified proteins that were not observed in the apical plasma membrane preparations derived from noncapacitated sperm cells are noteworthy. On membranes isolated from capacitated sperm, the emergence of spermatid-specific heat shock protein 70 and arylsulfatase A as well as the acrosomal proteins acrosin, acrosin inhibitor, acrosomal vesicle protein 1, IAM 38, SP10 was reported [148], which may all derive from the acrosomal membrane. Thus, the possibility that membrane preparations from capacitated or acrosome reacted sperm samples contain larger proportions of acrosomal membrane proteins should be checked with robust ultrastructural techniques such as those shown in Fig. 3.2. In general we believe it is of crucial importance to take care on interpretations of changes in the capacitation sperm surface as isolation nitrogen cavitation (Fig. 3.2) or detergent resistance membrane fractions (Fig. 3.3) or any other membrane isolation method will likely result in a more substantial co-isolation of the docked outer acrosomal membrane [147, 148, 150]. In this light manuscripts, describing the emergence of proteins at the capacitating sperm surface [12, 35, 36, 48, 88, 110, 122, 143, 147, 160] should be interpreted with care.

Human sperm surface preparations are usually made after a hypo-osmotic incubation followed by sonication and differential centrifugation (see for instance [30]). The purity of such membrane preparations for sperm plasma membrane material is not well documented, and contamination with intracellular membranes is likely.

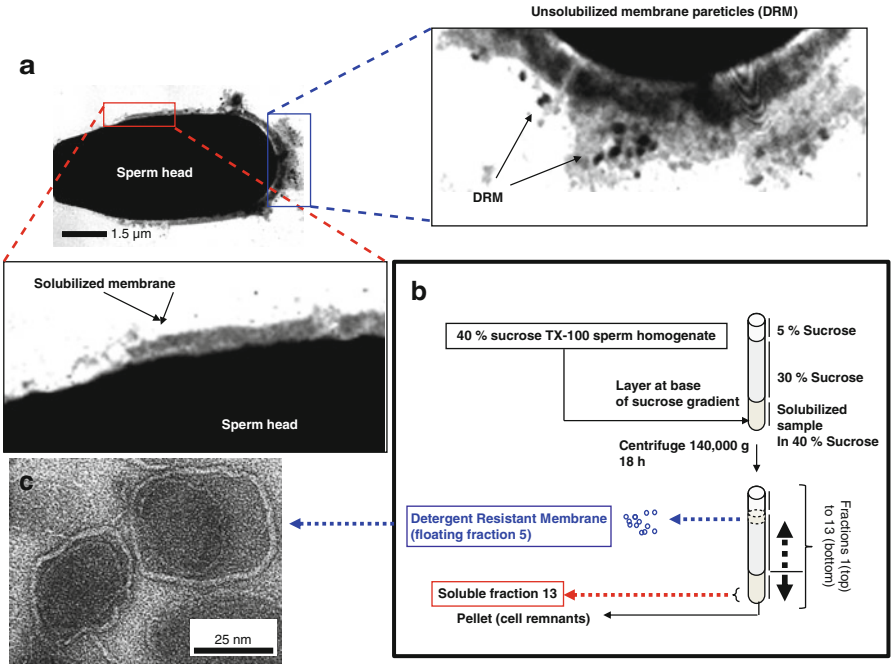


Fig. 3.3 Procedure to isolate the detergent-resistant membrane fraction (DRM) from porcine sperm cells. (**a**) The *upper left panel* shows the sperm cells head imaged by transmission electron microscopy. The *boxes* in this panel refer to either solubilized membrane (*lower zoom panel*) or nonsolubilized membrane (*upper right panel*). (**b**) Separation of the solubilized or the DRM fraction is achieved by layering the solubilized sperm sample in Triton X-100 in a final concentration of 40 % sucrose; on top of this a layer of 30 % sucrose is added and the last layer consists of 5 % sucrose. After centrifugation for 18 h at 140,000 g, the DRM is present in the interface between 5 and 30 % sucrose (Adapted and extended from introduction chapter of the PhD thesis of Dr. A. Boerke [26]). (**c**) Note that it is likely that due to stable acrosome docking the DRMs isolated from capacitated sperm contain more acrosomal material when compared to DRMs isolated from unprimed sperm samples. In fact a negative staining micrograph of a DRM fraction 5 isolated from capacitated sperm cells shows beyond the multilamellar outside membrane (cf. what was observed for DRMs isolated from unprimed sperm and on DRMs derived from two epithelial cell lines [150]) also the inclusion of an additional unilamellar membrane

Note also that in contrast to, for instance, porcine semen (typically with > 95 % life and fully matured normal morphology and motility spermatozoa), human semen is of much poorer quality with a large number of defective and contaminating cells. The high sperm surface purification by a factor in the hundreds is not to be expected for human semen samples under any conditions.

Another method to isolate surface proteins is to make use of lectins immobilized to beads. Lectins can bind to specific sugar residues at the extracellular domain of integral membrane proteins. Some marker lectins exclusively bind to the sperm plasma membrane. Therefore, affinity chromatography using immobilized lectins can be used to extract surface proteins [124]. A comprehensive profiling of

accessible sperm surface glycans using a lectin microarray has been described recently [156]. These methods can also be employed on nitrogen cavitated and solubilized sperm plasma membranes. Noteworthy is also the phenomenon of (de-)glycosylation which takes place on sperm surface proteins during sperm maturation ejaculatory transport and in the female genital tract [42].

Finally membrane raft isolation procedures can be employed to isolate microdomains from sperm (see Fig. 3.3 [6, 28]). Most methods use detergents at low temperature (4 °C) to isolate the detergent-resistant membrane fraction. Our group has identified that this DRM fraction after capacitation becomes highly enriched in GPI anchored proteins and in proteins involved in zona binding and the acrosome reaction [79, 146, 149]. With the use of phosphatidylinositol-specific phospholipase C GPI anchored proteins can be cleaved of the DRM (enriched in these proteins) or in untreated sperm [29, 79]. Possibly the treatment of sperm with such lipases may result in the liberation of a very specific subclass of integral sperm plasma membrane proteins, and clearly such proteins play an important role in capacitation-specific membrane surface alterations related to sperm-zona binding [151, 152] as well as the induction of the acrosome reaction [146–148, 158]. We have data that DRM from entire sperm contains intercellular (acrosomal) membrane material beyond the surface membrane material [26, 29, 63, 148]. The DRM fraction of whole sperm contains components that could be labeled with marker lectins for the outer acrosomal membrane. DRMs from purified plasma membranes did not show any labeling with this lectin. The best explanation for these results is that the outer acrosomal membrane also contains lipid rafts, which may explain the results of [35, 36, 111, 114] or that this membrane is stably docked to the capacitating raft aggregating sperm surface as discussed earlier [147]. Ultrastructural studies on the DRM fraction indeed showed that this insoluble membrane fraction appears as multilamellar membrane vesicles [150] and that DRMs derived from capacitated sperm show additional mono-lamellar membrane inclusions (see Fig. 3.3c).

As stipulated above, in general we advise any researcher working on sperm surface-specific proteins to have appropriate ultrastructural controls regardless of what type of membrane isolation technique is used. This will enable the exclusion of intracellular membranes especially when working on surface protein changes that may occur during sperm capacitation as this coincides with multiple synaptic docking of the outer acrosomal membrane (see also Fig. 3.2).

3.3 Detection of Sperm Surface Proteins

3.3.1 Tagging of Sperm Proteins and Peptides

Other chapters in this book describe a number of protein separation and mass spectrometric techniques mentioned that are key or of relevance for detecting and identifying amino acid sequences of peptides and proteins of sperm samples [101, 127]. The most popular present day proteomics approaches are summarized in Fig. 3.4,

and these and other approaches are reviewed more extensively [23]. Here, we will focus on strategies for the proteomics analysis of surface sperm-specific proteins. First of all it is important to clarify that a number of proteomics protocols studying differential expression of proteins in biological specimens under experimentally manipulated conditions are not possible with sperm. Specifically those techniques that make use of the fact that cells are fed with amino acids that are used for translation are not possible in sperm as sperm are transcriptionally and translationally silent (the translational machinery has shut down in the last phase of spermatogenesis) [27]. The most common approach involves control cells cultured with normal amino acids, while the experimental conditioned cells are fed with stable isotope label tags (SILAC; stable isotope labeling by amino acids in culture) and uses labeled hydrogen, carbon, or nitrogen in a number of amino acids [23]. Most of these techniques can also be used to detect translational capacities of cell extracts in vitro.

The lack of transcription and translation in sperm implies that variations in surface protein composition are either due to the changing environments the sperm faces en route to fertilizing the oocyte (Sect. 3.1.3) or due to aberrations in the sperm formation process in the testis. While approaches such as SILAC are not feasible in sperm, a number of surface labeling techniques have been used for proteomics analysis of sperm surface proteins. In human sperm, for instance, ^{125}I labeling of sperm surface proteins or biotinylation of surface proteins has been employed to detect immunodominant sperm surface antigens [128, 130]. This method turned out to be not completely "membrane proof" as some intracellular proteins were also iodinated.

Beyond SILAC there are a range of labeling approaches in general use for quantification in proteomics workflows. iTRAQ (isobaric tags for relative and absolute quantification) and TMT (tandem mass tags) are the two most extensively used proprietary methodologies [23, 159]. These approaches rely on peptide labeling post trypsin digestion. The tags are isobaric and have an amino-specific protein reactive group which will label all peptide fragments and enable detection of differential peptide (and hence protein) expression in 4–10 samples depending on the product. These approaches could be used on the isolated and solubilized membrane protein fractions and might be useful to detect changes in protein composition of sperm surface under various physiological and in vitro conditions (for instance, the release of decapacitation factors during in vitro fertilization treatment or alterations of sperm surface proteins of sperm collected at different regions of the epididymis). To date several studies have been published using either iTRAQ or TMT on whole cell lysates, but none have been reported on membrane fractions [5, 6, 9, 98].

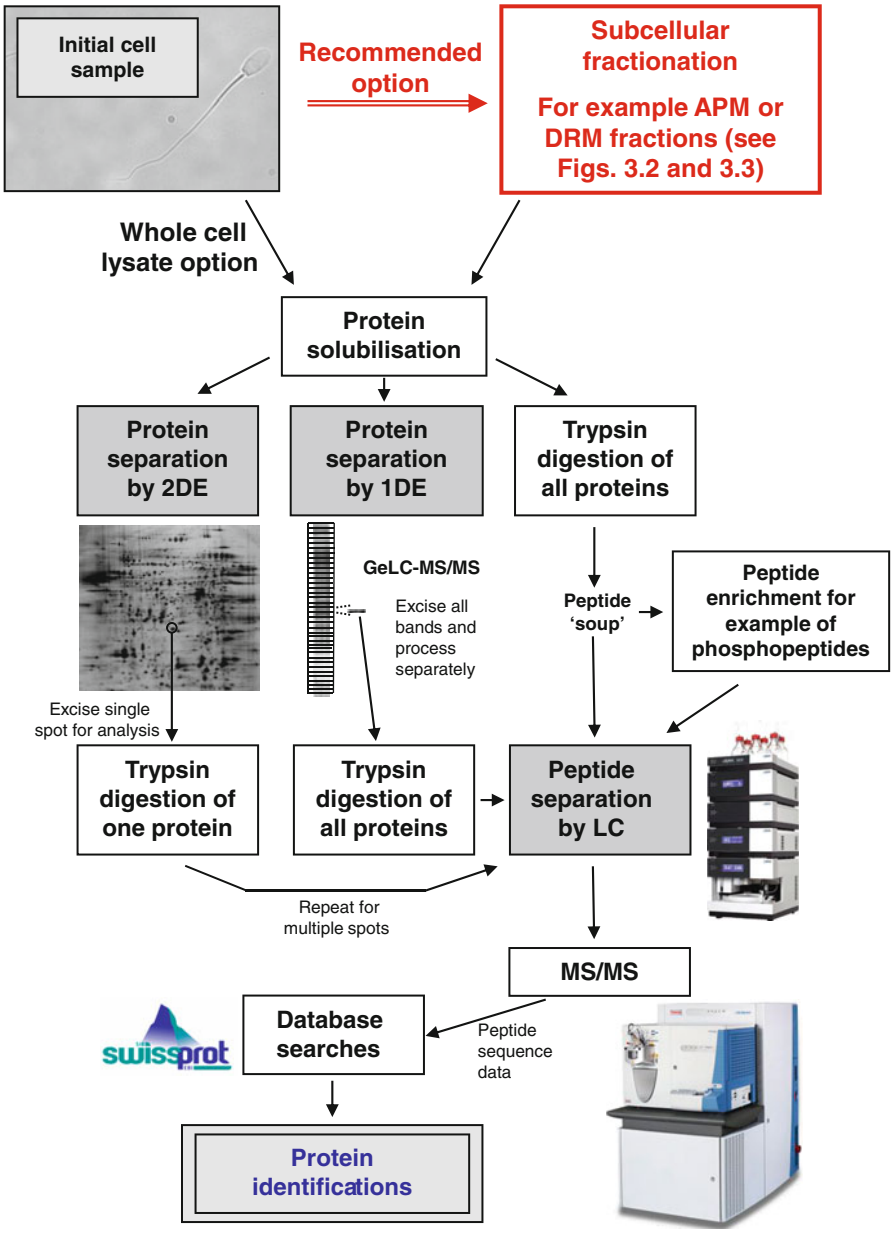
Posttranslational changes of the sperm surface membrane can also be detected [7, 57, 119]. In another study, a biotin-switch assay was employed to detect protein S-nitrosylation in human sperm [97] which provided fundamental new insights in NO-mediated sperm signaling under in vitro capacitation conditions. The modifications of these proteins take place intracellularly, and only a surface-specific membrane protein preparation can provide insights into surface posttranslational proteins.

3.3.2 Surface-Specific Considerations for Sperm Proteomics

Section 3.3.1 dealt with the applicability of a number of proteomics strategies to study sperm proteins. However, a number of additional considerations have to be taken into account when studying sperm surface proteomes. The researcher should be sure about the surface topology of the proteins under investigation. A first step into this direction is to isolate the membrane of interest (see Sect. 3.2). However, additional scrutiny is needed in ruling out the high amount of proteins that only interact indirectly with the sperm surface since they can easily become co-isolated and identified. To this end, sperm can be labeled with membrane impermeable tags prior to membrane subfractionation. Most commonly a biotinylated tag is used which is covalently bound to the sperm surface proteins [78, 85, 131, 136]. A streptavidin immobilized affinity column can be used to isolate the biotinylated proteins. After isolation the tag can be cleaved enzymatically, and the proteins can be digested into peptides for MS/MS analysis. Importantly this technique has some drawbacks as one has to be sure that only surface-oriented proteins are labeled. If sperm cells deteriorate during biotinylation, intracellular proteins will become biotinylated because they are accessible for the tag. For extracellular matrix components, this will always be the case even for intact sperm. Moreover, sperm also contains a certain number of endogenously biotinylated proteins. Finally, nonlabelled proteins may interact with the biotinylated proteins and thus may also be immobilized into the streptavidin columns. Indeed, many studies using immuno-purified surface-labeled membrane samples report the identification of a large number of nonmembrane proteins. There are

Fig. 3.4 Strategies for the global identification of proteins. Traditionally proteins are solubilized from entire cells to produce whole cell lysates, but subcellular fractionation is strongly recommended to enrich for proteins of particular biological interest and to achieve localization information. One option is the preparation of sperm apical plasma membranes (APMs; see Fig. 3.2) or isolation of the detergent resistance membrane fraction (*DRM*; see Fig. 3.3) of sperm). Following solubilization, protein separation may be achieved by two-dimensional electrophoresis (2DE), and this remains popular in low throughput studies. An individual separated protein is removed as a gel plug, trypsin digested, and the resulting peptides are separated on the basis of charge and relative hydrophobicity by nanoscale liquid chromatography (*LC*). Amino acid sequence of these peptides is then determined by tandem mass spectrometry (*MS/MS*), and these sequence data are used to search existing protein databases to achieve a match and therefore a protein identification (*ID*). In order to identify many (or all) of the separated proteins, it is necessary to excise and process multiple gel plugs from the 2D gel. For global analysis, it is more appropriate to trypsin digest the solubilized protein mixture to produce a peptide "soup" from all the proteins in the sample. Peptides are then separated by LC before extensive MSMS and database searches to identify many (ideally all) of the proteins in the original sample. Beyond this it is also possible to first separate proteins by one-dimensional electrophoresis (*1DE*; SDS-PAGE) before subjecting individual protein bands to digestion and LC-MS/MS (the so-called geLC-MS/MS workflow). It is also possible to enrich for peptides of a particular type, for example phosphopeptides, to study a particular group of proteins. In addition to the workflows illustrated, there are many other options. Protein rather than peptide enrichment may be used and peptide isoelectric focusing (IEF) as an additional step within the usual LC-MS/MS workflow is also a valid option for increased numbers of IDs (Adapted and modified from Brewis and Gadella [24])

many ways to reduce the amount of this contamination. For reviews around this topic see [52]. Besides the two steps mentioned here (labeling of the sperm surface and subsequent membrane isolation), the resulting preparations need to be treated with high salt media to get rid of adhering extracellular matrix and cytosolic components. The resulting membrane sample is highly enriched in integral membrane proteins.



Another important issue for integral membrane proteins (that is, those with (multiple) alpha helices spanning the membrane or with beta sheet barrels) is that such proteins have highly hydrophobic domains. This property of a major portion of membrane proteins often prevents solubilization under conditions compatible with 2D electrophoresis. A number of reviews provide an excellent overview of techniques that can be employed to identify these integral membrane proteins [52, 142]. Those workflows that first rely on trypsin digestion of protein mixtures overcome many limitations by digesting a specific isolated sperm surface protein fraction and analyzing the derived peptides with LC-MS/MS.

3.4 Comparison of Sperm Surface Proteomics in Different Species

A number of considerations for studying the sperm surface proteome have been summarized in this chapter. They need to be carefully considered in order to make proteomics databases of sperm surface protein composition more useful or meaningful. In this section, more emphasis is put on how existing sperm proteomics libraries should be interpreted and where appropriate some comments will be made on the suitability or originality of approaches used to decipher protein compositions of the sperm surface.

A number of groups have successfully analyzed the sperm proteome in a range of different species using either whole cell lysates or different fractions. Table 3.1 summarizes those studies that are the most noteworthy either from the point of view of the high numbers of proteins identified or the rigor of the sample preparation. From the perspective of this review, it is noteworthy that the majority are on whole cell lysates and that very few proteomics studies have focused on the sperm surface or membrane fractions. When browsing through such data, one needs to be critical in how the protein samples were prepared in order to understand how meaningful the proteomics libraries generated actually are for the sperm surface. (1) Sperm membranes are often isolated by the method of [30] in which sperm are first incubated in a hypo-osmotic environment followed by sonication and differential centrifugation. However, the purification for plasma membrane marker proteins over possible contaminating intracellular membranes is not tested convincingly for human sperm. (2) Indirect reacting proteins for instance from the extracellular matrix or the cytoskeleton may also be identified when the isolated membrane preparations were not subjected to high salt [52]. (3) Other groups use surface modification techniques to study sperm surface membrane proteins [85, 128]. The labeled proteins are supposed to originate from the sperm surface, but this approach can lead to the iodination or biotinylation of many intracellular proteins. (4) The isolated or labeled proteins are routinely solubilized and subsequently separated using protein gel-electrophoresis. The drawback of this technique is that an important group of integral membrane proteins due to their hydrophobic properties is not suitable for 2D gel-electrophoresis [52] and other approaches, such as geLC-MS or peptide IEF, may be required to enable full surface proteome coverage [23, 55].

Table 3.1 Summary of the major proteomics studies in mammalian sperm cells

Species	Sample proteins	Separation method	Total IDs	References
Boar	Whole cell lysate	Protein 2DE, peptide LC	310	(Brewis and Gadella, *unpublished data*)
	Lipid raft	Lipid raft preparation and protein 2DE or peptide LC and peptide LC or just peptide LC	34	[152] (Brewis and Gadella, *unpublished data*)
	Apical plasma membrane and docked outer acrosomal membrane	Subcellular fractionation and protein 2DE or peptide LC	63	[14, 148] (Brewis and Gadella, *unpublished data*)
Bull	Cytosolic tyrosine kinase	Subcellular fractionation, 1DE and peptide LC	130[a]	[94]
	Membrane fraction	Peptide LC	419	[38]
Human	Whole cell lysate and surface labeled	Surface protein labeling, 2DE and peptide LC	267[b]	[51, 129]
	Whole cell lysate	Protein DDE, 1DE and peptide LC	1056[c]	[15]
	S-Nitrosylated	Protein enrichment, 1DE and peptide LC	240	[97]
	Nuclear extract	Protein 2DE, 1DE and peptide LC	403	[49]
	Whole cell lysate	Peptide LC	348	[98]
	Whole cell lysate	Peptide LC	1157	[4, 5]
	Whole cell lysate	Peptide LC	1975	[102]
Mouse	Flagellum accessory structures	Protein DDE, 2DE and peptide LC	50	[39]
	Sperm acrosome	Subcellular fractionation, protein 1DE and peptide LC	114	[136]
	Whole cell lysate	Peptide IEF and LC	858	[14]
	Lipid raft	Lipid raft preparation, protein 1DE and peptide LC, peptide LC	100	[11]
	Whole cell lysate	Peptide LC	2850	[41]
	Whole cell lysate	Protein IDE and peptide LC (geLC-MS)	1234	[132]
Rat	Whole cell lysate	Peptide IEF and LC	829	[13]
Rhesus macaque	Whole cell lysate	Protein IDE and peptide LC (geLC-MS)	1247	[133]
Stallion	Whole cell lysate	Protein IDE and peptide LC (geLC-MS)	1130	[141]

This table is adapted from Brewis and Gadella [24] and has been updated and modified to include the most noteworthy studies. *Key*: *DDE* differential detergent extraction, *IDs* protein identifications, *LC* liquid chromatography, *MS/MS* tandem mass spectrometry, *1DE* one-dimensional electrophoresis, *PMF* peptide mass fingerprinting by MALDI-TOF MS, *2DE* two-dimensional electrophoresis, *geLC-MS* current terminology for IDE and peptide LC. All studies are based on MS/MS data except for [94] and [11] which additionally includes PMF data

Three published studies with high numbers of IDs are excluded from this list. Peddinti et al. [116] report 2814 IDs on bull whole cell lysates, but the presented data do not support this assertion. Johnston et al. [81] report 1760 identifications in human whole cell lysates, but the protein IDs and MS/MS data were not reported. Wang et al. [155] report 4675 IDs in human whole cell lysates, but the inclusion criteria used were not sufficiently robust

[a]Total number of proteins identified (4 were protein tyrosine kinases)

[b]John Herr, personal communication 2015

[c]Updated to 1223 by Baker et al. [14]

These points of attention are valid for sperm surface proteomics studies independent of the mammalian species under study. However, there are also a number of species-specific advantages and disadvantages in studying the human, mouse, and porcine or bovine sperm surface, which will be dealt with the next sections for these species.

3.4.1 Human Sperm Proteomics

Referring to Table 3.1, it is clear that there have been many studies on human sperm, and indeed there are many smaller studies not included in this table. For a very elegant summary of human sperm proteomics, the reader is referred to [5] which reported a total of 6198 proteins predominantly from studies on whole cell lysates. However, focusing on the sperm surface proteome, some specific limitations that are intrinsic to human sperm need to be considered and perhaps they explain why there has been relatively little focus on this region of the cell in humans. (1) Humans (and some primate species) produce semen with a rather high content of abnormal sperm (immature, deteriorated, or morphologically aberrant). Even in the ejaculate of fertile men, the proportion of deteriorated sperm is >40 % [76, 86], whereas the ejaculate of a fertile boar (male porcine) has only <5 % aberrant sperm [65]. When assessing human sperm with the strict Tygerberg criteria, in semen only 15 % morphologically normal sperm is the value for normal fertilization rates and morphology scores rarely were higher than 30% for most fertile men [89]. In stark contrast in porcine sperm, this morphology score is rarely below 85 % [65]. The problem with human sperm is that the surface of aberrant sperm is also labeled and/or isolated following the above-mentioned methods (Sects. 3.2 and 3.3). Therefore, the resulting protein mixtures will contain more proteins from malfunctional sperm and intracellular labeled proteins compared with porcine or mouse sperm. On the other hand, the relative abundance of abnormal sperm in ejaculates from males with reduced fertility characteristics are of use for diagnostic proteomics comparisons [51, 113]. With respect to the theme of this book, sperm antigens have been detected and characterized by comparing sperm proteins from healthy and infertile men. (2) For proper sperm surface isolation, one needs to have large amounts of sperm cells. This is not the case for the commonly used method to isolate total sperm membranes using the hypo-osmotic treatment followed by sonication and differential centrifugation. For sperm cavitation and subfractionation of sperm membranes, one needs much more starting material. However, the amount of sperm released in a human ejaculate (from a healthy fertile donor) is less than 200 million sperm [99], while for porcine (and bovine) sperm this number is approximately 100–200 times higher [31, 100].

3.4.2 Mouse Sperm Proteomics

Proteomics data obtained from mouse sperm need to be viewed with extra care. (1) When mouse sperm is collected by (electro-stimulated) ejaculation, they will almost immediately deteriorate due to the spermicidal coagulation plug in which the

sperm become entrapped during collection (in contrast to the in vivo situation where the sperm remain separated from the coagulation plug). Therefore, mouse sperm for IVF purposes or for studying sperm surface are routinely obtained by aspirating the epididymis [134]. Obviously this influences the quality of such specimen as epididymal sperm may not be fully matured and the amount of sperm collected is not sufficient for proper membrane subfractionation studies. (2) Specific problems to sperm surface isolation are related to the hook-shaped morphology of the mouse sperm head. Probably related to this, only one attempt has been described to strip the plasma membrane from mouse sperm with nitrogen cavitation [103] without data on the purification degree of the cavitate. The other sperm surface isolation method of blunt hypotonic sonication resulted in only low purification of mouse sperm plasma membranes 4–10 times [17]. (3) Obviously the mouse species also has specific advantages over human and porcine species for sperm surface proteomics. Like for human, the complete genome and proteome of mouse are available [84]. (4) Because the mouse is an important laboratory animal model, species-specific genetic breeding lines are available. When compared to human (also valid to some extent for porcine samples [124]), the advantage is that within a specific breeding line relative low biodiversity exists which will result in much more repeatable data [83]. (5) Of course the mouse is also a model of choice for generating genetic knock out or silencing phenotypes for validating the function of certain translation products identified in proteomics [44, 112]. Due to the fact mouse give birth to nests (multiple off spring) and have a relatively short generation time, this laboratory species is very well suited for obtaining fertility data that can be related to proteomics data bases to verify the functionality of certain proteins in fertilization. Genotypic manipulation of humans is of course not permitted.

3.4.3 Porcine and Bovine Sperm Proteomics

The major potential of porcine and bovine sperm is noteworthy. (1) Each ejaculate contains an overwhelming amount of mature and morphologically intact functional sperm [31, 100]. (2) Moreover, for both species a reliable method has been described for purification of the apical plasma membrane (or further subfractionation to obtain surface specific of membrane microdomains) [60, 93]. Therefore, much more reliable surface membrane protein samples can be obtained from these species compared to human and mouse. (3) Both in porcine and bovine species, most offspring is produced by artificial insemination. Over the past decade or more, all large AI-industries have set up huge fertility data sets of individual male animals, collection time, female animals inseminated, nonreturn rate, birth rate, and litter size (for pigs) [32, 126]. The enormous amounts of data for each sperm-producing animal can be used to get very relevant correlations between sperm characteristics and fertility potential. In collaboration with the AI-industries, these data sets can become accessible to correlate the presence of certain sperm surface proteins in certain sperm donors to the fertility performance of the boar or bull [25, 33, 115]. To a lesser extent, this is also possible for equine sperm [80]. (4) The equine and bovine

species are mono-ovulatory and therefore have a reproductive physiology that resembles the human reproductive physiology more than the laboratory animals or pigs which are poly-ovulatory mammals [50, 108]. (5) Porcine and bovine breeding is performed on a very large-scale worldwide. The offspring is of course relevant for delivery of milk for dairy products and for our need for animal food and animal-derived materials from those animals. At a certain moment, animals will be slaughtered to harvest these materials. For veterinary scientists, it is possible to obtain fresh materials from those animals at the slaughter line continuously. This enables the researcher to obtain materials of >6000 animals per day. In our setting, we were for instance able to isolate 5000 ovaries with ovulatory follicles from adult pigs in one collection session [59]. From this material, we isolated 500,000 oocytes with a mature diameter size and a functional zona pellucida. We were able to isolate zona ghosts that were not contaminated with other proteins as was verified on solubilized zona material on 2D electrophoresis [151]. This zona material was used to identify isolated apical plasma membrane proteins. A number of integral membrane proteins originating from the testis (such as fertilin beta) and GPI anchored proteins attached to the sperm surface when traveling through the epididymis (spermadhesins) were identified [28]. Although a number of proteins were not identified, this direct primary zona binding approach could not have been carried out with mouse or human material as such an amount of purified mature and prefertilization zona ghost material cannot be prepared from these species. In addition, due to their larger size farm animals are easier to approach for internal genital tract processing of the sperm surface. Examples are of epididymal surface remodeling or of in vitro manipulation of the sperm surface in the oviduct [47, 80, 137]. (6) Although technically possible, it is very expensive to perform genotypic silencing of farm animal species. This is due to the larger size of these animals compared to laboratory animals: Both the housing of animals and the relatively long generation time in larger animals make these types of studies less suitable. We should note here that fertility data from molecular manipulated mouse experiments can only to a limited manner be extrapolated to other mammalian species. This has to do with the fact that proteins involved in reproduction show a very rapid evolutionary diversification. There is a lot of redundancy in proteins within one species (in porcine sperm there are >10 zona binding proteins [150]) and between species; completely different sets of proteins are involved in the same processes related to fertilization due to rapid evolutionary diversification of proteins [77, 149]. For this reason, phenotypically altered mice may not always provide insights to understand the role of sperm surface proteins identified in other mammalian species.

3.5 Implications for Future Research

3.5.1 Proteomics and Male Fertility

Much of the research on mammalian sperm that has benefited from proteomics technology has been interested in better understanding molecular events and how they

affect the biological function of the sperm cell. Proteomics has also been used closer to the clinic to investigate potential human sperm defects that contribute to infertility. John Herr's group has been interested for many years in characterizing immunogenic surface epitopes to further understand the role of antisperm antibodies in infertility and to potentially provide insights for the development of contraceptive vaccines. For a recent overview in the use of "omics" for human male infertility, see [40]. Other studies have used proteomics to characterize functionally defective sperm (sperm that fail to fertilize at IVF, are asthenozoospermic, or are correlated with DNA damage/protamine content) [18, 20, 48, 49, 56, 107, 109]. Candidate proteins that are differentially expressed in patient samples compared with normozoospermic samples have been identified, but much work still needs to be done to properly validate these early candidates. Some may prove to be protein biomarkers of specific male infertility (sperm dysfunction) phenotypes, but in all likelihood much more rigorous analysis needs to be undertaken before such biomarkers are realized [117, 121]. Recently, sperm proteomics data have also been used to relate fertility properties of male animals (in pigs, horses, and cows), and both proteins were assigned to relate with higher fertility and with infertility characteristics [46, 61, 90, 91, 98, 140].

3.5.2 Quantification of the Proteome

The sperm research community has been slow to adopt the now gold standard approaches for relative protein quantification in proteomics. Such approaches will be key to the discovery of protein biomarkers of male infertility and in further understanding sperm dysfunction and function at the molecular level. In the past there have been some useful studies using difference gel-electrophoresis (DIGE) (fluorescently tagged samples are multiplexed, separated by 2D electrophoresis, and quantified with confocal laser scanning) [16, 125]. This approach has been superseded by the previously mentioned iTRAQ or TMT tagging workflows. To date there have been very few studies published in sperm that have used either of these tagging approaches and these have generally been on whole cell lysates [4, 9, 98]. One interesting exception to this is the study of Asano et al. [6] who have used iTRAQ to characterize the expression of certain proteins in different microdomains. Liu et al. [102] have used a label-free approach, which is a newer tagging-free mass spectrometric quantification approach to study changes involved in asthenozoospermia on whole cell lysates. Finally it is also possible to quantify phosphorylation on a larger scale using an alternative MS-based labeling approach (Fisher esterification of phosphopeptides using differentially deuterated methyl alcohols), and this was employed in an elegant study comparing capacitated and noncapacitated cells [61].

3.5.3 Protein and Peptide Enrichment for Proteomic Studies

As an alternative to subcellular fractionation, another option is to enrich for protein types of interest from a whole cell lysate. Several studies on sperm have investigated

protein phosphorylation on a proteomic scale as the this phenomenon is known to be very important to a number of aspects of sperm function, including epididymal maturation and capacitation. The first proteomics studies involving both the identification of multiple phosphoproteins [145] and the sites of phosphorylation were conducted by Pablo Visconti and colleagues on human sperm, and the same group has published widely in a number of species. For a recent review on sperm phosphoproteomics, see Porambo et al. [120]. Currently phosphoproteomics studies are generally performed using peptide affinity-based approaches with the enrichment of phosphorylated peptides by immobilized metal affinity (IMAC) chromatography or titanium dioxide, and indeed these prefractionation approaches are essential.

Conclusions

Antigens at the surface of sperm are of considerable interest compared with intracellular antigens as the latter are only accessible for immune responses when the integrity of sperm is compromised. When immune responses are elicited towards the sperm surface of intact sperm the fertilization potential of such sperm may be altered by the immune response. Thus, proteomics studies that focus exclusively on sperm surface material are very relevant for immune infertility studies. A number of considerations have been dealt with in this chapter to ensure that only the proteins of sperm surface membranes are isolated or labeled. Very few of the noteworthy proteomics studies to date have focused in the cell surface, and this remains a key challenge for this field. It is difficult to compare the surface proteome of human, mouse, and farm animals as the sperm surface proteome is highly species specific, and each mammalian species has its own drawbacks and advantages for studying the sperm surface proteome. The functional relevance of genotypic silencing experiments of mouse sperm proteins for human reproduction is therefore also questionable to a certain degree. The major drawbacks for studying the human surface proteome are the limited amount of material that is present in an individual ejaculate, the high incidence of aberrant sperm (both are no issues for farm animal species). Another drawback is that genetic manipulation of man is not permitted (this is not an issue for murine species and it is possible but very expensive and time consuming for farm animals).

Finally in many studies, the specificity of labeling methods and sperm surface separation from intracellular and extracellular components have not been analyzed or at least not with high enough scrutiny. For functional sperm surface proteomics, it will be of fundamental interest to have specific sperm surface protein preparations. In addition, the interacting structures should be purified to a satisfactory level. Somatic cells and fluids from the male and female genital tract are involved in the relevant surface modifications to achieve fertilization. Finally the complex and domain-dynamic organization of the sperm surface needs to be considered when studying the protein composition of the fertile surface of sperm. With this respect, it is noteworthy that sperm membrane proteins form complexes at different places on the sperm surface with specific functions in mammalian fertilization.

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Sperm Functions Influenced by Immune Reactions

4

Walter K.H. Krause

Abstract

Antisperm antibodies (ASA) may interfere with different steps of the fertilization process. The dysfunction evolving depends on the cognate antigens of the ASA. The usual technique for their identification is 2D-Western blot and MALDI protein analysis. A great variety of antigens are responsible for sperm agglutination, sperm apoptosis and inhibition of sperm motility. Impairment of migration in the cervix mucus appears mostly to be independent from the cognate antigens of ASA, but to be a consequence complement activation and cell lysis. Influences of ASA on acrosome reaction reveal a special problem, since a large variety of cognate antigen are liberated only after acrosome reaction. The cognate antigens of ASA inhibiting zona binding and oolemma binding are the most promising structures for immunocontraception. Also pronucleus formation may be influenced by ASA, but the clinical relevance of the antigens involved remains unclear.

Identification of cognate antigens of ASA has a number of practical consequences: (i) ASA inhibiting the fertilization process may be identified by an ELISA or a RIA using specific proteins or peptides; (ii) the identification of functionally relevant antigens is a prerequisite for treatment options; (iii) the identification of immunogenic proteins facilitates studies on immune contraception.

An overview over the sperm antigens involved in the steps of the fertilization process which were identified as cognate antigens of ASA gives Table 4.1. They are further reviewed in the following chapters.

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Table 4.1 Defined antigens of naturally occurring human ASA

Antigen	Localization	Source of ASA	Effect of ASA	Authors
Acrosin	Acrosome	Infertile women	Inhibition of acrosin effects	Veaute et al. (2009) [58]
ACTL7a	Acrosome	Vasectomized men	Agglutination	Fu et al. (2012) [22]
Calpastatin	Acrosomal region, partly flagellum	Infertile women	Inhibition of hamster-oocyte penetration, no inhibition of motility, agglutination	Koide et al. (2000) [31]
Caspase 3	Sperm surface	Seminal fluid	Induction of apoptosis	Bohring et al. (2001a) [8]
Catsper1	Sperm surface	Experimentally	Agglutination, motility	Li et al. (2012) [38]
Clathrin, heavy chain	Sperm tail	Female patient with lupus erythemasus	Agglutination	Domagala et al. (2011) [18]
CD52	Sperm surface, inserted into the sperm membrane during the epididymal passage	Infertile women	Inhibition of motility	Hasegawa and Koyama (2016) [25]
c-kit	Acrosomal region	Polyclonal rabbit	Agglutination, inhibition of acrosome reaction	Feng et al. (2005) [20]
CRISP-2 (TPX-1)	Equatorial section	Infertile women	Inhibition of penetration of zona-free hamster oocytes	Brunner-Agten et al. (2013) [10]
ER60, disulfidisomerase	Acrosomal region	Seminal fluid	Acrosome reaction	Bohring et al. (2001a) [8]
FA-1	Sperm surface, specifically reacting with zona protein 3 (ZP3)	Infertile women	Inhibition of capacitation and acrosome reaction	Menge et al. (1999) [43]
hSMP-1 (PubMed locus U12978)	Acrosome, sperm surface	Infertile women	Agglutination, inhibition of acrosome reaction, inhibition of zona binding	Koide et al. (2000) [31], Cheng et al. (2007) [15]
HSP60	Sperm surface		Inhibition of cervix-mucus penetration	
HSP70	Sperm surface	Seminal fluid	Apoptosis	Bohring et al. (2001a) [8], Naaby-Hansen and Herr (2010) [44]

LDH-C_4	Sperm surface	Seminal fluid	Unknown	Bohring et al. (2001a) [8]
Izumo	Acrosomal region following acrosome reaction	Infertile women	Inhibition of sperm-oocyte fusion, highly conserved	Inoue et al. (2005) [28]
NASP (human nuclear autoantigenic sperm protein)	Sperm surface	Vasectomized men	Unknown	Batova et al. (2000) [5]
Peptide NT (80 kDa-HSA)	Sperm surface	Infertile women	Agglutination of epididymal sperm	Bandivdekar et al. (2001) [2]
P36 (triosephosphate isomerase)	Acrosomal membrane	Seminal fluid	Inhibition of penetration of zona-free hamster oocytes	Auer et al. (2004) [1]
PH-20, glycerolphosphatidyl-inositol-linked hyaluronidase	Sperm surface	Experimentally	Inhibition of zona binding, inhibition of penetration of zona-free hamster eggs	Chan et al. (1999) [14]
Proteasome complex	Seminal fluid	Seminal fluid	Inhibition of motility	Bohring et al. (2001) [8]
SLLP-1	Acrosomal region	Experimentally	Inhibition of hamster-oocyte penetration	Wang et al. (2004) [61]
SP10	Acrosomal membrane	Experimentally	Inhibition of sperm-oocyte fusion, highly conserved	Hamatani et al. (2000) [26]
Sp17	Testis, head and tail of ejaculated spermatozoa	Vasectomized men	Inhibition of acrosome reaction, highly conserved	Lea et al. (1997) [35]
SPAG6	Sperm tail	Infertile male	Inhibition of motility	Neilson et al. (1999) [48]
SPRASA	Acrosome	Infertile male	Inhibition of acrosome reaction	Chiu et al. (2004) [16]
YLP12	Acrosomal region	Infertile women	Agglutination, immobilization, inhibition of penetration of zona-free hamster oocytes	Naz et al. (2000) [47]
YWK II	Equatorial region	Infertile women	Agglutination, inhibition of sperm-oocyte fusion, inhibition of zygote development	Koide et al. (2000) [31]

4.1 Sperm Agglutination

Influence of ASA on sperm agglutination seems feasible, since observation of agglutination is a proven method of ASA detection. A first investigation of cognate antigens binding sperm agglutinating ASA was published by Koide et al. [31]. The ASA were obtained from the blood serum of infertile women. Among the antigens identified were:

(i) SMP-B, a sperm tail component with 72 kD, recognized by ASA from the serum of infertile women. The gene was expressed only in spermatids. The human analogue (hSMP-1, see below) is coded by the HSD-I gene, which is located on human chromosome 9, region p12-p13 [33].
(ii) Calpastatin, a 17.5 kDa protein, being localized by immune staining with polyclonal antibodies in the acrosomal region and slightly on the tail. The cDNA consisted of 758 base pairs, having 99.7 % homology with the gene coding calpastatin. The gene was found to be transcribed only in spermatids. Calpastatin binds calpain, a Ca-dependent cysteine endopeptidase (see below).

Domagala et al. [17] described agglutination between sperm tail tips by antibodies from an infertile female patient suffering from systemic lupus erythematosus. Using proteomic analysis, the cognate antigen was identified as the heavy chain of clathrin, the main structural coat protein of coated vesicles which play a key role in the intracellular transport between membranous organelles. By immunofluorescence, it was localized in the principal piece and the cytoplasmatic droplets.

A polyclonal antibody from the rabbit against the human c-kit peptide was able to inhibit acrosome reaction in human sperm and to increase sperm agglutination. By immune fluorescence, the localization of the c-kit peptide in the acrosomal region was demonstrated, but the staining was absent in acrosome reacted sperm. Thus the c-kit peptide may be involved in acrosome reaction ([19]; Fig. 4.1).

Norton et al. [50] engineered a recombinant single-chain variable fragment (scFv) antibody binding to a tissue-specific carbohydrate epitope located on human sperm agglutination antigen-1 (SAGA-1), the sperm glycoform of CD52. The recombinant anti-sperm antibody (RASA) was expressed in E. coli HB2151 cells. RASA aggregated human spermatozoa in a tangled (head-to-head, head-to-tail, tail-to-tail) pattern of agglutination [64]. For further details of CD52, see Chap. 11.

Bandivdekar et al. [2] described antibodies binding to a human sperm-specific antigen of about 80 kDa, which agglutinated epididymal spermatozoa. The partial N-terminal amino acid sequence of 80 kDa HSA (peptide NT) and its peptides obtained by enzymatic digestion with endoproteinase Lys-C (peptides 1, 2, 3 and 4) and Glu-C (peptides 5 and 6) did not show sequence homology with any of the proteins in Gene database. In a further study, the authors showed that antibodies from the rabbit against this protein could cause infertility in mice [3].

Fu et al. [21] demonstrated a marked reduction of fertility in female mice by autoantibodies to ACTL7a from vasectomized men. The protein ACTL7a plays an important role in spermiogenesis, in particular in the morphogenesis of spermatozoa,

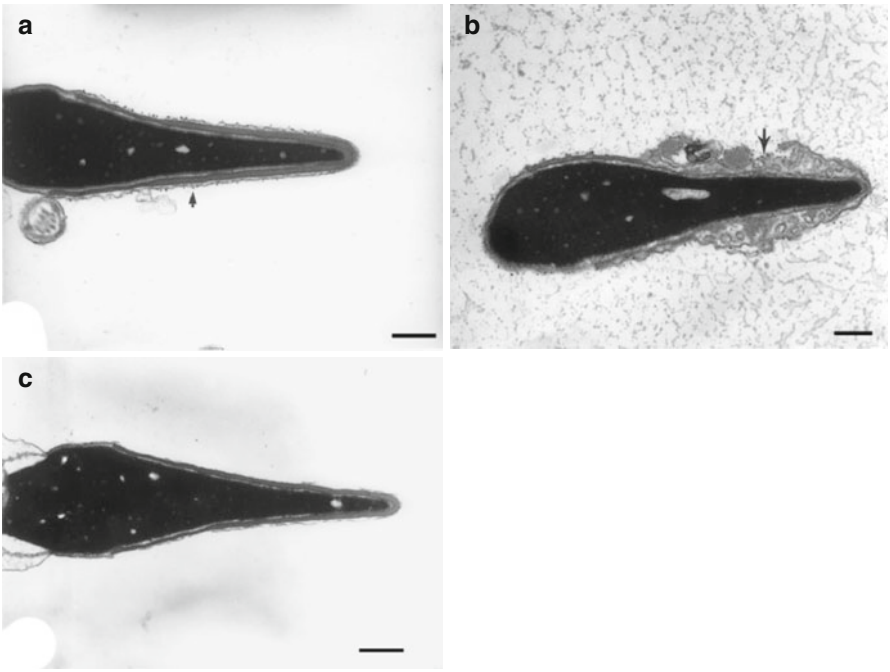

Fig. 4.1 Immunolocalization of c-kit receptor in human spermatozoa by electron microscopy. (**a**) The immunogold particles were located on the plasma membrane (PM) surface (*arrows*) of the acrosomal regions in the acrosome intact spermatozoa. (**b**) After the acrosome reaction, gold particles remained associated with the acrosomal vesicles (*arrows*), presumably in the PM components of the vesicles. (**c**) No gold label was observed on the acrosome-intact spermatozoa in incubated with normal rabbit serum sperm (**c**). Bar 0.5 mm (Reproduced from Feng et al. [19]; with permission)

but its functional role was not yet described. In spermatids, it forms a complex with other components of the cytoskeleton. In human spermatozoa, this protein has been located in the acrosome. The antibodies caused a marked agglutination of sperm in vitro.

Antibodies to Catsper1, one of the proteins of the cationic channel of sperm, experimentally induced sperm agglutination and inhibited fertility in the mouse [38]. Since Catsper1 is clearly associated with sperm functions, ASA against Catsper1 might be able to impair fertility. Evidence for this mechanism, however, is lacking up to now [55].

4.2 Sperm Apoptosis

Several proteins of the signal transduction pathways of apoptosis are present on the sperm surface, e.g. the externalization of phosphatidylserin, CD 95, and some caspases [51]. On the other hand, spermatozoa do not stain with Fas protein

antibodies [12], thus it is questionable whether the complete instruments of apoptosis are present in spermatozoa and whether these proteins are functionally active.

Inflammasome components and end-product cytokines are present in semen. Caspase-1 in sperm fractions and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) in seminal plasma and sperm fractions could be identified. Immunocytochemistry revealed that ASC was located in the acrosome, equatorial segment, and midpiece, and caspase-1 in the midpiece [67].

Reports on ASA binding to functional proteins involved in apoptosis in the literature are scarce. A binding of ASA to the inactive form of caspase-3 and to HSP70 as cognate antigens were demonstrated in our group [8]. Naaby-Hansen and Herr [45] described antibodies to HSP70 that blocked fertilization in vitro.

4.3 Sperm Motility

A special feature concerns sperm immobilizing ASA. They were demonstrated exclusively in the sera of infertile women. They appear to activate the complement system; their presence is frequently associated with impaired penetration of the cervical mucus. The antigen was identified as human CD52 antigen, which is inserted into the sperm membrane during the epididymal passage. Details on this topic are discussed in Chap. 11.

In general, it is hard to explain how other ASA will interfere with sperm motility, since it is likely that ASA bind to antigens of sperm membranes, while sub-cellular structures will not be reached by ASA in the living cell.

Neilson et al. [49] used serum from an infertile male with high titers of ASA to identify a novel human sperm antigen (SPAG6) by screening of a testis expression library. The human gene encodes 1.8- and 2.8-kb mRNAs highly expressed in testis but not in other tissues tested. The deduced amino acid sequence of the full-length cDNA revealed striking homology to the product of the Chlamydomonas reinhardtii PF16 locus, which encodes a protein localized to the central pair of the flagellar axoneme. Antibodies raised against the peptide sequences localized the protein to the tails of permeabilized human sperm.

The results of Inaba et al. [26] using immune electron microscopy suggested that flagellar movement of sperm is also modulated by proteasomes, which regulate the activity of outer dynein arm by cAMP-dependent phosphorylation of the 22 kDa dynein light chain. In our group, we were able to demonstrate ASA binding to the component 2 and to the zeta chain of the proteasome complex [7]. Complement regulatory proteins such as C1-INH, CD55, CD46, and CD59 has been found to be expressed on sperms [29]. IgG antibodies to these proteins significantly reduced sperm motility in general and other parameters of motility.

Applying Catsper1-antibodies to spermatozoa, as already mentioned in the previous chapter, was able to inhibit total motility and progressive motility. The mechanism of this inhibition remained unclear. CatSper1 expression has been found to be positively related to progressive and hyperactivated (HA) motility, men with

asthenozoospermia showed a reduced expression of CatSper1 in the spermatozoa [39]. Also antibodies binding to the voltage-gated anion channel protein (VDAC) showed impact on sperm motility, possibly by influencing the Ca+ influx into the cells [40]. However, there is no evidence of ASA binding to the two channel proteins up to now.

4.4 Cervix Mucus Penetration

Immunglobulin concentrations in the cervix mucus are generally low, thus ASA are rarely detected. Kamieniczna et al. [30] described a frequency of 3.2 % in infertile women, compared to 10.4 % of seminal samples of infertile men. In particular, women with immobilizing antibodies may display different titers also in the cervix mucus, which inhibit sperm migration [54] and result in poor post-coital test and reduced fertility.

However, the impairment of sperm penetration into the ovulatory cervical mucus is largely independent from ASA. During their residence in the cervical mucus, spermatozoa are exposed to complement activity, although the complement activity in cervical mucus amounts only to approximately 12 % of that in serum [23]. Immunoglobulins attached to the sperm surface activate the complement cascade, initiating cell lysis and a phagocytotic process. The complement-induced cell lysis depends on the immunoglobulin class of the antibody concerned, IgM is far more effective than IgG, while some IgA subclasses are unable to interact with the early complement components.

Another mechanism explaining the impairment of cervical mucus penetrating ability and the induction of the shaking phenomenon by ASA, in particular those of the IgA class, appears to be mediated through the Fc portion of the IgA [16], [28]. Sperm recovered after mucus penetration displayed a reduced binding to IgA immunobeads [61]. Experimentally, Bronson et al. [9] showed that IgA bound to the sperm surface, which was degraded by an IgA protease from *Neisseria gonorrhoeae* did no longer inhibit mucus permeation.

4.5 Acrosome Reaction

The loss of the acrosome including the release of the acrosomal content in order to enable the spermatozoa to permeate through the zona pellucida is called acrosome reaction. There is a large data pool on antigens involved in acrosome reaction and antibodies to these antigens.

In general, the majority of ASA increase the number of acrosome-reacted spermatozoa. In our group we showed that a number of spontaneous occurring ASA was able to enhance the number of acrosome reacted sperm [7], but none of them was able to inhibit acrosome reaction in vitro. In our study all patients, whose ASA bound to the acrosome region of the donor sperm, showed abnormal acrosin activity in their own spermatozoa, indicating a functional relevance of the cognate antigens.

In contrast, Feng et al. [18] could not demonstrate an increase in the rate of acrosome reacted spermatozoa after incubation with ASA-containing serum.

When seminal plasma samples containing ASA or spermatozoa loaded with ASA were adsorbed with fertilization antigen-1 (FA-1), the percentage of immunobead-free swimming sperm increased on an average of 50 % [44]. The rate of spermatozoa undergoing acrosome reaction as induced by the calcium ionophore A23187 showed improvement in 78 % of the sperm samples after FA-1 adsorption.

Calpastatin, a 17.5 kDa protein, is an integral part of the acrosomal cytoplasma. Using polyclonal antibodies to calpastatin, immunstaining was seen over the acrosomal region and slightly on the tail. The calpastatin gene was found to be transcribed only in spermatids. The inhibition of calpastatin leads to a premature acrosome reaction [31]. Calpastatin binds calpain, a Ca-dependent cysteine endopeptidase, fromwhich at least two isotypes exist. Antibodies to calpain bound to the region between the plasma membrane and the outer acrosomal membrane of sperm. Following the acrosome reaction, the anti-calpain antibodies labeled the acrosomal shroud presenting acrosomal contents, suggesting that calpain is located in the cytoplasmic area between the two outer sperm membranes. Calpain is relocated from cytoplasm to plasma membrane, where it cleaves spectrin, one of the proteins of the cytoskeleton, and thus facilitating the acrosome reaction [4].

Auer et al. [1] isolated a protein P36 as a cognate antigen of ASA, which was identified as a glycolytic enzyme. P36 was not detectable at the surface of live non acrosome-reacted sperm cells. It was characterized as human triosephosphate isomerase (TPI), which catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Its functional role is unclear, but may be independent of the catalytic activity, as demonstrated already for other sperm enzymes (moonlighting proteins, see following section).

Cheng et al. [14] found ASA from an infertile female patient being specific for a human sperm membrane protein (hSMP-1, PubMed locus U12978), a testis-specific protein. Polyclonal antibodies against a fragment of the mouse protein homologue showed intense hSMP-1 immune reactivity on the acrosome of human sperm. hSMP-1 is also active in the zona binding (see below).

Wang et al. [60] described the sperm lysozyme-like protein 1 (SLLP-1), which is a unique nonbacteriolytic, c-lysozyme–like protein and is present in the acrosome of human spermatozoa. Antisera to SLLP1 were shown to block binding of sperm to hamster oocytes. The occurrence of ASA binding to this antigen was not described up to now.

Chiu et al. [15] described two men with high concentrations of ASA, which bound to a novel protein localized in the acrosome called SPRASA. They were able to determine the peptide sequence of the protein by MALDI-MS and could show that it was a theoretical protein, XP-085564 encoded by the lysozyme/alpha-lactalbumine gene family. Only ASA from infertile men reacted with SPRASA, suggesting that this novel protein may be important in the processes of fertility. Later, it was demonstrated that SPRASA is also expressed in ovarian follicles and corpora lutea. Spontaneous antibodies to SPRASA were found only in infertile women, but not in fertile women, indicating its role also in female immune infertility [58].

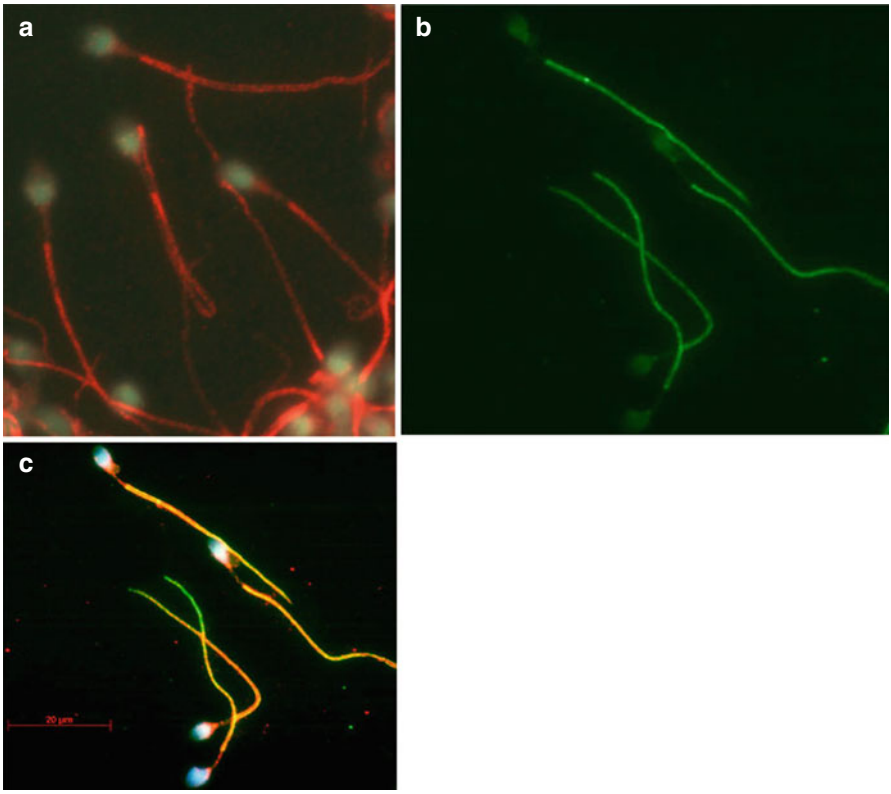

Fig. 4.2 Immunofluorescence localization of Sp17 and AKAP3. (**a**) Immunofluorescent localization of Sp17 in human spermatozoa. Sp17 is found in the principal piece, middle piece, and in scattered patches throughout the head region. The nuclei are DAPI stained. (**b**) Immunofluorescent localization of AKAP3 in human spermatozoa. AKAP3 is located predominantly in the principal piece of the flagella. The nuclei are DAPI stained. (**c**) Co-localization of Sp17 and AKAP3 in human spermatozoa. AKAP3 (*green*), located in the principal piece, co-localizes with Sp17 in some areas (*yellow*) of the principal piece but not in others. Sp17 (red) is also located in the middle piece of the tail and head regions. The nuclei are DAPI stained. Scale bar is 20 µm for **a**–**c** (Reproduced from Lea et al. [36]; with permission)

A very interesting protein localized in the acrosome reaction is sperm protein 17 (Sp17). It is a highly conserved protein localized in the testis and in the head and tail of ejaculated spermatozoa, but also in other cilia within the fibrous sheath, which contains A-kinase anchoring protein (AKAP) 3 and 4 (Fig. 4.2). Sp17 was additionally localized in human neoplastic cell lines, thus it is designated as a cancer testis antigen [37]. Sp17 was sequenced and cloned from human sperm [36], and from baboon, mice, rabbit, and rat sperm [22, 32]. There is a high degree of homology within these species. It is a three-domain protein that contains: (1) a highly conserved N-terminal domain that is 45 % identical to the human type II alpha regulatory subunit (RII alpha) of protein kinase A (PKA); (2) a central sulphated

carbohydrate-binding domain; and (3) a C-terminal Ca++/calmodulin (CaM) binding domain. Epitopes of SP17 appear to be suitable for the development of vaccines in cancers which express the antigen (lung, ovarian, hepatocellular; [63]).

Sp17 is a cognate antigen to naturally occurring ASA. Lea et al. [35] constructed an ELISA using recombinant human Sp17 for the determination of Sp17-ASA in serum of men following vasectomy. Addionally, the B cell epitopes of Sp17 were determined. The sera from men after vasectomy contained ASA against Sp17, the linear B cell epitopes were found to be amino acids 52–79 and 124–136. Eleven percent of infertile men and women displaying sperm antibodies (ASA) were positive for anti-SP17 antibodies in an ELISA [65].

The epididymal protease inhibitor (Eppin) is located in the acrosome and the sperm tail. Antibodies to eppin inhibited significantly the acrosome reaction in a dose-dependent manner. Spontaneaous ASA binding to eppin was not described until now [66].

4.6 Zona Binding

The binding of the spermatozoa to the zona pellucida (ZP) occurs via specific receptors localized over the head region of the spermatozoa. Immunologic characterization and reports on ASA of zona-binding proteins is available in the literature.

Mahony et al. [42] observed patient expressing ASA in their sera that bound to the sperm surface, most specifically the head region, and that reduced zona pellucida tight binding of spermatozoa as assessed by the hemizona assay (HZA). The responsible protein was not characterized. Liu et al. [41] described similar results. In their study, they confirmed that ASA of the patients studied interfered predominantly with sperm-zona pellucida binding. They concluded from their observations that the inhibition of oolemma binding may not be the major cause of failed fertilization with sperm autoimmunity.

One of the possible antigens involved in zona penetration is PH-20, a glycerolphosphatidyl-inositol-linked hyaluronidase. In the guinea pig, two regions of this enzyme (res. 94-119 and res. 424-444) were highly immunogenic. Since PH-20 is present in the spermatozoa of many species and also in the human, it may be a cognate antigen of ASA in the human [13]. However, these potential ASA were not described up to now.

Naz et al. [48] described a dodecamer sequence, designated as YLP (12) that is involved in sperm-ZP recognition/binding. Anti-YLP(12) Fab' antibodies of natural occurring ASA recognized a protein band of approximately 72 ± 2 kDa only in the lane of testis homogenates. In a later study, 29/67 ASA from the serum of infertile women reacted positively with the YLP12 dodecamer peptide [62].

In mice, a sperm antigen designated as fertilization antigen 1 (FA-1) was identified [68]. The authors cloned and sequenced the cDNA and were able to translate a protein, which was a novel protein not included in protein databases up to that time. The protein specifically reacted with zona protein 3 (ZP3) of oocyte zona pellucida. When polyclonal antibodies were generated, they completely blocked sperm-zona

pellucida interaction in mice. Similar results were found in the human system. In the same study as quoted above [62], 41.8 % of the sera from immunoinfertile women reacted positively with the peptide 82-97aa derived from hFA-1.

Human sperm membrane protein (hSMP-1), a testis-specific protein expressed during human sperm development (see above), was immunologically localized in the acrosome. Naturally occurring antibodies against hSMP-1 were found in the sera of infertile women. The treatment of mice with antibodies against a recombinant protein significantly decreased the average number of sperms bound to each egg in the process of fertilization. This observation indicated a role of hSMP-1 in zona binding [14].

The analysis of proteins binding to ASA which compromise the zona binding of spermatozoa is complicated by the fact that several candidates have multiple function, they are moonlighting proteins [53]. This holds true for phosphokinase type3 (PK3), enolase 1 (ENO1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldolase A (ALDOA) and triose phosphate isomerase (TPI), GSTM, phospholipid hydroperoxide glutathione peroxidase (PHGPx) 4, voltage-dependent anion channel 2 (VDAC2).

Veaute et al. [57] found anti-acrosin antibodies in about 20 % of sera of infertile women. The specifity of the antibodies was proven with an ELISA using recombinant peptides from human proacrosin.

4.7 Oolemma Binding and Sperm-Egg Fusion

Several antigenic proteins are involved in the process of sperm-egg fusion. The knowledge on these proteins mainly originates from the experimental binding of human spermatozoa to zona-free hamster oocytes.

Francavilla et al. [20] studied the effect of ASA on the hamster egg penetration assay. They added ASA from patients to motile donor sperm, but they did not found ASA with the ability to reduce the rate of acrosome reacted sperm as well as ASA with the ability to reduce the hamster egg penetration rate.

Hamatani et al. [24] isolated and characterized SP-10, a sperm intra-acrosomal protein, which is produced specifically in the testis, but expressed in human spermatozoa only after acrosome reaction. A mAb to this protein inhibited sperm-oolemma binding in the zona-free hamster egg penetration test, but it did not inhibit sperm-zona binding in the hemizona assay. Margalit et al. [43] demonstrated that SP-10 is a member of the prostate and testis expression (PATE)-like proteins (Fig. 4.3). A polyclonal antibody against the PATE protein did not influence zona binding, but inhibited sperm-oolemma fusion in an appropriate assay. A vaccine evolved against several recombinant human acrosomal proteins including SP-10 was able to reduce fertility in female macaces [34]. *PPT Margalit downloaded*

Testicular protein TPX-1, also known as CRISP-2, is a cysteine-rich secretory protein specifically expressed in the male reproductive tract. After in vitro capacitation and ionophore-induced AR, TPX-1 is demonstrable in the equatorial segment of the acrosome. When a hamster-oocyte penetration test was performed in the

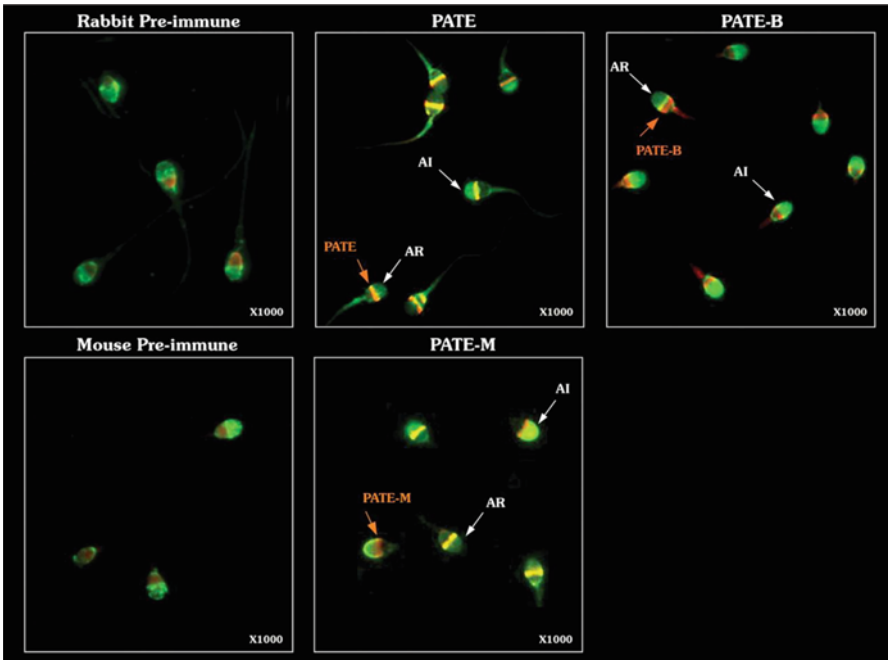

Fig. 4.3 Expression and localization of PATE, PATE-B, and PATE-M on acrosome intact (AI) and acrosome-reacted (AR)-ejaculated human sperm cells. Sperm cells were incubated with polyclonal antibodies directed against each PATE-like protein and with PSA-FITC (*green color*) for acrosome staining. Antibodies that bound the PATE-like proteins were detected with rhodamine-conjugated secondary Ab (*red*). The acrosome was considered to be intact when the anterior half of the head of a sperm cell was fluorescent bright green. Relevant negative controls (pre-immune sera) for the rabbit anti-sera and mouse anti-sera are shown. A band-like pattern was detected in the equatorial zone for PATE and PATE-M and at the post-acrosomal region for PATE-B in AR and in the AI sperm cells. No similar staining of PATE-like proteins was seen in the control cells, and only a non-specific smear was occasionally seen in the heads of control sperm cells. Magnification ×1000 (Reproduced from Margalit et al. [43]; with permission)

presence of anti-TPX1, the percentage of penetrated hamster oocytes was decreased, without affecting sperm motility [11]. Brunner-Agten et al. [10] described a surprising co-incidence: the ASA binding to CRISP-2 cross-reacted with a specific venom of wasps (VES v5).

The Izumo protein is a specific integral part of spermatozoa, which was firstly shown to be an essential sperm membrane protein for sperm-egg-fusion. Inoue et al. [27] identified a new antigen by separation of the crude extracts from mouse sperm by two-dimensional gel electrophoresis and subsequent immunoblotting with a monoclonal antibody that specifically inhibited the sperm-oocyte fusion process. It was a testis (sperm)-specific 56.4-kDa antigen, the corresponding protein in humans had a size of 37.2-kDa. They termed the antigen ‘Izumo’ after a Japanese shrine dedicated to marriage. The registered DNA sequence was confirmed by sequencing after polymerase chain reaction with reverse transcription (RT–PCR) with total RNA prepared from the

testis. A human homologue was found as an unverified gene in the NCBI database (accession number BC034769). The gene encodes a novel immunoglobulin superfamily (IgSF), type I membrane protein with an extracellular immunoglobulin domain. By immunofluorescence, the Izumo protein was not detectable on the surface of fresh sperm, but only after the acrosome reaction. When a polyclonal anti-human Izumo polyclonal antibody was added, no fusion of human spermatozoa to zona-free hamster eggs was observed. The folate receptor 4 (folr4) was identified as the counterpart for binding of Izumo in the mouse egg, and it was proposed to denominate it as Juno [6].

The relevance of the Izumo protein for fertilization was underlined by the experiments of Naz [47], who was able to show that immunization of female mice with peptides derived from Izumo and other sperm-specific proteins were able to induce antibodies exerting a long-term contraceptive effect.

4.8 Pronucleus Formation

Oocytes fertilized with ASA-bound sperm demonstrated abnormal cleavage of the embryos. The antigens involved had a low molecular weight of 14, 18, and 22 kD [46]. At gamete fusion, the sperm tail is incorporated into the ooplasm, and the centriolar region forms the sperm aster. ASA against proteins of the centrioles may be responsible for mitotic arrest [52].

The testis form of the human nuclear antigenic sperm protein (tNASP) is a testicular histone-binding protein of 787 amino acids to which most vasectomized men develop ASA. In a study using recombinant deletion mutants spanning the entire protein coding sequence 20/21 sera had ASA to one or more of the NASP fusion proteins. These may be the cognate antigens of ASA in vasectomized men. The clinical relevance of this antigen as well of their antibodies remains unclear [5].

The antibody against NASP may result in reproductive failure. In the mouse, ASA inhibited sperm-egg binding and fusion and there was a significant antifertility effect of these ASA in vivo [59].

Conclusions

As practical consequences of the research on ASA related sperm proteomic those ASA will be identified, which decrease male fertility by inhibiting sperm functions that are essential for fertilization (Fig. 4.4). The presence of antibodies in a biological substrate (serum, seminal plasma) that bind to specific antigens may be visualized by an ELISA or a RIA. In contrast to the earlier immunoassays, however, the antigens used will be defined proteins or peptides. Since ASA of an individual patient bind to up to ten different proteins, in a patient with a significantly positive MAR test or IBT up to ten different ELISA's have to be performed in order to decide whether the patient suffers from immune infertility.

The identification of functionally relevant antigens is a prerequisite for treatment options. At time, no antibody-specific treatment of autoimmune diseases is possible, but the treatment is based on the suppression of antibody production in

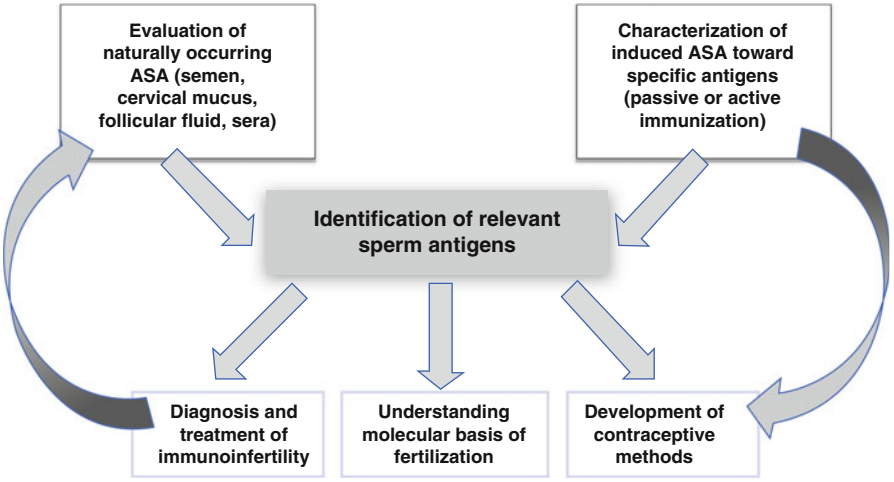

Fig. 4.4 The identification of sperm antigens relevant to fertilization by the binding of naturally occurring ASA in semen, follicular fluid, or serum contribute to the understanding of the molecular basis of fertilization, an improvement in the diagnosis and treatment of immunoinfertility, as well as to the development of contraceptive methods (Reproduced from Vazquez-Levin et al. [56]; with permission)

general. Increasingly, however, the use of monoclonal antibodies in autoimmune diseases is described. It may be speculated that this procedure also may be adapted to the treatment of autoimmune infertility.

The analysis of the cognate antigens of ASA involved in the process of fertilization is important from another point of view: it improves the identification of immunogenic proteins being candidates for immune contraception, i.e. which allow the artificial induction of antibodies in male or female inhibiting fertilization. Some of the possible approaches will be discussed in Part 4 of this book.

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Part II

Antisperm Antibodies (ASA)

The Immune Privilege of the Testis

5

Monika Fijak, Sudhanshu Bhushan,
and Andreas Meinhardt

Abstract

The onset of spermatogenesis at puberty represents unique challenges to the immune system as neoantigens of meiotic and haploid germ cells appear long after formation of systemic self-tolerance. The protection of germ cells from autoimmune attack, the "immune privilege" of the testis, was originally attributed both to the existence of the blood-testis barrier and to a failure of the testicular immune system to respond to antigens. Recent research has now shown that the testis is by no means ignorant, but can mount well-balanced immune deviant responses that can protect the gonad from damaging inflammatory responses to pathogens. Moreover, an excessive immune response can lead to inflammatory-based male factor infertility. The mechanisms controlling immune privilege seem to involve factors that also control spermatogenesis and steroidogenesis. They appear to include androgens, a delicate balance of immunomodulatory molecules such as cytokines and chemokines and a polarizing capacity of the testicular interstitial fluids towards a tolerogenic M2 phenotype.

5.1 Introduction

Male germ cells enter meiosis beginning their complex transition into highly specialized spermatozoa at the time of puberty after the establishment of immune competence. During the process, a myriad of surface and intracellular proteins is

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expressed, yet these new autoantigens are tolerated by the testis. The immunogenicity of the proteins is not diminished, as shown by their ability to induce strong autoimmune reactions when injected elsewhere in the body [1, 2], rather it is the testis itself that confers protection. Initial suggestions that the testis was an immune privileged site were substantiated experimentally when histoincompatible allo- and xenografts placed into the interstitial space of the rat testis survived and prospered for indefinite periods of time [3]. Similarly, ectopically transplanted allogeneic Sertoli cells not only survive, but when co-transplanted with allogenic pancreatic islets, resist rejection without additional systemic immunosuppression in animals [4]. More recently, the transplantation of spermatogonia in germ cell depleted testis could restore spermatogenesis even across species borders in some instances [5]. There is general agreement that immune privilege is an evolutionary adaptation to protect vulnerable tissues with limited capacity for regeneration, thereby avoiding loss of function [6, 7]. For the testis, this means safeguarding reproductive capability. Notwithstanding its immune privileged status, the testis is clearly capable of mounting inflammatory responses, as proven by its effective response to viral and bacterial infection. In pathological circumstances, the misbalance between the tolerogenic and the efferent limb of the testicular immune response can lead to the formation of autosperm antibodies and in rare instances, autoimmune or granulomatous epididymo-orchitis in humans. All facets of immune infertility together are now estimated to be a considerable cause of childlessness in couples seeking medical assistance [8–12].

The most common used models for the investigation of inflammatory infertility are (1) infection models mimicking acute epididymitis and orchitis by injecting live bacteria or lipopolysaccharide and (2) models of sterile chronic, progressive inflammation, namely, experimental autoimmune orchitis (EAO), a rodent model based on active immunization with testicular homogenate and adjuvants [13]. The clinical term “orchitis” is particularly attributed to acute, symptomatic disease due to local or systemic infection, whereas subacute or chronic, asymptomatic inflammation of the testis including noninfectious disease is difficult to diagnose and therefore likely to be ignored [14]. Infection-based epididymitis and orchitis mainly represent manifestations of sexually transmitted diseases such as gonorrhea or *Chlamydia trachomatis* or pathogens typically causing urinary tract infections such as *Escherichia coli* pathovars with uropathogenic *E. coli* as the most prevalent [15, 16]. The most common cause of viral orchitis is mumps. On balance, these data clearly indicate that the mechanism underlying immune privilege in the testis and its disruption by pathological alterations are matters of clinical importance and hence continued scientific interest. The following chapters highlight some of the mechanism that are associated with the establishment, maintenance, and disruption of immune privilege.

5.2 Mechanism of Maintenance and Disturbance of Testicular Immune Privilege

5.2.1 Role of Blood Testis Barrier and Sertoli Cells

The formation of the blood testis barrier (BTB) by Sertoli cells has long been assumed to be the most critical, if not sole factor, determining testicular immune

privilege. The BTB is formed by tight junctions connecting adjacent SC and gap junction proteins, namely, junctional adhesion molecules (JAMs), claudins 1 and 11 with claudins 3-5 and claudins 7-8 also identified in the testis besides occludin [17]. The BTB barrier divides the seminiferous epithelium into two distinct compartments: the basal compartment containing the spermatogonia and preleptotene spermatocytes, while more advanced spermatocytes, secondary spermatocytes, haploid spermatids, and spermatozoa are found in the adluminal compartment. The main task of the BTB is protecting or sequestering auto-immunogenic germ cells from the systemic immune response.

In addition to BTB formation, SC maintain the immune privilege of the testis by skewing immune responses by producing immunosuppressive molecules such as PDL1, Tyro3, Axl; MER and IDO [18–22]. The immunoregulatory repertoire of SC also contains constitutively expressed immunosuppressive cytokines like transforming growth factor (TGFß) and activins [23, 24]. Mechanistically, elevated levels of tumor-necrosis factor (TNF)-α and transforming growth factor (TGF)-β, found in systemic and local testicular inflammation [25–28], have been shown to perturb the assembly of the tight junctions in cultured Sertoli cells probably by downregulating occludin expression [29, 30]. Despite the junction's ability to isolate meiotic and postmeiotic germ cells from circulating antibodies and leukocytes, it is now accepted that the blood-testis barrier alone does not account for all the manifestations of the testicular immune privilege. It was supported by the findings that germ cell autoantigens that germ cell autoantigens are present in the basal compartment in spermatogonia and early spermatocytes, which are not protected by the blood-testis barrier [31, 32]. Moreover, the blood-testis barrier is incomplete in the rete testis, a location where immense numbers of spermatozoa with newly adapted surface molecules traverse towards the epididymis, making it a particularly susceptible region for the development of autoimmune orchitis. Furthermore, Head and Billingham [33] showed extended survival (i.e., no immune response/attack) of allografts that were placed under the organ capsule in the testicular interstitium. Therefore, some other mechanism, beside physical separation, must exist to maintain testicular immune privilege, which requests more robust protection of the tolerogenic environment of the testis.

5.2.2 Macrophages

Besides playing a crucial role in innate immune response, macrophages are also essential for maintenance and regulation of organ homeostasis. In steady state condition, testicular macrophages (TM) are exclusively found in the interstitial space; in human they can be located also in the tubular wall, but never within the seminiferous epithelium. In fetal testis development, TM have nonclassical functions in organogenesis by regulating the vascularization of the organ and spermatogenic chord formation [34]. In addition, TM seem to show a direct effect on spermatogenesis by influencing spermatogonial stem cell differentiation [35]. TM maintain the immune privilege of testis by secreting substantially lower amounts of pro-inflammatory cytokines (TNF-α, IL-12) in response to inflammatory stimuli, whereas concomitantly high amounts of the archetypical anti-inflammatory cytokine IL-10 are

produced [36]. Of note, TM constitutively express low levels of the toll like receptor gene family and suppress the pro-inflammatory NF-kB signaling pathway by blocking the degradation of IkB alpha (inhibitor of p65). While blocking NF-kB signaling effectively, a more moderate innate immune response is triggered alternatively by activation of AP-1 and CREB signaling pathways [36]. Taken together, TM play an important role in normal function and development of the testis. There is little doubt that macrophages take a central role in the establishment and maintenance of the testicular immune privilege. This supposition was first substantiated by in vitro studies, in which TM displayed a reduced capacity to synthesize IL-1β and TNFα compared to macrophages from other tissues and exhibited overall immunosuppressive characteristics [37–40]. In the rat testis, at least two subsets of macrophages can be discerned. This heterogeneity has functional implications as in the testis the ED1+ "inflammatory" subsets, but only few ED2+ resident macrophages, express MCP-1 and iNOS in untreated and LPS challenged rats [41, 42]. The ED2+ resident population of testicular macrophages does not participate in promoting inflammatory processes; it is thought to have an immunoregulatory role in maintaining immune privilege and trophic functions, particularly on Leydig cells. Clear evidence points out that the ED1+ ED2− monocytes/macrophages are involved in the testicular inflammatory response, and it is the influx of ED1+ monocytes during acute and chronic inflammation, which drastically alter the composition of the macrophage population and shift the cytokine balance in favor of an inflammatory response with the potential to overcome the immune privilege [41–43] (Fig. 5.1).

5.2.3 Dendritic Cells (DC)

DC belong along with macrophages to the most important antigen presenting cells (APC) and play a major role in the initiation and orchestration of primary immune responses. DC not only activate lymphocytes, but also tolerize T cells to antigens, thereby minimizing autoaggressive immune responses [44].

In normal mouse, rat, primate, and human, testis DC are found in very limited numbers [45–49]. In contrast, in the inflamed testis, the numbers of DC are significantly upregulated as shown in a rat model of experimentally induced autoimmune orchitis (EAO), where elevated levels of DC were found in the interstitial space of the testis and in testicular granulomas and lymph nodes draining the testis [46, 47, 50]. Similarly, in human azoospermic testis with chronic inflammation, higher numbers of IL-23 producing CD11c+ DC could be detected [49].

Immature DC have the highest capacity to internalize antigens, but low T cell stimulatory activity, whereas mature DC downregulate their endocytic activity and are excellent T lymphocyte stimulators [51]. Interestingly, the levels of co-stimulatory molecules (CD80, CD86) and MHC class II molecules on the DC surface from normal and inflamed rat testis are similar [46]. However, the expression of chemokine receptor CCR7 responsible for the migratory behavior to the lymph nodes was upregulated in DC from inflamed testis. Furthermore, the expression of IL-12p35 and IL-10 mRNA was detectable only in DC from EAO testis and draining lymph nodes pointing to their

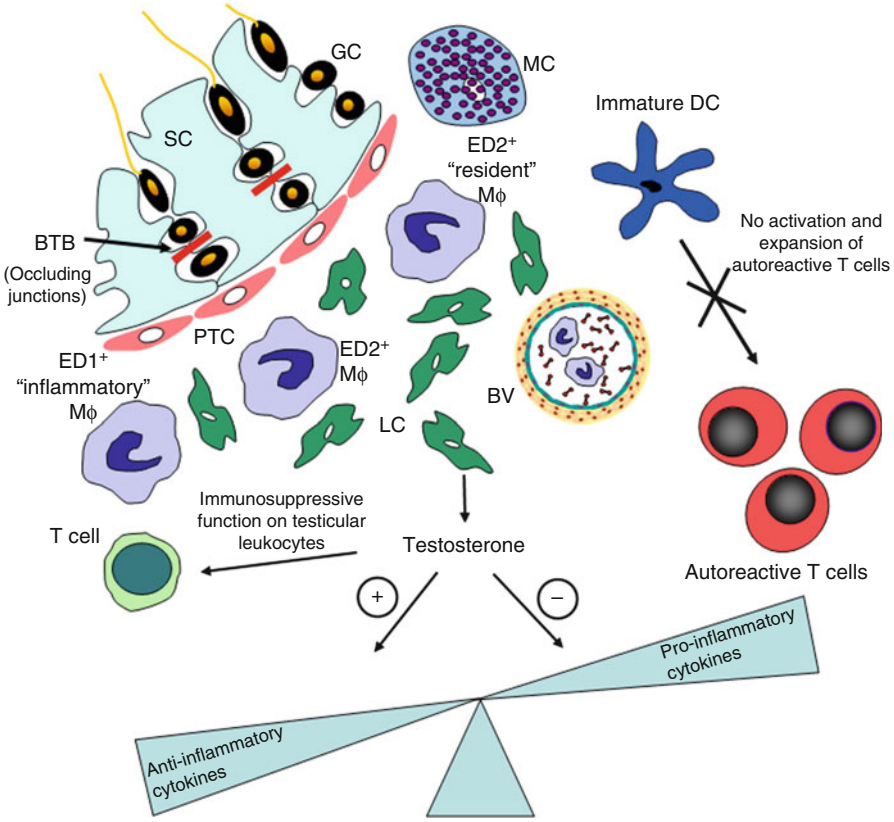

Fig. 5.1 Hypothetical model of factors maintaining the testicular immune privilege. The blood-testis barrier (*BTB*) connects neighboring Sertoli cells (*SC*) and segregates the majority of neoantigen expressing meiotic and postmeiotic germ cells (*GC*) from the testicular immune system. In the interstitial space, the ED2+-resident type of macrophages (*Mφ*) with its immunoregulatory and trophic functions constitutes the largest subpopulation of leukocytes, whereas the ED1+ "inflammatory" macrophage cohort is much smaller in number. Most likely the phenotype of testicular dendritic cells (*DC*) in normal testis inhibits an activation and expansion of autoreactive T lymphocyte clones. The concentration of testosterone in the testicular interstitial fluid synthesized by the Leydig cells (*LC*) is about 8–10 times higher than in serum. Recent data point to an increasingly important immunosuppressive role of androgens by inhibiting leukocyte function and by reducing of pro-inflammatory cytokine expression. *BV* blood vessels, *PTC* peritubular cells, *MC* mast cells

mature immunogenic state [46, 50]. At the functional level, DC isolated from EAO testis and draining lymph nodes significantly enhanced the proliferation of effector T cells compared with control DC, suggesting a more tolerogenic phenotype for DC in normal testis function, thereby maintaining immune privilege (Fig. 5.1) [46, 50]. Based on existing data, it seems that during development of testicular inflammation DC acquire a functional mature phenotype, take up testicular antigens, migrate to the lymph nodes, and stimulate antigen-specific T cell responses, thus initiating autoimmune responses in the testis leading to immunological infertility.

5.2.4 T Lymphocytes

In normal testis of human and experimental animals, the population of lymphocytes comprises 10–20 % of total leukocytes. Testicular T cells consist mainly of CD4+, CD8+, and CD4+ CD25+ Foxp3+ regulatory (Treg) T cells [48, 52–54]. Immunosuppressive factors produced locally in the testis such as IL-10, TGF-β, activin A or lyso-glycerophosphocholine are believed to promote diminished responses of testicular T cells leading to prolonged tolerance [48, 55–57]. A hallmark of disturbed immunological balance during testicular inflammation is significantly increased numbers of CD4+ and CD8+ T cells as well as Treg cells [58–60]. There is increasing evidence that testicular CD4+ CD25+ Foxp3+ Treg are important players in the maintenance of testicular immune privilege. Interestingly, data from our own in vivo and in vitro studies showed a stimulatory effect of testosterone on the expansion of Treg cells in the testis [59, 61]. Moreover, factors produced by cultured Sertoli cells like TGF-β trigger de novo differentiation of fully functional Treg cells [62].

5.3 Endocrine Regulation of Testicular Function and Immune Privilege

In addition to the well-established anabolic and spermatogenic effects, a role for androgens in downregulating pro-inflammatory responses has now been shown in both experimental and in clinical studies. Incubation of several immune and nonimmune cell types with testosterone resulted in the suppression of adhesion molecules and cytokines such as IL-1, IL-6, and TNFα and increased production of anti-inflammatory cytokines such as IL-10 [63–69]. Testosterone is also involved in T cell apoptosis [70]. A direct connection between sex steroid levels and testicular immune privilege was shown by Head and Billingham [71], when in transplantation studies, rats pretreated with estrogen to suppress Leydig cell testosterone production, promptly rejected intratesticular allografts in contrast to tolerance towards the grafts in untreated cohorts. In interventional studies, testosterone supplementation of hypogonadal patients with Crohn's disease led to reduced protein C levels and inhibition of chronic inflammation [72, 73]. These findings were supported by evidence delivered from our earlier studies, showing that substitution of reduced testosterone levels during rat EAO inhibited the disease development and caused reduction of TNF-α, IL-6 and MCP-1 levels in testis. Furthermore, the number of TM and CD4+ T cells was significantly decreased with concomitant increase of Treg cells as compared to untreated EAO animals [59]. Further in vitro studies confirmed an inhibitory effect of testosterone on inflammatory responses in Sertoli and peritubular cells [61]. These studies indicate that the high local testosterone concentrations characteristic for the testis seem to play an important role in the maintenance of testicular immune privilege.

What can be surmised from the available data is that androgens appear to exert their immunosuppressive function on testicular leukocytes by stimulating the differentiation

of Treg cells and by regulating the balance of pro- and anti-inflammatory cytokine expression in Sertoli, Leydig, and peritubular cells.

Conclusions

There is now widespread agreement that the immune system, spermatogenesis, and steroidogenesis, and the intrinsic testicular functions are intricately linked by a network of complex interactions. The importance of the delicate balance needed between the suppression of the immune response to protect the germ cells from autoattack on the one hand and the ability to an active immune response to prevent damage from infection, trauma, and cancer on the other is reflected by the fact that in the human male about 12–13 % of all diagnosed infertility is related to an immunological reason. It needs to be considered that the incidence can well be higher as the contribution to idiopathic infertility (31 % of all cases) still remains elusive [9–12]. The mechanisms responsible for testicular immune privilege are still far from being understood, but it is apparent that the identified factors involved are multiple. According to recent progress, androgens and possibly other steroid hormones are likely to play a major role [61, 74]. Overall, long regarded as a peculiar side issue of testis function, immune privilege is now established as part of the general scheme of male gamete formation and successful reproduction. Further research in the area will not only help to improve diagnosis and treatment of immunological based male infertility, but will also open new avenues in contraceptive development and transplantation medicine.

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Immune Chemistry of ASA

6

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Abstract

Immune chemistry of ASA encompasses all the types of antisperm antibody reactions in humans that are ontogenically driven by (a) 'natural' antibodies, (b) pre-pubertally derived ASA due to testicular failure, (c) adult ASA in males and females, and (d) carbohydrate-mediated epitopes. Both sexes (with exception of boys before puberty) present active immune tolerance to male gametes which allows to resume a natural intercourse as well as permanent gamete differentiation in spermatogenesis. 'Natural' antibodies are developed as a part of immune history of the individual but they are also essential elements of innate immunity in both sexes. ASA developed before puberty are well characterized by proteomic approach and deserve more attention. ASA reactions in males and females are fairly well studied while carbohydrate-mediated chemistry of ASA activity is still a complex issue under study.

6.1 Introduction

Antisperm (ASA) antibodies can be recognized as a strange immunological phenomenon. First of all, the immune response to spermatozoa is rarely sperm-specific; on the other hand, it cannot be also considered as a part of polyorgan type of

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reaction since rarely such cross-reactivity was ever found. In the older literature, ASA were occasionally linked to react with kidney (rabbit) or thyroid (beagle dog); however, such cross-reactions were not generally confirmed in mammals as long as sperm antigens were not enhanced during vaccinations in female immunocontraceptive trials where it was found the molecular similarity of sperm (PH20) protein to kidney antigens [25]. Furthermore, antisperm antibody reaction is different in ontogenesis since only during puberty sperm antigens seem to appear. ASA can be then considered as the enhancement of naturally existing 'natural' antibodies. Since the onset of puberty we may treat ASA as the mature response to gametes of adult individuals which may interfere with infertility. Discrepancy of ASA reactions, however, still exists between female and male subjects as well as regarding its prepubertal phenomenon. This brings us to the serious question on a nature of ASA, are they unique or too common (from immunological point of view), why are they so rarely sperm-specific, are they mostly mediated through the carbohydrate moieties? The first subdivision in their chemistry of reaction (antigenic epitopes) would be therefore ontogenically based on (a) circulating 'natural' autoantibodies, (b) antisperm antibodies developed pre-puberty, (c) ASA developed in females, (d) ASA developed in males, and (e) carbohydrate-mediated ASA antibodies with different degree of molecular identity (including 'molecular mimicry').

6.2 Natural Autoantibodies

Investigating both the female sera and their genital secretions it was found the presence of the antibodies with broad specificity to the so-called 'public' antigenic determinants. These were often consisted of carbohydrates that have properties to induce IgM, pentameric antibodies reactive to the range of cells carrying common epitopes (carbohydrates). It has been then discovered through the technique of monoclonal antibodies that very often that antibodies of IgM class were the most prominent ones in detecting common sugar structures although affinity of such antibodies was not of a very high magnitude. Thus spermatozoa with a richly developed glycocalyx were one of the cells commonly reactive with such antibodies. One of the hypotheses of female genital tract formulated by Hancock regarded such 'natural' broadly cross-reactive antibodies as the one of the elements of female isotolerance to sperm [23]. Although a definition of 'natural' autoantibodies was questioned in existing literature at least several times they were subsequently re-discovered by Paradisi et al. [45, 46], where at the opportunity to detect novel sperm entities by Western immunoblotting they found at least two strange phenomena, (1) intensity of reactions from fertile and infertile (but not infertility-mediated) polyclonal human sera were different with fertile vs infertile sperm, and (2) fertile sera represented certain fraction of positive reactions with sperm originating from in vivo fertile males. Such phenomena were then frequently reported by various other groups which reported positive reactions both by Western immunoblotting and 2-D electrophoresis when using polyclonal human sera from some of the ASA-negative donors that precipitated distinct spots [15, 42, 50, 51]. In our observations with Western

immunoblotting technique [11, 13], we have found quite broad phenomena of positive reactions presented by ASA-negative human sera at the same time identifying the activities both to sperm as well as to human lymphocytes or erythrocytes, proving once more the character of previously formulated reactions by 'natural' antibodies. Although the development of such antibodies would have most probably take place through 'molecular mimicry', it is clear that these 'public' determinants may mask the more specific immune autoantibody response when sperm would appear around puberty.

This could have also reflected potential history of immunological reactions towards variety of infectious diseases becoming remnants of earlier immunological history and dependent both on constitutional MHC predisposition as well as geographical region inducing different profiles of bacterial strains and vulnerability to them [43]. According to Paradisi et al. [47], some of these specificities represented by 'natural' antibodies may be enhanced through the breakdown of physiological tolerance what may augment such reactions to the pathological level, so he formulated some of these 'public' specificities as the ones belonging to the both groups ('natural' and the 'pathological' ones), thus identifying three categories of sperm antigens: (1)'natural' ones (appearing in fertile individuals), (2) the infertile ones (assigning immune pathological reactions), and (3) enhanced reactivities derived from the first category. This could be particularly true (category 3) while looking at the antibodies reacting with sperm, which at the same time recognize antigens on lymphocytes and erythrocytes. They can be molecularly differentiated based on antigenic (carbohydrate) isoforms, which may then dissociate into antisperm and anti-lymphocytic reactions (CD52 antigen). It is unquestionable that both types of CD52 may have completely different functional meaning [12].

Another proof for 'natural' antibodies originated from the studies on antisperm antibodies developed pre-puberty. Although became recently evident that even prepuberty we may find testicular specific antigens responsible for immunological reactions initiated by testicular pathology (cryptorchidism, torsion, mobile testis(es)), it was previously found in mammals that puberty (sperm appearance) induces physiological reactions which all of sudden become enhanced just at this age. Brilliant documentation of this phenomenon was done in rodents model [18] (most of it in rats) where it was indicated that serum antisperm antibodies increased only after sexual maturation what may suggest that some differentiation antigens on sperm are processed and presented to the immune system under the normal circumstances (at least in some strains). Interestingly, it may be added that in our studies we indicated enhanced (as comparing to adults) antisperm antibody reactions prepuberty (in normal boys) while Tung et al. [57] reported it in both sexes (surely through 'molecular mimicry' mechanism to microorganisms expressing similar carbohydrate groups). Physiological, sperm antigenic 'leak' documented in variety of rodents (guinea pig, rats, rabbits, dark mink) could be an important mechanism reassuring immune tolerance to newly appearing cells (sperm).

Considering cell mechanisms of immunological tolerance mainly developed at puberty (germ cells appearance), it cannot be excluded that the certain level of antisperm antibodies being subfraction of 'natural' antibodies can precipitate sperm

antigens allowing them to be typed by 2-D electrophoresis including mass spectrometry identification. In one of our recent papers [44], we have managed to identify 13 sperm entities detected by sera samples delivered from male, fertile individuals.

Finally, coming back to the original statement, it has to be emphasized that the level of 'natural' antisperm antibodies is ontogenically driven and independent on the level of auto- and isoimmune reactions to sperm in adults. It begins from the relatively high values (until puberty) declines thereafter in order to reach another peak post-40 and finally declines with very advanced age (>88 years) what illustrates the general immunity phenomenon and its regulatory feedback (possibly through Treg population) [29].

6.3 ASA Antibodies Developed Pre-puberty

In the scientific literature, we may find only few reports (often contradictory) on the existence of ASA in pre-pubertal boys with testicular failures [10, 41, 53]. Since until now there are undefined objective 'markers' allowing prognosis of future fertility status in these boys, it is postulated to carry on surgical procedures (orchidopexy) in cryptorchidism, undescendent testis or mobile testis at the earliest opportunity as well as to suppress (possible with steroids or anti-globulin therapy) the immune response to eliminate a risk of high levels circulating ASA that may persist until puberty. Urry and co-workers [58] have observed circulating ASA in infertile males suffering at childhood on one or both undescendent testis(es). ASA were present in 66 % of such infertile males when comparing to 2.6 % or 2.8 % in groups consisting of infertile individuals without testicular failures or in vivo fertile men. In the patients with cryptorchidism in the past, it was also observed low sperm quality (low sperm count, low sperms with progressive motility). It could be therefore suggested that developed pre-puberty antibodies may mature during different episodes of ontogenesis (including somatic mutations) and then may react with antigens of mature spermatozoa being a reason for low sperm quality connected with infertility [4].

Undescendent testis(es) are located in the conditions of elevated temperature [40] comparing to scrotum, so it might induce the development of degenerative changes in spermatogenesis or may lead to unique exposure of membrane (integral) antigens in germ cells (spermatogonial stem cells) already present in the gonads. It can be also considered the change in Leydig cells functioning at elevated temperature what may alter the local synthesis and secretion of sex hormones (testosterone) [39]. Altogether, this may induce destructive immune response that could be initiated in favoring conditions of the lack of appropriate populations of T regulatory cells that are not triggered due to the absence of spermatozoa. It is also well known that location of the testis in abdomen may cause enhanced activity of proto-oncogenes what may result in increased frequency of testicular cancer [54].

As we know, the blood-testis barrier is not formed until the signaling from the initiated spermatogenesis, thus circulating ASA antibodies induced through the elements of testis may freely traverse the pre-pubertal gonad and such phenomenon seems to be a dose-dependent when testicular failure has been observed [31].

Interestingly, very often after the removal of the one gonad (involved in pathological process) it is observed the significant decrease of the circulating ASA titre in comparison to pre-operative stage. It could be speculated that gonads are both inducive as well as the sorbent elements of the ongoing immune response. It could be also reasonable to speculate that persisting ASA may be enhanced at the time of puberty (appearance of sperm) while being on the wrong side of the barrier. At the same time, the 'molecular mimicry' due to the past experience of infections ('mumps') may be still an active component. It is quite reasonable therefore to warn against the using of bacterial wall non-specific lysates as the form of vaccination in boys around puberty [36].

It has to be pointed out that 'natural antibodies' can be enhanced and/or their background may be the significant obstacle in the proper detection of the pathognomonic levels of ASA during that period of time. There is not established a reliable 'cut off' value that would argue for suppression of immune response in such 'threatened' boys, specifically that widely accepted immunobead test (IBT) does not serve to the purpose. Conversely, new assays are required that would bring positive predictive value for the later observed decrease in sperm quality. In our studies on ASA developed pre-puberty, we have used at least five assays (IBT, MAR test, immunosorbent assay, flow cytometry, Western immunoblotting) to conclude on the nature of the observed ASA [10] due to visible and significant inter-individual variability in ASA levels. The sperm epitopic changes must be taken under consideration due to the subtle mechanisms of low sperm immunogenicity and possibly low affinity at the initial stages of immune response development. Sperm partial denaturation (fixation reagents) as well as assays sensitivity and specificity should be carefully defined in each out of applied tests. Western immunoblotting versus flow cytometry on live sperm could be interesting to be reciprocally validated. Furthermore, additional risk factors determining appearance of antisperm antibody before puberty must be considered concomitant to testicular failures inducing ASA. Recently, we have considered different allelic gene variations belonging to Class II HLA system in boys with cryptorchidism and associated ASA [37]. Some alleles (DRB1*04) were predisposing while other protective (DRB1*06) in respect to ASA development and their frequency.

The notion that deserves the very careful attention is the fact that apart of the possibility raised towards the 'natural antibodies' enhancement' in pre-puberty cases one has to admit that mass spectrometry made out of some of the spots reactive with polyclonal sera (from pre-pubertal boys) indicated several sperm-specific entities [15]. This may argue against no biological meaning of low affinity circulating ASA antibodies and its putative limited interference with reproductive events.

6.4 ASA Antibodies in Females

Immunological tolerance has been also an important and strong factor in females. The mechanisms of quick elimination of deposited spermatoza belong to the arm of the innate immunity. The occurrence of so-called 'natural antibodies' (see, above)

is one of them. Influx of leukocytes after semen deposition (clearing mechanism), opsonization of sperm and engagement of local lymph nodes (rather in favor of tolerance than immunization) should be here mentioned [24]. However, we have to realize that both in males and females, the reproductive system is in the close contact with exogenous antigens (microorganisms, antigens of natural and artificial nature, also haptens and allergens), so both arms of immunity, the innate and adaptive one have to be incredibly precise in order to differentiate between the pathogenic antigens and sperm which periodically (but in big numbers) enter the reproductive tract. No wonder that all the stimuli reaching cervix and cervical canal may affect the labile environment of selective immune tolerance. Yet, whatever local disorder is created as cervical infection, its erosion, neoplasma and hysterectomy provides condition of the immediate danger to initiate the response towards spermatozoa.

The chemistry of female isoimmune response is absolutely intriguing and is distinctly different than autoimmune response in males. First of all, whatever we would like to say about the common carbohydrate structures on sperm and other cells, the female isoimmune response does not follow the normal pattern of reaction. Such a difference is reflected, first of all, with some readiness to differentiate among the partners [61] but at the same time not being a sperm-specific. In our earlier work we aimed to analyze ASA-positive infertile individuals of both sexes, finding numerous false-positive reactions in female sera (but at the same time not being able to trace the polyclonal ASA in cervix) and not being able to core immune-reactive spots present in 2-D to the mass spectrometry (the rate of success was far more less than for male sera). Yet, recently some evidences appear that fractionated sera of women with fertility disorders may become a tool for sperm private-entity identification [3]. In this particular study family of antigens belonging to heat-shock proteins, some of them private-sperm isoforms were detected. Another immunodominant sperm antigen (alpha enolase) strongly appears in several studies to be repeatedly documented [3, 15]. Yet, very recently, we have managed to identify three antigens involved in fertilization recognized by immune sera of infertile women [44] among then: TCP-1-theta (T complex protein 1 subunit theta isoform 1), arylsulfatase-A and arrestin domain containing protein 5 (ARRDC5). Arrestin contains chains that was previously reported to be reactive (and sperm-specific) on women suffering from systemic lupus erythematosus [14].

At the same time, we have to be careful about the effective local immune response, as the frequency of ASA in cervical mucus (>10 %) is greater than in serum (5–8 %) when analyzing the same Caucasoid populations [5]. Speaking of ability of female local immune response to differentiate among the individual protein isoforms at the same time not being so much sperm-specific creates a space for the statement that most of this immune response in females interfere with sperm on the basis of ‘molecular mimicry’. Yet, we may find a lot of differences between subtypes of female immune response regarding carbohydrate-mediated reactions as sperm agglutination and immobilization. As it was, in relatively old literature reported, the lectin-mediated sperm agglutination was mostly induced through terminally positioned common sugar residues while sperm-immobilization test [27] appears as the

far more selective one, therefore not being considered as a good ASA screening assay. Yet, sperm-immobilizing antibodies also react to sperm carbohydrates.

The most representative cycle of experiments of female ASA isoimmune response originates from the work of Isojima and Koyama groups dissecting the molecular epitope of one of the glycoprotein, coded as CD52, present on both sperm and somatic cells (lymphocytes). Three classes of highly specific antibodies were developed to this epitope (first originated from woman with circulating sperm-immobilizing antibodies) [26]. Originally obtained MAb of heterohybridoma cell line was coded as H6 3C4 (This epitope can be removed by N-deglycosylation). Similarly, S-19 antisperm monoclonal antibody developed in Herr group was generated against sperm CD-52-specific N-glycan's and not cross-reactive to lymphocytic CD-52 [7]. Next two antibodies, 1G12 (IgM mouse monoclonal antibody with sperm-immobilizing activity) and Campath-1 (IgM monoclonal antibody) were then obtained, both recognized CD52 molecule, albeit with different specificity. The first one (1G12) reacted with structure formed by the GPI anchor and/or peptide portion while Campath 1 epitope included COOH-terminal three amino acid sequence on the core peptide. It was then provided (by 2-D electrophoresis) different pattern of reaction, including (in case of 1G12) most possibly the reaction with three spots of O-linked carbohydrates while in case of Campath-1 carbohydrates were not involved therefore both lymphocytes and spermatozoa were recognized. It is extremely interesting that when antiserum has been produced by immunization with CD52 core protein then it reacted to sperm causing sperm agglutination and complement-dependent sperm immobilization as well. Thus, different portions of CD52 molecule including carbohydrate moieties, core peptide and GPI anchor may induce ASA which may interfere with sperm. So when the studies showed that glycosylation pattern included both N-linked and O-linked carbohydrates on mrtCD52 (mrt – male reproductive tract) and they both contributed to the heterogenous negative charge of CD52 – such a structure may prevent the cells from auto-agglutination and non-specific adherence to adjacent tissues. Consequently as the female genital tract is often subjected to frequent infections with various pathogens (bacteria, viruses, protozoa) such protection from auto-agglutination seems to be a quite helpful evolutionary mechanism for sperm transport. However, the mechanism by which mrtCD52 induces ASA in female genital tract is unknown. Furthermore, it seems that N-linked carbohydrate structures are not well-recognized immunogens (similar studies on N-linked moieties were performed on auto-antibodies in males), therefore sperm immobilizing antibodies are not the most often encountered type of ASA antibodies. In summary, we may underline odd mechanistics in women raised antisperm antibodies. They could be classified to several separate types. First, due to active immune tolerance being neutral to one trillion of sperms designed for life time intercourses women rarely mount active response to free spermatozoa. However, having some T helper cells in female reproductive tract they may direct responses according to differentiated cytokine pattern [38] specifically involving IFN-y which would activate macrophages and contribute to pro-inflammatory cascade [60] against spermatozoa, specifically when they have been coated with bound male ASA.

Second, when maintained intercourse at the presence of common microbial and cross-reactive sperm carbohydrates takes place [6, 59] in both sexes. ASA may readily develop. This hypothesis has been formulated long ago in our pioneering work reporting on such cross-reactivities when characterizing big set of monoclonal antihuman sperm antibodies [35, 36].

Third hypothesis would be related to specific female predisposition when mounting autoimmune reactions (e.g. lupus erythematous) with hyperactivated humoral response as it has been previously reported [14, 55].

Finally, as we have said before, a plethora of factors may enhance immunological responses in the female reproductive tract, and although they can be led by 'natural' antibodies at first, they may fall easily (through any type of 'molecular mimicry' enhancement) to the "category 3 antigens" proposed by Paradisi [47].

6.5 ASA Antibodies in Males

Although antisperm antibodies may start to develop together with the other autoantibodies as the often quoted example of beagle dogs and associated autoimmune thyroiditis, it could be also a misleading phenomenon. This has been, however, repeated in females, in whom antisperm antibodies are mostly appearing among the other reactivities, mostly associated with thyroid, and then with the other organs as it can be encountered in women approaching IVF or those with polycystic ovary syndrome (PCOS) or premature ovarian failure (POF). These types of reactions did not become, however, a rule in respect to autoantibodies in males. It could follow well in line with lack of specificity of ASA reactions in women but it is not the essence of auto-reactions in men [15]. Indeed it is somehow a mystery that despite of the abundantly glycosylated sperm surface we can observe the formation of specific ASA (among the autoantibodies). It became very fortunate in man that unlike in the other species (guinea pig, rabbits, monkey) we may notice a certain type of protection against the ASA autoreactivities initiated by vasectomy procedure that has never became the inducer of the polyorgan type of autoimmunity. This observation did not exclude, however, the epidemiology of ASA along the other pathologies including infectious microbial agents. Interestingly in some reports, it was found the opposite type of reactions concerning males and females in respect to *Listeria monocytogenes* (mouse model) which was then associated with differential interleukin-10 production [48]. It was also more often noticed in females than in males the antisperm antibodies appearance together with chlamydial infections (often associated with infertility) [8]. It may favor the previously presented suggestion that females prefer to react more readily with 'common' (public) epitopes than the males which tend to be rather sperm-specific what can be logical taking into account the presence of sperm in males (and its absence in females). Males may drive humoral response into more mature antibodies (somatic mutations) with better affinity to sperm antigens [22] although the affinity process of ASA formation has never been directly observed (even more this would be an urgent issue when concerning the sex differences). Another

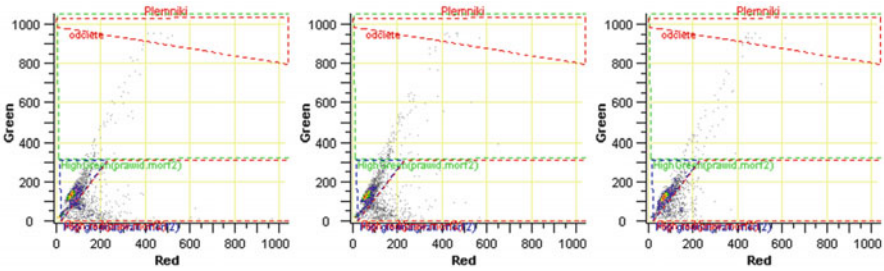

Fig. 6.1 Antioxidant therapy gradually diminishes sperm DNA fragmentation with concomitant ASA disappearance

finding was the cross-reactivity of antisperm antibodies with filarial nematode (*Litomosoides sigmodontis*) [28] as well as the development of ASA circulating antibodies in diarrhoeal diseases (shigellosis, salomonellosis) [30] or their co-existence with *Helicobacter pylori* [16] and in patients with ulcerative colitis [9]. Several questions or hypotheses might be then raised concerning the initial point of ASA formation in males. First, it was indicated the interesting pattern of elevated cytokines (innate immunity) associated with general inflammatory reactions that occurred in case of lethal filariosis which became associated with interferon (IFN) gamma, tumor necrosis factor (TNF) alpha, interleukin 12 and 6. It has to be immediately noticed the negative effect of these cytokines per se on sperm quality (IFN gamma), as well as the appearance of most of them in male reproductive tract inflammations (TNF, IL-12 and 6) associated with oxidative stress [20, 49] or with sperm apoptosis [21]. It could be therefore easily formulated the hypothesis that apart the blood-testis breakdown together with insufficient immunosuppressive mechanisms of active tolerance to sperm, the chemistry of ASA antibodies in males must have its origin (at least in some cases) in acute, chronic or latent infections of male reproductive tract together with involvement of oxidative stress. This could be a basis for autoimmune reactions that could be driven either to integral sperm antigens or 'coated' antigens or more specifically to prostasomes in case of autoimmune prostatitis [52].

Recently, further confirmation of reactive oxygen species (ROS) co-existed with inflammatory reaction and ASA development was delivered [1, 2]. In our observations (Fig. 6.1), co-existence with DNA fragmentation and ROS culminated in antisperm antibody presence which subsequently disappeared when antioxidant therapy was provided. Microbial induction as element to trigger such a cascade of events should be likely considered.

As far as the cross-reactivity of ASA antibodies is concerned, our earlier detailed work indicated that plethora of mouse monoclonal antisperm antibodies showed specificities to 'common' carbohydrates which mediated the observed reactivities both to sperm and microbial agents as *Staphylococcus aureus*, *Streptococcus viridans*, *Escherichia coli* and *Salmonella typhi* [35]. This early work suggested already 'molecular mimicry' as the important 'trigger' point for

ASA autoantibodies development. The mentioned bacteria were next analyzed in terms of in situ frequency and occurred to be (with the exception of *Salomonella)* one of the most common strains responsible for male reproductive tract infections in our cohorts as well as the potent inducers of oxidative stress reactions [19].

Apart from the fact that terminal common sugar moieties were often encountered in sperm epitopes studied (galactose, N-acetylgalactosamine, terminal acetylglucosamine, alpha-L-fucose, alpha-D-mannose) [36], it has to be again emphasized that enhancement of immune response and affinity maturation process may convert initially developing low-affinity antibodies into sperm-specific response severely affecting sperm structures [15, 22].

It is also worthy to note that the obtained through human-human hybridoma technology antisperm antibodies revealed very interesting specificities. First, Fab homology indicated antibodies similar to those reactive with HIV gp 41/120 (fusion proteins) as well as anti-CD55 (complement relevant functions) and with anti-beta galactosidase activities (the last one may be one of the sperm-oocyte receptor mediators) [17].

6.6 Carbohydrate-Mediated ASA Antibodies

The chemical organization of ASA antibodies has become a complex matter. After pioneering work of molecular mapping of the sperm epitope reacting with female immobilizing antibody [55], we have also found common carbohydrates both linked with protein and lipid carriers on human sperm [35]. An interesting conclusion coming up from these studies was a bimodal curve received with virtually all antisperm monoclonal antibodies after sperm periodate oxidation, while more gentle N-deglycosylation carried out with cocktail of enzymes seemed to open sperm glycocalyx allowing to penetrate antibodies uncovering the previously seen cryptic sperm determinants [35]. Unlike, however, in Koyama and co-workers paper [33] where by using chemical deglycosylation (trifluoromethyl acid with sodium periodate) they abrogated all ASA reactivity; in our hands, high amounts of periodate did not remove the reactivity completely, so we could speculate that some antigenic portions were hidden beneath the cell membrane or alternatively there was retained binding mediated through the protein (lipid) carrier. While Tsuji [56] has managed to strip off (by strong oxidation) all the carbohydrates, the conclusion was that immobilizing properties strongly depend on carbohydrates while the remaining activity of antibodies recognized the portion that was digested off by proteolytic enzymes.

The intriguing observation on sperm N- and O-deglycosylations led us to explore the partial carbohydrate digestion, although the revealed pattern by Western blotting and immunoprecipitation (reaction of antibody with denatured or ‘native’ antigenic conformations) was quite confusing; see Fig. 6.2 and Table 6.1).

After immunoprecipitations obtained with various families of antisperm antibodies (from infertile individuals), three types of reactions can be seen. For example, O-deglycosylation procedure in same cases did not change the pattern of reaction

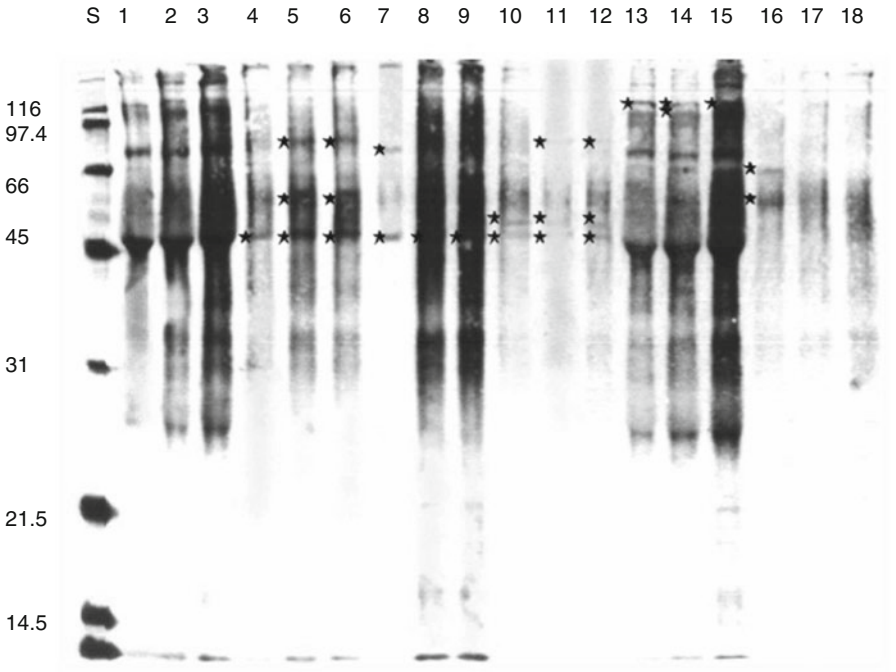

Fig. 6.2 Immunoprecipitation of glycosylated and deglycosylated sperm surface antigens by ASA contained in sera samples after in situ sensitization.
S Molecular weight standards (kDa × 10^{-3})
Lanes 1–3 control (ASA-negative) sera
Lanes 4–18 ASA-positive samples
Lanes 1, 4, 7, 10, 13 and 16 sera obtained by using N-deglycosylated sperm antigens
Lanes 2, 5, 8, 11, 14 and 17 sera obtained by using O-deglycosylated sperm antigens
Lanes 3, 6, 9, 12, 15 and 18 sera obtained by using glycosylated sperm antigens
* – specific immunoprecipitated antigens

Table 6.1 Sera samples and ASA-reactive sperm antigens

	Sperm antigens containing sugar moieties inducing antisperm antibodies		Non-glycosylated antigenic epitopes
Technique	O-linked	N-linked	
Western immunobloting	82, 70, 68–65, 63–61, 59–56, 53, 52, 33–30, 27–29	64, 59, 56, 53	76, 74, 68
Immunoprecipitation	160, 119, 77, 23, 19	160, 119, 108, 38, 23	111, 101, 45, 38

Molecular weights (kDa) were obtained from two independent methods: Western immunoblotting and immunoprecipitation techniques

when comparing to N-deglycosylation or the 'native' sperm extract (lanes 1 vs. 2 vs. 3). In some variants, O-deglycosylation procedure (lanes 4, vs. 5 vs. 6) produced more bands with ASA polyclonal sera than after N-deglycosylation, and in some

cases (lanes 16, 17, 18), O-deglycosylation abolished the antibody reaction when comparing to the other applied sperm extracts (N-deglycosylated or 'native' ones).

In the next series of experiments, reactions were performed when the sperm antigens were selectively or simultaneously deglycosylated and antisperm antibodies reacted both in Western immunoblotting and immunoprecipitation techniques (Table 6.1). The relatively less number of positive reactions revealed in N-deglycosylation sperm does not allow to infer highly sensitive N-linked binding since technical difficulties in protocols for liberation N-glycans have not been yet satisfactorily solved [32].

However, it can be concluded (from the Western immunoblotting) that there is more (sensitive) O-deglycosylated sites on human sperm than N-deglycosylated ones what could indirectly confirm our earlier reports on enhancement of ASA antibodies binding due to limited N-deglycosylation [35]. Further, it can be clearly concluded that N- and O-deglycosylation procedures applied simultaneously significantly diminished the number of bands precipitated or immunoblotted in comparison to each of the applied procedures alone.

It should be further underlined upon glycemic and proteomics analyzes the high amounts of fucosylated glycoconjugates in reproductive compartment [34] of both sexes concerning, e.g. glycomics of uterine fluid or glycemic gradient complexity in different regions of epididymis. The carbohydrate sequences involved in reproductive tracts must take part in creation: (a) immune privilege in the human reproductive system; (b) immunologic interface between self (female reproductive tract epithelium) and non self (placenta, sperm cell components); and (c) operating milieu for effective response to pathogens. All these tasks an incredible to be precisely fulfilled without any errors if both arms of innate and adaptive immunities are in full action. Yet, in reproductive age relatively low number of errors occurs resulting in anti-gametic response formation. This may be at the expense of immunological deviations arising after reproduction is completed opening the space for malignancies in already redundant compartment.

Conclusions

We may again emphasize the diversity of antisperm antibody reactions which recognize abundantly glycosylated human sperm entities. It seems that there is sufficient data to underline once more a complex nature of ASA reactions (1) being mediated by carbohydrate epitopes (with background of 'natural' antibodies), (2) sex-dependent differences in sperm recognition expressed by auto- and isoimmune reactions, and (3) genuine characteristics of ASA reactions developed pre-puberty that deserve further epidemiological interest.

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Sperm-Specific T Lymphocytes

7

Michael Hertl and Walter K.H. Krause

Abstract

Sperm antigens, which are able to induce antibodies, may also be able to induce antigen-specific T cells. The knowledge on antigen-specific T lymphocytes in immune infertility is scarce, but the involvement of T cellular immune reactions to sperm antigens is likely. T lymphocytes in close association with spermatozoa were first observed in men after vasectomy within the so-called sperm granulomas. Specific T cell clones were generated by the incubation of peripheral lymphocytes with extracts from the testis and of spermatozoa in the rat. The only report on specific T cells reactive to sperm antigens in humans is related to sperm protein 17 (Sp17), a cancer-testis antigen.

T lymphocytes are present also among the normal population of leukocytes in semen. Little is known about autoreactive T cells in semen. Some studies provided evidence that their occurrence may be the consequence of immune reactions to prostatic antigens (autoimmune prostatitis). The presence of T lymphocytes in semen, however, may be of relevance due to their secretion of cytokines which may influence sperm functions.

7.1 General Characteristics of T Lymphocytes (From Janeway's Immunobiology [12])

Sperm antigens, which are able to induce antibodies (the effector molecules of humoral immunity), may also be able to induce antigen-specific T cells (the effectors of cellular immunity). T cells are formed in the thymus from precursor cells

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originating from the bone marrow. They differentiate into T cells expressing α/β or γ/δ receptors. T cells which are not able to recognize MHC (human: HLA) molecules expressed on somatic cells or those which react with self-peptides undergo apoptosis (clonal selection). T cells surviving the process may leave the thymus as naive T cells. Specific T cells arise from the interaction of naïve T cells with antigen-presenting dendritic cells in the peripheral lymphoid organs. The dendritic cells are bone-marrow-derived and reside in tissues that are involved as the primary contact sites of the antigen(s) concerned. The migration of naïve T cells into the lymphoid organs is guided by the chemokine receptor CCR7, and L-selectin expressed by naïve T cells attracts T cells to the specialized surfaces of high endothelial venules. Facilitated by the expression of ICAM-1, the diapedesis and migration of T cells into the T cell zone is achieved, where the naïve T cells meet antigen presenting dendritic cells. Due to the kind of participating toll-like receptors (TCR) of cytokines and other mediators, the T cells differentiate into the various subtypes of T helper cells (TH1, TH2, TH 17). The differentiation of the T cells requires at least three types of signalling: specific recognition of the MHC- (HLA-) protein-complex by the TCR, the presence of costimlating molecules and the cytokines involved, which drive differentiation into a particular T cell subset.

Naïve T cells will respond to antigen only when the antigen-presenting cell presents a specific antigen in association with costimulatory molecules such as B7 (which interacts with CD28 on T cells). The activation of naïve T cells leads to their proliferation and differentiation which is promoted by the production of IL-2. When antigens are presented without costimulatory molecules, effector T cells become anergic or die. Antigen-stimulated T cells develop into effector T cells which require continuous antigen recognition in association with MHC (HLA) class I or II molecules for continuous activation.

Upon antigen recognition, T cells secrete distinct cytokines, i.e., Th1 cells secrete cytokines such as IFN-γ and TNF-α that activate macrophages and induce cell-mediated immune response while Th2 cells secrete the cytokines, IL-4, IL-5, and IL-13, which induce B cell activation. Cytotoxic T cells, which are commonly CD8 positive, kill their target cells via different mechanisms, including secretion of granzymes perforin and granulysin, as well as by Fas-Fasl-mediated cytotoxicity.

7.2 What Is Known About Antigen-Specific T Cells in Immune Infertility?

The knowledge on antigen-specific T lymphocytes in immune infertility is scarce, but the involvement of T cellular immune reactions to sperm antigens is likely. T lymphocytes in close association with spermatozoa were first observed in men after vasectomy within the so-called sperm granulomas. Sperm granuloma represents a dynamic structure and a site of spermatozoal phagocytosis. Intraluminal macrophages (“spermatophages”) absorb degradation products, rather than whole sperm. Besides the well-known formation of antibodies, also a modest T lymphocyte activity is observed. However, the contribution of T lymphocytes and antisperm antibodies to testicular damage after vasectomy is far from being clear [14].

Following experimental vasectomy in the ram, Saravanamuthu et al. [24] identified T- and B-cells infiltrating the resulting sperm granulomas. They found MHC-II-restricted lymphocytes in the early granulomas, and MHC-I-restricted lymphocytes in the late granulomas. They assumed that the lymphocytes represented sperm-specific T- and B-cells. Mathur et al. [13] also suggested the existence of sperm-specific T lymphocytes since lymphocytes of men with antisperm antibodies (ASA) showed enhanced reaction to lectin-triggered stimulation by sperm antigens compared to men without ASA.

Observations of Munoz et al. [17] indicated that a proliferative response of γ/δ^+ T cells accompanies the development of ASA. Men with ASA fixed to the sperm surface had higher numbers of γ/δ^+ T cells and α/β^+ T cells in the semen than men without ASA. The number of peripheral blood T cells was not different among men with and without ASA. After incubation of blood lymphocytes of men with/without ASA with spermatozoa, the number of γ/δ^+ T cells increased only in the men with ASA, while the T cells from men without ASA showed no proliferative response.

Yule and Tung [31] generated specific T cell clones by the incubation of peripheral lymphocytes with extracts from the testis and of spermatozoa. These T clones induced an autoimmune orchitis in syngeneic mice. This experiment demonstrated unequivocally the existence of T cells specific for sperm antigens. The absence of an immune reaction to testicular antigens in the healthy testis is obviously due to immunological tolerance by yet unknown mechanisms, but it is not due to the separation of the testicular tissue from the immune system. Evidence for suppressor mechanisms comes from the observation that the immune rejection of foreign tissues upon transplantation into the testis is delayed compared to other organs. The observation that orchitogenic T cell clones are able to induce autoimmune orchitis strongly suggests that regulatory immune mechanisms rather suppress the afferent phase and the effector phase of an immune response. In humans, the etiology of autoimmune orchitis is likely to be multifactorial, including testicular inflammation, infection, or trauma, which eventually induces proinflammatory T cell responses resulting in blood-testis-barrier permeability alteration, ASA production, and apoptosis of spermatocytes and spermatids [27].

In the experiments of Qu et al. [21], immunization of syngeneic mice with testicular germ cells induced an immune response against antigens of spermatids, resulting in autoimmune orchitis. This inflammatory response was characterized by a lymphocytic infiltrate of the tests. Subsequent vasectomy blunted the inflammation in the testis, but provoked epididymitis in the caput involving CD4+ T cells, CD8+ T cells, B cells, and macrophages. Surprisingly, although the sperm antibodies in mice without vasectomy were reactive to round and elongated spermatids, those in mice undergoing vasectomy were reactive with the acrosomes of mature spermatozoa. Thus, the site of activity of autoreactive lymphocytes determines the nature of the target antigens. Further studies of the same authors [18] demonstrated a resolution of the lymphocytic infiltration, but the disturbation of the seminiferous epithelium persisted. The authors noted maturation arrest, deposits of IgG in the seminiferous tubules, and a thickened basement membrane, similar to the tubular wall fibrosis observed in human seminiferous tubules of infertile patients.

The situation is complicated by the fact that presentation of antigens to T cells by antigen-presenting cells appears to be divergent in the testis as compared to other tissues. Some costimulatory molecules, such as CD80 and CD86, are lacking in the testis. The immune privilege of the testis may thus be in part due to an anergy of T cells in this environment, although antigen-presenting macrophages are active in the testis [22]. In addition, CD4+ CD25+ regulatory T (T_{reg}) cells strongly influence the autoimmune responses to meiotic germ cell antigens in vasectomized mice. Within 24 h of unilateral vasectomy, the epididymis underwent severe inflammation and granuloma formation, but immune responses to germ cell antigens failed to appear. If Treg cells were depleted, a specific autoimmune response to these antigens was observed. Obviously, the tolerance to germ cell antigens depended on a rapid de novo Treg cell response after vasectomy. The antisperm antibodies after vasectomy develop independently of the T_{reg} cell response [23]. Noteworthy, a reduced percentage of peripheral CD4(+) CD25(+) Foxp3(+) Treg cells at the late follicular phase was associated with artificial insemination by donor (AID) sperm failure and can be a potential biomarker for predicting AID-induced failure [11].

Experimental results questioning the existence of sperm-specific T cells were published by O'Rand et al. [20]. These authors sequenced and cloned a sperm antigen in the rabbit designated as Sp17. Mice which were immunized with this antigen developed antibodies, but did not show a proliferative response of T lymphocytes.

The only report on specific T cells reactive to sperm antigens is provided by a previous study of Chiriva-Internati et al. [8] relating to sperm protein 17 (Sp17). Sp17 is a specific protein of spermatozoa, which is also expressed as a cancer-testis antigen by about 30 % of patients with multiple myeloma. Sp17-specific human leucocyte antigen (HLA)-A1 and B27-restricted cytotoxic T lymphocytes (CTLs) were successfully generated from peripheral blood mononuclear cells of a healthy donor. Effects on spermatogenesis are not reported since the focus of the study was on treatment of multiple myeloma.

The interaction of autoaggressive T helper cells and B cells in the course of immune infertility remains to be clarified. Autoaggressive T cell clones should be isolated and expanded ex vivo and the immunodominant T cell epitopes need to be characterized. Moreover, the T cell dependent activation of autoaggressive B cells remains to be demonstrated. In immune infertility, it is unknown whether autoaggressive T cells of a given epitope specificity interact with autoaggressive B cells leading to T cell help for the induction and perpetuation of antibody production. Sperm epitope-specific T cells may be identified in vitro upon coculture with synthetic peptides of a known cognate antigen of ASA (e.g., HSP70). The influence of epitope-specific T helper cells on antibody production remains to be studied.

7.3 T Cells in Semen

Among the normal population of leukocytes in semen at a range of up to $1 \cdot 10^6$/ml, granulocytes are the most prevalent type with 50–60 %, followed by macrophages (20–30%) and lymphocytes (2–5 %). The lymphocytes were further divided into

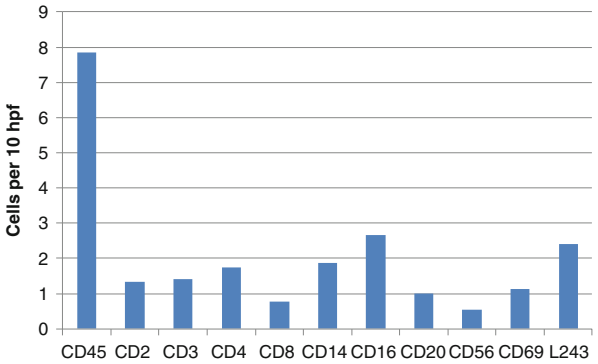

Fig. 7.1 Various populations of leukocytes in semen, as differentiated according to surface molecules. The surface molecules characterize the following cell populations: CD2 (T cell in general), CD3 (T cells in general), CD4 (T helper cells), CD8 (T cytotoxic cells), CD14 (monocytes), CD16 (granulocytes), CD20 (B cells), CD45 (pan leucocyte), CD56 (natural killer cells), CD69 (activated T and B cells). *Hpf* high power field (Depicted from Seshadri et al. [26])

CD4 positive T cells (2.4 %) and CD8 positive T cells (1.3 %). B lymphocytes were not present in healthy men, but only in men with seminal inflammation [30]. The percentage of T lymphocytes is enhanced in men with spinal cord injury [2]. Seshadri et al [26] again analyzed the presence of various types of leukocytes in semen, differentiating them by means of antibodies (Fig. 7.1). Their study intended to show different leukocyte populations in various semen qualities, i.e., normozoospermia, oligozoospermia, and others, but no significant differences could be demonstrated.

Little is known about autoreactive T cells in semen. Some studies provided evidence that their occurrence may be the consequence of immune reactions to prostatic antigens (autoimmune prostatitis). T cells proliferate in response to proteins of seminal plasma of men with autoimmune prostatitis [1, 3, 7]. Witkin and Goldstein [29] performed lymphocyte and monocyte counts in the semen of 14 men with intact vas deferens and 13 men who had undergone vasovasostotomy. In both groups, the number of lymphocytes and monocytes cells was identical with 10^3/ml. However, in men with intact vasa, T suppressor/cytotoxic cells predominated. In contrast, in the vasovasostomized men, the levels of CD8+ T cells were significantly reduced and CD4+ T cells predominated in their semen. The authors thus speculated that damage to the excurrent ducts was responsible for the alteration in T cell regulation leading to a decrease of CD8+ T cells and loss of tolerance permissive for the formation of ASA.

Seminal plasma possesses immunosuppressive activity. In vitro, large molecules of the seminal plasma were able to suppress the B cell proliferative response induced by the Nocardia mitogen, while small molecules suppressed the T cell proliferative response to phytohemagglutinin A (PHA). Purification of the B cell suppressor factor identified a protein with a molecular weight of 180 kD. This molecule may be able to suppress ASA formation in females, as well as autoantibodies in men [6, 28].

More recent experimental data supporting this hypothesis is still lacking. The immunosuppressive effect, however, is of relevance in the regulation of the immune activity against spermatozoa in the female genital tract (see Chap. 10)

Munoz et al. [17] determined the number of α/β+ and γ/δ+ T cells in serum and semen of 23 men. In a cohort of 7 men with ASA, the mean numbers of γ/δ and α/β T cells were 3,560 and 3,230 cells/ml semen, respectively. In contrast, a group of 16 men with no evidence of autoimmunity to sperm showed a mean number of 350 γ/δ+ T cells and 610 α/β+ T cells/ml semen. The numbers of γ/δ+ and α/β+ T cells in the peripheral blood of the identical men were unrelated to their antisperm antibody status. Thus, γ/δ+ T cells in human semen comprise a larger proportion of the total T cell population than of the T cells in blood. The number of γ/δ+ T cells appeared to be elevated in the semen of men with evidence of localized autoimmunity to their own sperm. These results suggest that the proliferative response of T cells with a γ/δ+ TCR favors an autoimmune response to sperm. The higher proportion of lymphocytes bearing a γ/δ+ antigen receptor in the testis as compared to peripheral blood was also confirmed by Bertotto et al. [5]. The rise of γ/δ+ T cells was mainly due to an overexpansion of cells expressing Vδ1 gene-encoded determinants on their surface. This finding points to a special immune milieu in the semen.

T cells in semen appear to be target cells of HIV infection. Bernard-Stoecklin et al. [4] investigated seminal leukocytes in macaques after infection with SIV and found infection of $CD4^+$ T lymphocytes together with macrophages. The lymphocytes had a mucosal phenotype and expressed activation and migration markers. Thus, seminal T cells may facilitate sexual transmission of the immunodeficiency virus.

The presence of T lymphocytes in semen may be of relevance not only regarding their specificity to sperm antigens, but also due to their secretion of cytokines which may influence sperm function. Sperm cells express IFN-α and IFN-γ receptors. IFN-α, IFN-γ, and other cytokines have deleterious effect on sperm motility and fertilizing ability [19]. This may concern also female lymphocytes within the genital tract [9]. Seminal fluid induces an inflammatory reaction in the female genital tract, which results in the secretion of cytokines from the female side that in turn have a range of effects on conception and pregnancy, e.g., the priming of the female immune response to paternal antigens to promote T cell-mediated immune tolerance [25].

In addition, the membrane cofactor protein (MCP), also known as CD46, is a link between T cells and sperm function. CD46 is a multitasking molecule in complement regulation and as a costimulatory molecule for T cell activation. It exists as multiple isomeric forms while human spermatozoa express only an isoform comprising the four short consensus repeat (SCR) domains, the short Ser/Thr/Pro-rich domain C, and the Cyt2 variant of the cytoplasmic tail. Spermatozoal CD46 is identical to the previously described acrosome-restricted spermatozoal protein trophoblast-leukocyte common antigen. Because of its acrosome-restricted expression pattern, spermatozoal CD46 has been utilized as a specific acrosome marker in humans [15, 16]. From further studies it appears that also other CD molecules associated with the immune system may be involved in the fertilization process, such as CD9, CD29, and CD49 [10].

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Site and Risk Factors of Antisperm Antibodies Production in the Male Population

8

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Abstract

Inflammatory and/or infectious (inflammatory/infectious) diseases of the male reproductive tract (MRT), varicocele, genital trauma, testicular tumors, and testicular sperm extraction (TESE) between others have been proposed as risk factors for the generation of antisperm antibodies (ASA). However, as demonstrated in numerous clinical trials, the association between these entities and the presence of ASA is still controversial, indicating that the understanding of these conditions in the development of ASA is incomplete. However, there seems to be a consensus in the literature on three important facts: First, vasectomy followed by vasovasostomy (VV) is the only clinical condition that shows almost permanently high titers of ASA in numerous clinical series. Second, circulating ASA do not play an important role and do not show any negative influence on the fertility prognosis of the affected men. In contrast, local ASA act negatively on the motility of spermatozoa, on their ability to pass through the female genital secretions and/or on the fusion of gametes, which is the key event of fecundation. Third, the lack of a standardized method and an established cut-point for ASA detection in the ejaculate makes the comparison difficult among different clinical trials. Taking into account the increased prevalence of the above mentioned diseases in an uroandrological setting, this chapter evaluates the existing data trying to identify the possible risk factors and sites of ASA production in the MRT.

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8.1 Introduction

During the last 30 years, conflicting data regarding the risk factors and site of antisperm antibodies (ASA) formation in men have been published. Inflammatory and/or infectious (inflammatory/infectious) diseases of the male reproductive tract (MRT), varicocele, genital trauma, testicular tumors, and testicular sperm extraction (TESE) between others have been proposed as risk factors for the generation of ASA deteriorating the fertilizing capacity of the affected men (Table 8.1) [1]. However, as demonstrated in numerous clinical trials, the association between these entities and the presence of ASA is still controversial, indicating that the understanding of these conditions in the development of ASA is incomplete. Although many questions regarding the site and risk factors of ASA production in males remain unanswered, there seems to be a consensus in the literature on three important facts: First, vasectomy followed by vasovasostomy (VV) is the only clinical condition that shows almost permanently high titers of ASA in numerous clinical series [2–5]. Second, circulating ASA do not play an important role and do not show any negative influence on the fertility prognosis of the affected men. In contrast, local ASA act negatively on the motility of spermatozoa, on their ability to pass through the female genital secretions and/or on the fusion of gametes, which is the key event of fecundation [6]. Third, the lack of a standardized method and an established cut-point for ASA detection in the ejaculate makes the comparison difficult among different clinical trials [7]. Taking into account the increased prevalence of the above mentioned diseases in an uroandrological setting (i.e., inflammatory/infectious diseases of the MRT, varicocele, TESE, etc.) and the negative impact of

Table 8.1 Suggested risk factors for ASA formation

Chronic obstruction of the MRT
Congenital
Congenital bilateral absence of the vas deferens (CBAVD)
Müllerian prostatic cysts
Acquired
Vasectomy
Iatrogenic obstruction of the epididymis and/or ductus deferens
Inflammation and/or infection of the male reproductive tract
Varicocele
Cryptorchidism
Testicular trauma
Testicular torsion
Testicular surgery
Testicular sperm extraction (TESE) testicular biopsy
Organ-sparing surgery for testicular tumors
Testicular tumors
Homosexuality

ASA on fertility of these men, this chapter evaluates the existing data trying to identify the possible risk factors and sites of ASA production in the MRT.

8.2 Risk Factors and Site of ASA Formation

8.2.1 Chronic Obstruction of the MRT

Surgical interventions of the epididymis and vas deferens that cause an obstruction have demonstrated to be the only widely accepted conditions [2–5], demonstrating almost permanently high titers of ASA. Several reports suggest that between 50 and 100 % of men who undergo vasectomy subsequently have sera positive for ASA [4, 8] and the prevalence of ASA in the ejaculate of these men is also extremely high (70–100 %) [5]. It is not that only acquired obstruction of the seminal tract may trigger the formation of ASA. Bronson et al. [9] demonstrated that ASA were detected after the onset of puberty in a cohort of 35 men with cystic fibrosis and congenital bilateral aplasia of the vas deferens (CBAVD).

Following vasectomy, epididymal distension and sperm granuloma formation may result from raised intraluminal pressure. The sperm granuloma is a dynamic structure and a site of much spermatozoal phagocytosis by its macrophage population; however, it is not a permanent finding in patients who have undergone vasectomy [10]. In many species, spermatozoa in the obstructed ducts are destroyed by intraluminal macrophages, and degradation products, rather than the whole sperm getting absorbed by the epididymal epithelium. Humoral immunity against spermatozoal antigens following vasectomy would be secondary to the combination of a constant leak of sperm antigens that by far surmounts all known mechanisms against autoimmunity present in the epididymis and a chronic increase in intraluminal pressure.

The time-course for postvasectomy ASA production does not seem to be triggered exclusively by acute, sudden, and massive reabsorption of spermatozoa after vasectomy but also by slow, gradual, and late sperm antigen reabsorption. Data from a rat model suggests that IgM ASA develop within 2 weeks after vasectomy, decreasing in the next weeks followed by increasing titers of ASA IgG between 8 and 12 weeks [11].

Cellular immunotolerance mechanisms are also implicated in the ASA production of vasectomized patients, as demonstrated by Witkin and Goldstein [12] who described reduced concentrations of T suppressor lymphocytes in their semen when compared with undisturbed vasa.

The immunologic response to spermatozoa is polyclonal, so that the populations of ASA directed against different epitopes vary from individual to individual. As far as the antigenic structure of spermatozoa is concerned, several groups of specific substances have been studied: the ABO groups antigens (Ags), acrosin, HLA Ags, hyaluronidase, protamines, and DNA polymerase [13].

The strength of the ASA formation after vasectomy is variable but there are some patients who have a genetic predisposition to develop ASA [14]. It can be postulated

that a breakdown of sperm immune tolerance depends on an individual's immune responsiveness, the nature of the precipitating event, and the length of exposure of inoculum. The genetic predisposition for the development of an autoimmune sperm reaction has been demonstrated in monozygotic twins where the antisperm immune reaction is triggered by genetic predisposition rather than by the spermatozoa concentration [15].

Still under debate is the location in the genital tract where ASA transuded from serum and locally produced antibodies, respectively, become attached to the surface of the spermatozoa. In spermatozoa retrieved directly from the distal end of the vas in patients undergoing vasovasostomy (VV) and IgG and IgA ASA determined by the immunobead test were present in 78.6 and 32.1 % of the patients [16], respectively, indicating that in these patients, the epididymis would be the primary site of ASA local production and transudation from serum. The question whether the rest of the MRT contributes to ASA deposition in the ejaculate of these patients was addressed by Meinertz et al. [17], who performed the mixed antiglobulin reaction (MAR) for IgG and IgA in the whole ejaculate and the fractions of the split ejaculate of 11 men with history of vasectomy and successful VV. The MAR test revealed almost identical concentrations of ASA in the first and second fractions of the ejaculate. The results suggest that ASA in the ejaculate from VV patients are transuded from serum not only at the epididymal but also at the prostatic and seminal vesicle levels.

In conclusion, chronic obstruction at the level of the vas deferens constitutes a clear risk factor for ASA formation (Table 8.2). In these patients, the most probable site of ASA production is the epididymis; however, once autoimmunization happens transudation of ASA seems to occur also at other levels of the MRT (i.e., seminal vesicles and prostate). The pathophysiology of ASA formation in these patients would involve, among others, an increased intraductal pressure associated with chronic absorption of spermatozoa or sperm fragments and a decrease in the cellular immunomodulatory factors present in the seminal plasma, namely reduced concentrations of T suppressor lymphocytes. It seems logical that any pathologic condition that causes chronic obstruction of the MRT can constitute a risk factor for ASA formation through the same mechanisms.

8.2.2 Infection and/or Inflammation of the MRT as Cause of ASA Formation

Infection/inflammation of the MRT is a risk factor for ASA formation through four main mechanisms:

- Obstruction of the MRT because of inflammatory and postinflammatory changes
- Tearing of the blood–testis barrier because of local inflammation
- Decrease of the immunomodulatory factors (cellular and humoral) present in seminal plasma that normally prevent sperm autoimmunization
- Cross-reactivity between antigens of the microorganisms responsible for MRT infections (i.e., *Chlamydia trachomatis*) and sperm antigens

Table 8.2 Suggested risk factors and sites of ASA production in the MRT

Risk factor	Status	Most probable site of first immunization and ASA production
Chronic obstruction of the MRT	Confirmed risk factor	Epididymis
Inflammation/infection of the MRT	Not confirmed	Epididymis/prostate
Varicocele	Not confirmed	Testis
Cryptorchidism	Not confirmed	Testis
Testicular trauma	No risk factor (more evidence is needed)	–
Testicular torsion	No risk factor (more evidence is needed)	–
Testicular surgery	No risk factor (more evidence is needed)	–
Testicular tumors	Not confirmed	Testis
Homosexuality	Not confirmed	Gastrointestinal mucosa

Controversial data exist on the association between inflammation/infection of the MRT and ASA [7]. In a series of 79 infertile patients with inflammatory/infectious diseases of the MRT [5], the comparative results of the two tests for ASA detection in seminal plasma, the MAR and immunobead test, demonstrated no clear role of this association for male infertility. In a second series of 365 patients with documented inflammation/infection of the MRT, such as chronic bacterial prostatitis (CBP), inflammatory chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), noninflammatory CPPS, chronic urethritis, and chronic epididymitis, again we found no association between ASA formation and these diseases [7]. Also in patients with CP/CPPS, Hoover and Naz did not observe a higher presence of ASA in serum compared to healthy controls [18]. Controversially, Witkin and Toth [19], using an ELISA, reported a 48 % incidence of ASA in seminal plasma of men with a history of urethritis and CBP. Jarow et al. [20] also found a positive association between CP/CPPS and ASA using the gel agglutination assay in serum. Garolla et al. [21] reported significantly higher percentage of ASA in the semen of patients infected with human papillomavirus compared to healthy controls. The number of clinical series dealing with the detection of "significant" ASA levels in seminal plasma of men with inflammation/infection of the MRT is small; the tests used for ASA detection and the positive cut-points for the different methods vary between the different studies. All these factors may explain the fact that the relationship between these two conditions is still debatable.

From a pathophysiological point of view, the absence of a confirmed clinical association between infectious/inflammatory diseases of the MRT and ASA formation would rely on the fact that the four previously mentioned mechanisms, by which these diseases would constitute a risk factor for ASA formation, are not regular findings in patients with these conditions.

Even though both acute and chronic infection/inflammation of the MRT have been claimed as risk factors for partial and total obstruction of the MRT [6, 7], further reports have demonstrated that the link between these two conditions, with the exception of epididymal tuberculosis, is weak [22]. Especially, for CP/CPPS obstructive findings seem to be rare, either not evident or evaluable in less than 10 % [23]. Inflammatory or infectious diseases do not appear to be important causes of obstructions of the MRT [24].

Inflammatory-induced tears of the distal segments of the epididymal duct or efferent duct epithelium may occur in inflammatory/infectious diseases of the MRT breaching the blood–epithelial barriers [12]. This would activate the immunological defense and induce the production of ASA [25, 26]. As there are no sensitive markers for the disruption of the BTB and BEB, it is not possible to evaluate if this event really occurs in patients with inflammation/infection of the MRT or up to what degree it may be present. It is questionable if the presence of leukocytes in seminal fluid could indicate some degree of disruption; however, it is a known fact that the levels of seminal leukocytes in patients with inflammation/infection of the MRT are extremely variable and their exact role and meaning are not clear. Moreover, if this would be the case we [7] and other authors [27–31] have found no association between the presence of ASA and elevation of inflammatory parameters in seminal fluid, such as leukocytes and elastase. Associated with the fact that the disruption of the blood–epithelial barriers is a questionable event in the mentioned diseases, there is also the important fact that a breach of the barrier alone is not enough in many patients to trigger the formation of ASA. This finding was clarified by the studies of Komori et al. [32] and Leonhartsberger et al. [33], in which patients undergoing TESE and organ-sparing surgery for testicular tumors, where certainly a disruption of the BTB occurs, no increased risk of ASA formation was reported.

Even though the significance of white blood cells in the ejaculate remains a matter of debate, several authors have suggested that such cells are important in the modulation of an ASA response, in the sense that the role of the suppressor lymphocyte predominance over helper lymphocytes prevents the formation of ASA. Some reports support the idea that inflammatory/infectious diseases of the MRT would not only not promote granulocyte migration into the MRT but also the activation of B- and T-helper lymphocytes [34], modulating the physiological predominance of T suppressor lymphocytes over helper T lymphocytes [12]. Local production of cytokines at the epididymal epithelium would be an important factor for recruiting lymphocytes into the seminal fluid [35].

In agreement with this theory, Munoz and Witkin [26] postulated that an asymptomatic, undetected *Chlamydia trachomatis* infection of the MRT may induce the local activation of γδ T lymphocytes that are believed to comprise the first line of immunological defense against infection at mucosal surfaces. Once activated, these would react with those sperm antigens that do not require presentation by MHC class I or class II molecules, resulting in further amplification and activation of γδ T lymphocytes and increased cytokine expression. This in turn would activate gd T lymphocytes in the genital tract and lead to the induction of an autoimmune response to spermatozoa. However, as previously mentioned, several authors [27–30, 36]

have reported no relation between the presence of ASA and the number of leukocytes in the ejaculate, suggesting that despite the fact that both abnormalities are manifestations of an immunological response they are not interrelated. Moreover, Barratt et al. [37] reported that in men with ASA the predominance of T helper lymphocytes over T suppressor lymphocytes is very rare. Owing to their similarity, immunological cross-reactivity between antigens of the sperm membrane and *Chlamydia trachomatis* has also been proposed as a theory to explain the questionable association between infections of the MRT and ASA formation [7]. Immune response against stress proteins (i.e., heat shock proteins (HSP) – essential mammalian and bacterial stress proteins) can be highly cross-reactive. It has been suggested that the antibodies against conserved epitopes on chlamydial HSP 60 may cross-react with those on human HSP 60 and initiate an autoimmune response [38]. However, once again, clinical data fail to detect an association between chlamydial infections and the presence of ASA in seminal plasma [7, 39]. In the hypothetical scenario that inflammatory/infectious diseases of the MRT are associated with ASA formation, for anatomical reasons previously discussed the epididymis would be the most probable site of ASA formation (Table 8.2). However, the prostate gland is another site where a localized immune response can be induced, since prostatic fluids have been identified to contain specific IgA antibody against *Escherichia coli* and spermatozoa [19]. Some authors suggest that inflammation/infection of the MRT in some men may interfere with the complete closure of prostatic ducts during ejaculation, resulting in leakage of sperm into the prostate gland inducing an immune response [40]. Patients with inflammatory/infectious diseases of the MRT seem to bring together many favorable conditions for ASA formation; however, evidence in clinical studies indicates that the association between these two conditions is extremely weak. A probable explanation for this contradiction would be that all the favorable conditions for ASA formation, supposed to be present in these patients, do not seem to be as important or prevalent as usually considered.

8.2.3 Varicocele as Cause of ASA Formation

In 1959, Rümke and Hellinka [41] first suggested a probable association between varicocele and ASA; since then, the association between these two entities is a matter of debate. Clinical studies supporting an association have been based in the detection of ASA in serum and in the ejaculate of patients with varicocele. Using enzyme-linked immunoabsorbant assay (ELISA), Golomb et al. [42] and Gilbert et al. [43] found significantly higher levels of ASA in the serum of patients with varicocele vs. controls (90 % vs. 41 % and 32 % vs. 14 %, respectively). Both concluded that varicocele was a risk factor for ASA formation. During the 1990s, other authors [44, 45], testing ASA in the ejaculate by means of immunobead and MAR test, came to the same conclusion. Djadalat et al. [46] using the MAR test found a weak association between varicocele and ASA; moreover, he concluded that even though surgical treatment for varicocele may reduce the ASA level in some patients, it may increase it in others. In the same scenario, Bozhedomov et al. [47] found that

after varicocele surgery, ASA developed in 16 % of cases; they also reported that 3 months after surgery patients that were primarily ASA negative improved more sperm parameters compared to patients that were previously ASA negative. Contradicting the previous evidence, Oshinsky et al. [48] and Heidenreich et al. [49] reported in two different series of patients that varicocele is not a risk factor for ASA production. This finding was confirmed by Veräjänkorva et al. [50] who, using the MAR test, analyzed the predisposing factors for male immunological infertility in 508 patients that had been treated for infertility. Patients with a history of varicocele had statistically significant lower level ASA than patients without. In a large study that included 1729 men of reproductive age, varicocele was not significantly associated with ASA formation [51]. Basic research evidence is also controversial: Shook et al. [52] demonstrated in an animal model that a surgically induced varicocele triggers ASA formation. However, Turner et al. [53], working also with surgically induced varicocele model in rats, demonstrated that the BTB was not damaged in these animals, suggesting that the impairment of spermatogenesis in this disease is not immunologically mediated. Interestingly, in patients with varicocele and ASA in the ejaculate these immunoglobulins are also present in testicular biopsies, more specifically inside the seminiferous tubule, suggesting that if in fact there is an association between these two conditions, the most probable site of formation would be the testis [44] (Table 8.2).

8.2.4 Cryptorchidism as a Cause of ASA Formation

Cryptorchidism is defined as a condition in which one or both testes fail to descend to the scrotal position. The incidence of this condition varies from 1.4 to 2.7 % in male births and is increased in premature birth [54]. Several studies have reported an increased incidence of ASA (up to 28 %) in patients with history of cryptorchidism either treated or untreated by orchidopexy [55, 56]. However, most studies include prepuberal population where ASA has been only tested in serum, not addressing the important issue of the presence of ASA in the ejaculate. Moreover, there is a high probability that an undefined percentage of the patients who have undergone orchidopexy develop, as a complication of surgery, some degree of obstruction at the epididymal or ductal level. Those patients have a high probability of ASA formation, but the etiology would be falsely classified to cryptorchidism and not to chronic obstruction.

These later studies oppose to the findings of others [57–59], who evaluated ASA in the serum and ejaculate of patients with history of cryptorchidism, orchidopexy, and testicular biopsy not finding any association between the mentioned conditions and the presence of ASA. Clinical evidence in agreement with this last fact was published by Mirilas et al. [60, 61], who found no evidence of ASA in prepuberal boys with history of cryptorchidism.

In prepuberal population, the clinical evidence is even more conflicting, since before puberty, the absence of mature spermatozoa with its antigenic material should exclude any possible immune reaction against sperm antigens; however,

several studies report the presence of ASA in the serum of prepuberal boys with cryptorchidism [55, 62, 63]. Sinisi et al. [64] suggested that in these patients the sperm surface antigens are already present before meiosis and the BTB is either immature or impaired by heat due to the abnormal position. However, evidence in experimental rat models of cryptorchidism demonstrates that the BTB remains competent under this situation [65, 66].

As with other previous conditions, the association between cryptorchidism and ASA remains controversial (Table 8.2). If the association is real and the bias from surgical treatment complications, namely iatrogenic obstruction of the vas deferens, is excluded the most probable site of ASA production in these patients would be the testis.

8.2.5 Testicular Trauma, Surgery, and Torsion as Cause of ASA Formation

It seems logical that every condition where the BTB is breached should constitute a clear risk factor for ASA production, since the immune system establishes a direct contact with the antigens present in the sperm surface. Surprisingly, the data are not clear. Kukadia et al. [67] evaluated the presence of ASA in the ejaculate, using the direct immunobead test, in eight patients with a history of severe testicular trauma who underwent surgical exploration. Only one patient had detectable levels of ASA; all other patients were negative for ASA. He concluded that there is no association between these factors. Surgical procedures to the testis have also proved not to be a risk factor for ASA formation; successful TESE [32, 68], open and needle testicular biopsy [69], and organ-sparing surgery for testicular tumor do not constitute a risk factor for ASA production [33]. Surgeries that do not directly compromise the testis but the nearby structures, such as inguinal hernia repair with and without mesh, have also reported no association to ASA formation [70].

Testicular torsion is a surgical emergency, which requires prompt diagnosis and immediate treatment. One of the consequences that patients may face in the follow-up is a compromise of the exocrine testicular function (spermatogenesis) [71]. The generation of ASA because of the rupture of the BTB is claimed to be one of the possible causes of this exocrine impairment. Arap et al. [72] evaluated ASA formation in the ejaculate of 24 patients with history of testicular torsion; 15 were treated with orchiectomy and 9 were treated with orchidopexy. He used 20 proven fertile men as controls and found no significant differences in the ASA levels between patients and controls, regardless of the treatment applied. These results agree with the findings of Anderson et al. [73], who studied a similar population and were unable to find an increased rate of ASA detection in these patients. Identifying the risk factors for ASA production in a population of male patients, Heidenreich et al. [49] also concluded that testicular torsion is not associated with this condition.

Even though clinical evidences seem to be conclusive, studies in animal models generally confirm the presence of ASA [74]. However, animal models for testicular torsion may not exactly reproduce the conditions found in humans, so care should be taken in extrapolating these data [72].

With the available evidence, trauma, surgery, and torsion of the testis do not seem to constitute a risk factor for ASA formation; however, larger studies are needed (Table 8.2). Nevertheless, the BTB is clearly disturbed in all these cases; ASA formation is not regularly triggered, this fact demonstrates that the pathophysiology of ASA formation is still unclear.

8.2.6 Testicular Tumors and Microlithiasis as Cause of ASA Formation

Testicular tumors have been reported to be a risk factor for ASA formation. The incidence of ASA in these patients ranges from 18 to 73 %; however, most studies were biased in that only serum ASA have been evaluated and most importantly did not include a control group to evaluate if the detection rates were significantly higher [75–77]. Paoli et al. [78] evaluated a large series of 190 patients who underwent orchiectomy for testicular cancer; 1 month after surgery they found only a 5.8 % prevalence of ASA in serum, they concluded that testicular cancer might not be a posible cause of ASA formation. Regarding testicular microlithiasis, Jiang and Zhu [79] using direct immunobead test in semen samples from 22 patients with microlithiasis found no presence of ASA. With the available evidence, the link between testicular tumors and ASA remains questionable (Table 8.2); larger studies including healthy fertile controls are needed.

8.2.7 Homosexuality as Cause of ASA Formation

During the 1980s, experimental studies in rabbits demonstrated that nontraumatic weekly deposition of sperm in the rectum led to the formation of ASA [80, 81]. Taking into account this evidence, it seems logical that unprotected anal intercourse in homosexual men could constitute a risk factor for ASA formation. Wolff and Schill [36] evaluated the incidence of ASA in the serum of different groups of men. Four percent of dermatologic patients ($n=223$), 9.6 % of andrologic patients ($n=178$), and 28.6 % of homosexual men ($n=42$) were positive for IgG and/or IgM antibodies. They concluded that there was a high incidence of ASA among homosexual men, probably because of contact of spermatozoa with the immune system by passive anal intercourse. Five years later, Mulhall et al. [82] reported a 10 % prevalence of ASA in homosexual men and 17 % in those who had practiced unprotected anal receptive intercourse in the previous 6 months. They found no correlation between the presence of ASA and human immunodeficiency virus (HIV) infection. They speculated that rectal intercourse may be a risk factor for ASA formation, even though a comparison with a healthy fertile population was not performed. Contradicting the previous results, Sands et al. [83] found no significant difference in the serum ASA titers between sexually active heterosexual men and homosexual men with or without HIV infection, concluding that ASA levels are not higher in homosexual men.

In conclusion, there is not enough evidence to support homosexuality as a risk factor for ASA formation; however, taking into account clinical evidence and basic research studies, it seems highly probable that if this association exists the primary site of ASA production would be the distal gastrointestinal mucosa (Table 8.2).

Conclusions

The pathophysiology of ASA formation is still unclear; the old concept that a simple tear or breach in the BTB is enough to trigger ASA formation has been pulled down by clinical evidence. As the exact mechanisms operating in ASA formation remain to be elucidated, it is not surprising that many clinical conditions still have a questionable association with ASA formation. The only condition that is a confirmed risk factor for ASA formation is chronic obstruction of the MRT, especially after vasectomy. With the available evidence, testicular trauma, surgery, and torsion should not be considered as risk factors for ASA production; in all other conditions, the association is still questionable. In patients with chronic obstruction of the MRT, the most probable site of ASA production is the epididymis; however, once the immune reaction is triggered and the systemic production of ASA starts, immunoglobulins may enter the MRT at other levels (i.e., seminal vesicles and prostate).

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Biologic Substrates Containing ASA

9

Walter K.H. Krause

Abstract

Antisperm antibodies (ASA) are immunoglobulins. ASA present in the human biological fluids are predominantly of the IgG and IgA class.

ASA were observed in the blood serum of male and female patients. It has become evident that ASA in serum mainly are not a consequence of the contact to sperm antigens, but they are independently existing isoantibodies. Mainly, ASA found in the seminal fluid or attached to the spermatozoa are of relevance. Men expressing ASA in the seminal fluid usually also have ASA in blood serum. The IgG antibodies in semen originate from the serum IgG, but ASA of the IgA fraction originate from a local production. The main determinant for the concentration of immunoglobulins in semen is inflammation,

Immunoglobulin concentrations in the cervix mucus vary with hormonal conditions during the menstrual cycle and during pregnancy and with inflammation. The occurrence of ASA in cervical mucus is generally quoted to be rare, and their concentrations are not correlated to those in blood serum.

Follicular fluid contains lower or equal concentrations of immunoglobulin as compared to blood serum. ASA are found only in women with antibodies circulating in serum. ASA in the follicular fluid were able to induce the acrosome reaction, but it has to be taken in consideration that also normal follicular fluid is able to induce acrosome reaction, depending on the amount of progesterone present.

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9.1 ASA Are Immunoglobulins

There are five main types of these proteins, distributed in a specific manner and with a specific activity (see Table 9.1). IgM antibodies are the first immunoglobulins to be synthesized in the course of immune response. They form pentamers, which exhibit ten antigen-binding sites. Because of the large size, IgM concentrations in the extravascular space including seminal fluid are low. The immunoglobulins of glandular secretions and in the extraepithelial spaces are IgG and IgA. IgA is secreted in dimers, in which two molecules are connected by the secretory piece, a fragment of the IgA receptor of the epithelial cell (Janeway's Immunobiology, 2008)

Antibodies are secreted from plasma cells, which are derived from activated B lymphocytes. The activation of B cells requires both antigen-binding and the support by antigen-specific T helper cells. The B cells internalize the antigens which are bound to surface immunoglobulins and present it as peptide bound to MHC class II molecules to the helper T cells. Subsequently, T helper cells stimulate the B lymphocytes through binding different mechanisms including CD40 and TNF and finally induce differentiation of the clonally B cells into plasma cells. The antibody-producing plasma cells may be systemically active, but also topical activity is possible. This may explain different antigen specificities of antibodies present in the different compartments.

A study of the B-cell activation process producing ASA has been published by Dimitrova et al. [10]. They were able to produce three stable cell populations derived from transformed B-lymphocytes from infertile patients with ASA. The cDNA of the heavy chain of this immunoglobulin showed high homology to the DNA of immunoglobulins in general. Thus, the authors concluded that it was more likely that the ASA were natural antibodies (iso-antibodies) than that they were induced by stimulation of a specific sperm antigen. This is supported by the observation that only a minority of boys with cryptorchidism developed ASA and this group may be prone to autoimmune reactions [11].

ASA may be present in the human biological fluids – blood serum of both sexes, seminal fluid and fluid of male accessory glands, cervix mucus, and tubal and follicular fluid. They are also demonstrable after binding to spermatozoa or even to testicular progenitor cells ([21]; Fig. 9.1). These ASA are predominantly IgG and

Table 9.1 Activity and distribution of immunoglobulins

	IgM	IgD	IgG	IgA	IgE
Neutralizing antibody	+	–	+	+	–
Sensibilization of mast cells	–	–	+	–	++
Activation of complement	+	–	+	+	–
Transepithelial transport	+	–	–	++	–
Diffusion into extravascular space	–	–	+	+	+
Mean serum concentration (mg/ml)	1.5	0.04	13	2.1	30.000

From Janeway et al. (2001)

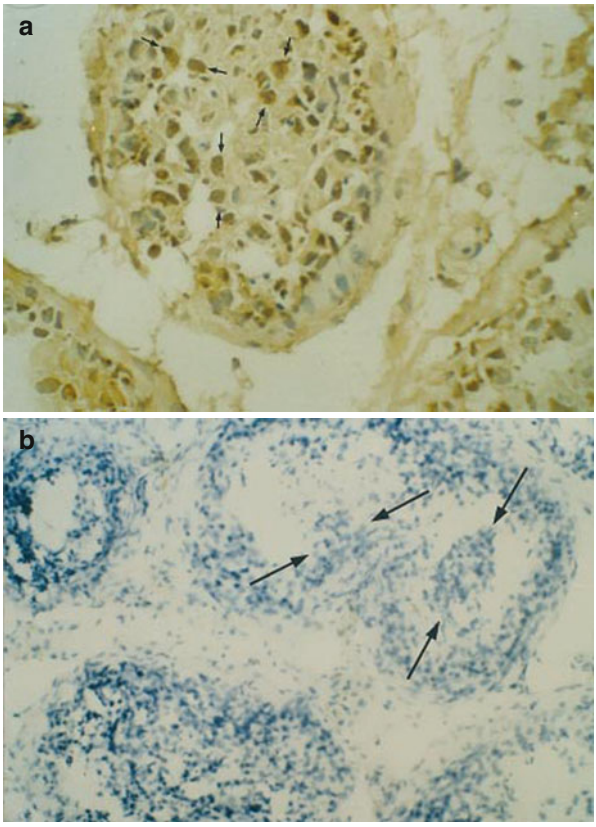

Fig. 9.1 (**a**) Direct immunofluorescence on seminiferous tubules. Mature spermatocytes (*arrows*) in the lumen from a patient with varicocele show the binding of ASA by the brown staining from the POPA method. (**b**) A slide from the control group without ASA binding (From Isitmangi et al., with permission)

IgA. Various tests used for the demonstration of ASA are able to differentiate between these immunoglobulins (see Chap. 13).

ASA will influence sperm function only when they are bound to spermatozoa. In general, antibodies may influence a cell function in different manners:

(i) The most relevant mechanisms are inhibition of function provided by that protein, which includes the cognate antigen (epitope). The proteins influenced by ASA binding will be outlined in the appropriate chapter.
(ii) The complement activation is of minor importance. Complement activating ASA are not effective in the seminal plasma, because it contains complement inhibitors. However, during their residence in the cervical mucus, spermatozoa are exposed to complement activity, which is approximately 12 % of that of serum [17]. But also here the spermatozoa themselves are protected against complement attacks mainly by CD46, the main complement-regulation protein ([18]; Fig. 9.2). Human spermatozoa express CD46 on the inner acrosomal membrane after the acrosome reaction.
(iii) Antibodies may activate accessory effector cells (phagocytes) or natural killer cells after binding of the Fc fragment of the immunoglobulin. Sperm-destructing

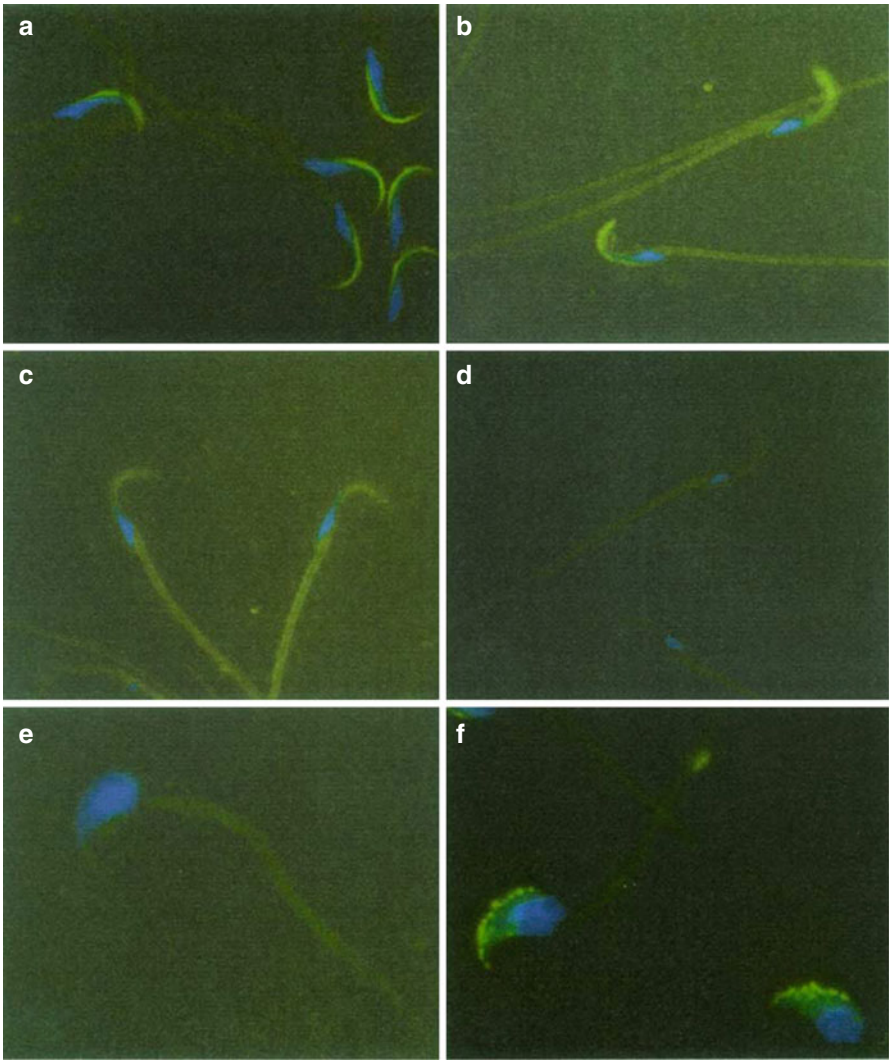

Fig. 9.2 Distribution of membrane CReg on rodent spermatozoa. CD46 expression in the rat is restricted to the acrosome (**a**), whereas CD55 is also found on the tail (**b**). CD59 in the rat is broadly distributed (**c**). CD59a in the mouse is broadly distributed (**e**), whereas CD59b is restricted to the head (**f**). (**d**) Negative control. Original magnification is 1000×, magnified a further three times electronically in (**e**) and (**f**) (From Harris et al. [18], with permission)

phagocytes (spermiophages) are normal constituents of seminal cells. They are, however, less the consequence of the destruction of spermatozoa bearing ASA [36, 37] than the elimination sperm undergoing apoptosis [38].

An important question is whether ASA influence the conception rate in general and ASA of which compartments are of greatest significance. Collins et al. [7] investigated 471 couples undergoing investigation for marital infertility. Among

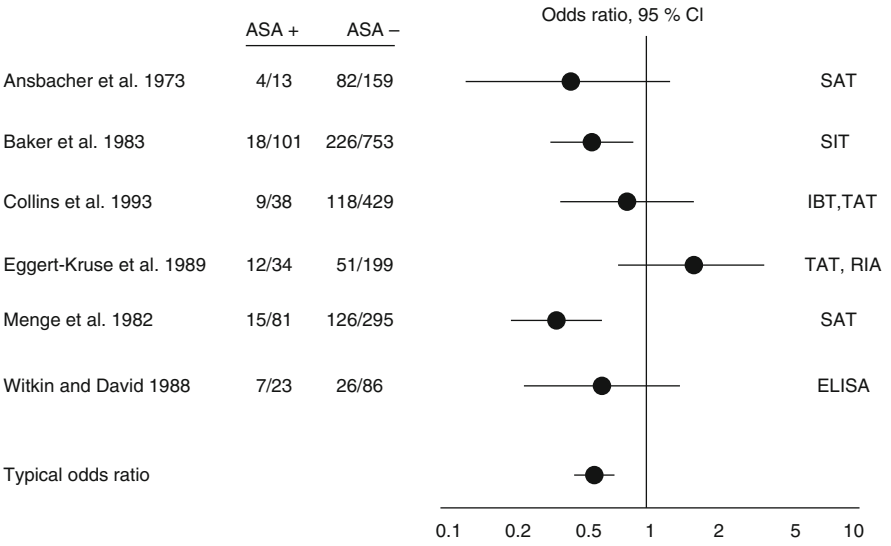

Fig. 9.3 The association of the presence of ASA in infertile men and the pregnancy rate in their partners as calculated on the basis of different studies. The *vertical line* in the middle indicates an odds ratio of 1, i.e., no association. The bars including this *line* indicate no significant increase or decrease of the odds ratio, the length of the *bars* indicate the 95 % CI (From Collins et al. [7], with permission). *SAT* serum agglutination test, *SIT* serum immobilization test, *IBT* immunobead binding test, *TAT* tray agglutination test, *RIA* radioimmunoassay, *ELISA* enzyme linked immunosorbent assay

them, they found 38 men and 6 women being positive for ASA in serum. In 23.7 % of the couples with male ASA in serum a pregnancy occurred, and in 27 % of the couples without ASA, the difference being not significant. Men with ASA, however, had a significantly longer time-to-pregnancy (TTP) and a significantly lower sperm concentration. The authors hypothesized that not ASA themselves might be the cause of subfertility, but the ASA were a consequence of errors in the spermatogenesis, which in turn decreased fertility. With proportional hazards analysis, however, antibody status in either partner was not a significant independent predictor of time to pregnancy (Fig. 9.3). Also Vujisić et al. [48] could not observe any correlations of ASA concentrations in semen, serum, and follicular fluid with the fertilization rate in IVF outcome in 52 couples.

9.2 ASA in Serum

ASA may occur in the blood serum of male and female patients. With the increasing knowledge on the cognate antigens of ASA and their biological relevance, it has become evident that some of the ASA in serum are not a consequence of the contact to sperm antigens, but they are independently existing isoantibodies. This concerns mainly antibodies in female serum, such as the sperm-immobilizing antibodies ([22], see Chap. 11), antibodies to the proacrosin/acrosin system [47], antibodies to

the fertilization antigen-1 or YLP12 [49], and antibodies to the Izumo proteins of human sperm [6]. Also the antibodies detected in cryptorchid boys may represent isoantibodies, and the cryptorchidism itself is not a risk factor for ASA in serum [11, 23, 32, 40]. This holds also true for the ASA detected in patients with testicular tumors [35].

Another hypothesis for the induction of antisperm antibodies (ASA) is based on the crossreactivity between antigens of spermatozoa and exogenous antigens. Common antigenicity has been established between spermatozoa and *Escherichia coli*, *streptococcal antigens*, *Trichomonas vaginalis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* [9]. Also a correlation of ASA testing and the presence of antibodies against chlamydia trachomatis has been described [8]. Since the antibodies have been detected only in the serum of patients with genital chlamydial infection, but not in those with ocular infection, it appeared likely that the ASA formation is a result of the chlamydial inflammatory process with genital localization, but not of cross-reactivity between sperm and C. trachomatis antigens [15]. Also the ASA observed in patients with colitis-ulcerosa might be provoked by the systemic inflammatory responses or by a polyclonal activation of B-cells [9].

9.3 ASA in Seminal Fluid

The main determinant for the concentration of immunoglobulins in seminal fluid is inflammation, whereupon acute inflammation increases the concentrations to a much higher extent than chronic inflammation ([4]; Table 9.2). Similar results were described by Marconi et al. [29], who have included also the results of ASA determination in seminal fluid in their study. There was no difference of the ASA prevalence between healthy men and men with inflammations.

Usually, men expressing ASA in the seminal fluid also have ASA in blood serum. Andreou et al. [2] have described a close correlation between the concentration of ASA fixed to spermatozoa (direct MAR test) and that of ASA solubilized in serum and seminal plasma (indirect MAR test). For IgG, a correlation was found between ASA in seminal plasma and in serum. Vujisić et al. [48], on the other hand, could not find a correlation between ASA concentrations in the different biological fluids.

Table 9.2 Concentration of different proteins in seminal fluid

	Healthy men [25]	Acute prostatitis [25]	Chronic prostatitis [37]
Albumin	0.59	4.7	1.6
Haptoglobulin	0	0.14	0.001
Transferrin	0.04	0.28	0.11
a-1 antitrypsin	0.08	0.22	0.12
a-2 macroglobulin	0	0.12	0.007
IgG	0.21	2.4	0.49
IgA	0.02	0.35	0.13

From Blenk and Hofstetter [4]

The studies indicate that ASA in semen predominantly are the product of locally active B lymphocytes. This is less pronounced in IgG, since IgG in semen is mainly derived from the serum IgG. ASA of the IgA fraction, however, clearly originate from a local production [2]. The conditions are complicated by the fact that human semen contains antibody-binding proteins with IgG-Fc affinity, which is not present in other compartments. The function of these proteins is unclear [5].

As a consequence of different B cell populations present in the different compartments, it appears that the ASA must not recognize identical antigens. Domagała et al. [13] have demonstrated that local antibodies in seminal plasma may bind to other cognate antigens than those in blood serum.

9.4 ASA in Cervix Mucus

The cervical fluid has no unique origin. It is a mixture of secretions from cervical vestibular glands, plasma transudate, and endometrial and oviductal fluids. As cellular components leukocytes are present, the molecular components include inorganic salts, urea, amino acids, proteins, and a number of fatty acids. Among the proteins albumin, transferrin, and immunoglobulins are demonstrable. The characteristic mucins are high molecular, which are heavily glycosylated glycoprotein products of the different mucin genes. They are similar to the mucins of other origin such as saliva, respiratory tract, and the gastrointestinal tract [45].

Immunoglobulin concentrations in the cervix mucus vary with hormonal conditions and with inflammation [39, 44]. During menstrual cycle, they are highest at the day of ovulation, while the levels outside this period are far lower (see Table 9.3). Eighty percentage of the IgA occur in the polymeric forms [28]. The concentrations also vary in the course of pregnancy. Immunoglobulin A remained stable during each trimester of pregnancy (26 mg/dL). Cervical mucus immunoglobulin G decreased from a first-trimester high of 44.4 mg/dL to lower levels in the second and third trimesters [27]. At term of pregnancy, levels of IgG [median 3270 μg/mL] and IgA [540 μg/mL], but not IgM [30.5 μg/mL], were significantly elevated compared to cervical mucus from nonpregnant women [20]. IgG and IgM originate mainly from serum, whereas a local synthesis provided total-IgA and secretory IgA [3].

The occurrence of ASA in cervical mucus is generally quoted to be rare. Stern et al. [42] compared retrospectively the concentration of ASA in serum and mucus by means of the indirect IBT in patients undergoing evaluation for infertility. They found that ASA levels in serum did not correlate with the ASA levels in mucus, which is not

Table 9.3 Immunoglobulin amount in cervix mucus (concentration multiplied by volume of mucus) at midcycle

	IgA	IgG	IgM	total Ig
Ovulation day–1	11.9 ± 9.2	29.2 ± 26.7	5.9 ± 3.3	47.0
Ovulation day–4	2.0 ± 1.5	4.8 ± 3.9	2.5 ± 1.4	9.3

From Kamieniczna et al. [29]

in line with the changing levels of immunoglobulins as described by Kutteh et al. [28]. They also could not demonstrate an alteration with the menstrual cycle. In those couples, in which ASA were demonstrable as a possible cause of infertility, ASA were found in serum in 58 % of patients, but in cervix mucus only in 25 % of patients [25].

Eggert-Kruse et al. [14] found among 192 infertile patients in only 2 % of cervical mucus samples significant ASA levels by means of the indirect MAR test. All ASA positive women had a negative outcome of the postcoital test, but a greater number of negative postcoital tests was independent of ASA. Among 48 patients of Domagala et al. [12] were only two CM samples (4.6 %), which yielded positive results in the indirect IBT. Among 155 infertile women, Kamieniczna et al. [24] demonstrated ASA in 3.2 % of cervix mucus samples by means of the IBT.

Menge and Naz [31] used a special ELISA for the detection of ASA directed to the fertilization antigen-1 (FA-1). In 32 infertile women, 10 sera were negative and 22 positive. Of the 22 CM samples from ASA-positive women, 9 were positive for IgG antibodies, 9 for IgA, 7 for IgA1, and 6 for IgA2.

An interesting question is whether insemination may induce local ASA. Friedman et al. [16] observed 51 women, which underwent 1 to 9 cycles of IUI. In these women, mucus or serum ASA titers did not increase. The observation indicated that the local immune response is not activated by intrauterine insemination. Consequently, this question was not resumed in later studies.

9.5 ASA in Follicular Fluid

Serum proteins and immunoglobulins in follicular fluid are of lower or equal concentration as in blood serum (Table 9.4); the interrelationship between the protein fractions, however, is similar to that in serum [33]. Additionally, also other cofactors of the immune system such as various cytokines such as SCF; IL-2 and IL-11 are present in the follicular fluid; and IL-6, IL-8, TNF-α, MIP-1α, and IFN-γ were detected in oviductal fluid [41]. A first analysis of the proteomics of follicular fluid has been published by Anahory et al. [1]. A 2D-electrophoresis revealed up to 600 protein spots. The proteome undergoes characteristic changes with age and hormonal status [19].

Kohl et al. [26] tested follicular fluid for ASA in 38 women by means of an ELISA. Positive results were found only in women with antibodies circulating in serum ($r=0.88$, $P<0.001$). There was no correlation between ASA in serum with sperm agglutination and the postcoital test. Neither was there any correlation between antibodies in follicular fluid and the postcoital test, the pregnancy rate or successful IVF. Nip et al. [34] described a higher prevalence of ASA in infertile women, and a relation between the concentration found in serum and follicular fluid. Marín-Briggiler et al. [30] demonstrated the presence of ASA in the follicular fluid, which were able to induce the AR in capacitated human donor spermatozoa. These ASA were also able to block the zona-binding of spermatozoa [46]. Vujisić et al. [48] found no association of ASA concentrations in the follicular fluid have with those of the blood serum. In their study, the authors also have described no association of ASA concentrations in the different biological fluids and with the fertilization rate.

Table 9.4 Follicular fluid and serum concentration of proteins (g/l) in relation to ovarian stimulation

	N	Clomiphene-hMG-hCG	*N*	hMG-hCG	Serum range
Total proteins	40	46.33 ± 7.26	20	43.66 ± 7.36	65–80
Fibrinogen	38	0.24 ± 0.17	17	0.23 ± + 0.15	2.0–4.5
α-2 Macroglobulin	40	0.20 ± 0.11	20	0.17 ± 0.09	1.75–4.20
α-l Antitrypsin	40	4.29 ± 1.66	20	4.22 ± 1.09	1.9–3.5
IgG	40	7.56 ± 1.87	20	8.02 ± 1.88	8–18
IgA	39	1.03 ± 0.52	19	0.73 ± 0.30′	0.8–4.5
IgM	7	0.44 ± 0.12	3	0.28 ± 0.03′	0.7–2.8

From Suchanek et al. [43]

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ASA in the Female

10

Gary N. Clarke

Abstract

This article briefly reviews our knowledge about antisperm antibodies (ASA) in females and outlines several hypotheses regarding the etiology of sperm immunity in women.

There is evidence that strong ASA in females can reduce the chances of conception and ASA from female sera have also been found to inhibit in vitro fertilization (IVF) in humans and some animal models. Several possible factors leading to the development of ASA in human females have been proposed, including cross-reactivity with microbial antigens, and the possible role of antibody idiotypes and interferon gamma-mediated potentiation of the antisperm immune response in women whose male partners have ASA in their semen. It is vital that more research is conducted in this area if we are to understand female immuno-modulation in response to sperm antigenicity.

10.1 Introduction

The main aim of this chapter is to review selected literature which is pertinent to understanding why some females develop sperm immunity, with primary focus on antisperm antibodies (ASA) detectable in serum, follicular fluid, or cervical mucus. Another important aim is to discuss several aspects/observations from animal models which have so far received little consideration from the clinical perspective with the objective of stimulating more research focus in these areas. Other chapters in

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this volume cover in detail the tests available for detecting ASA (Chap. 13), antigens (Chap. 2), impact on assisted reproduction (Chap. 15), and treatment of immune infertility (Chap. 16) and other important aspects.

10.2 Historical Background

During the first few decades of the twentieth century, many studies in animals had indicated that homologous or heterologous immunization of females with sperm or testis preparations could induce sperm antibody activity and infertility (see Katsh [1] for review). The considerable evidence derived from animal models, combined with preliminary evaluation of patients, provided stimulus for "clinical trials" involving immunization of women with their partner's semen with the aim of inducing immuno-contraception. Baskin [2] reported on a study of 20 fertile women immunized three times intramuscularly at weekly intervals, with their partner's whole ejaculate. All but one of the women showed sperm immobilizing activity in their serum by 1 week after the last injection which persisted for up to 1 year. One woman became pregnant after 12 months when the sperm immobilizing activity was no longer detectable in her serum. These trials demonstrated that women could be immunized to develop sperm immobilizing activity and that this was associated with reduced fecundity.

Further significant evidence for female ASA association with human infertility awaited the report by Franklin and Dukes in 1964 [3]. They found that 20.1 % of 214 women undergoing infertility investigations had detectable sperm agglutinating activity in their serum. Women with unexplained infertility had a much higher incidence (72.1 %) than women with organic causes for their infertility (8.4 %) or fertile women (5.7 %). It should be noted that this study found a very high incidence of ASA, and the results are not supported by recent studies using immunologically specific procedures such as the immunobead test (IBT). However, this report was notable from an historical perspective in that it stimulated significant interest in the idea that female immunological responses to sperm could be involved in the development of otherwise unexplained infertility and in the concept of an antisperm contraceptive vaccine.

10.3 More Recent Studies on ASA in Females

Since the early reports described above, a multitude of studies have examined the effects of ASA on sperm-cervical mucus penetration, in vitro fertilization (IVF) and infertility. Many review articles have described the clinical and experimental research in this area [4–7]. It is pertinent however to review some of the background information and studies which are relevant to explaining the pathogenesis of female immuno-infertility associated with ASA.

The uterine cervix is a highly competent mucosal immune site (for review, see [8]) which contains many IgA-positive plasma cells located in the subepithelial

layers of the endocervix. Most of the IgA in cervical mucus is secretory IgA consisting of two IgA monomers linked by J-chain and secretory piece. The secretory IgA antibodies directed against potential pathogens and occasionally sperm [9] can immobilize the invaders by cross-linking them to the cervical mucus strands, effectively blocking their progress to the upper reaches of the reproductive tract [10]. There are obviously mechanisms which normally prevent such immunological reactions to sperm in women. However, in a small percentage of couples, these are somehow circumvented or disrupted, resulting in local and often circulating ASA production and reduced chances of natural conception. In women with otherwise unexplained infertility, sperm antibody activity has been detected in cervical mucus in more than 10 % of cases [11–13].

Investigations using zona-free hamster eggs or salt-stored human zona pellucidae indicated that high level ASA might be expected to interfere with human fertilization [4], but this could not be adequately confirmed using fresh human oocytes until the availability of routine clinical IVF around 1985. Retrospective analysis of IVF results by Clarke et al. [14] provided some of the first evidence that ASA from female serum could inhibit the fertilization of viable human oocytes by human spermatozoa. They observed a fertilization rate of only 15 % for patients who had significant titers of IgG and IgA class ASA in their serum, which at that time was used as a supplement in the IVF culture medium, versus 69 % for those patients where replacement serum was used during the fertilization culture. Their later experimental results confirmed that very high titer ASA of IgG immunoglobulin class in female serum could effectively inhibit fertilization of fresh human oocytes [15]. Subsequent reports from other laboratories have also indicated that high level ASA can inhibit human fertilization [16–18]. In addition, more recent animal studies have also provided considerable evidence that experimentally induced sperm isoimmunity could have detrimental effects on fertility and in vitro fertilization [4]. Consequently, it is now generally accepted, at least with strong sperm immunity, that ASA can block sperm functions such as cervical mucus penetration and fertilization and thereby impair fertility.

10.4 Clinical Evaluation of ASA

It is strongly recommended that both the female and male partners should be tested for ASA during infertility assessment. The initial investigation of the male partner of an infertile couple should include a direct mixed antiglobulin reaction (MAR) screen for sperm-bound antibodies [7]. A positive result (>50 % of motile sperm being antibody coated) should be followed up with a repeat test and preferably mucus penetration testing to make an assessment of the potential functional significance of the antibodies. High levels of circulating antibodies in the female may severely reduce the chances of successful treatment by IVF [4] or donor insemination. Assessment of in vitro sperm-mucus interaction by means of the capillary (Kremer) test and/or the semen/cervical mucus contact test (SCMCT) may suggest the likely presence of sperm antibodies in CM, even though circulating ASA may

have been weak or undetectable. The presence of antibodies in CM can be confirmed by testing liquefied CM using the indirect MAR. The presence of high CM antibody levels and associated negative or low titer circulating ASA suggests a good prognosis for treatment of the couple by intrauterine artificial insemination. In contrast, the presence of high antibody concentrations or titers both locally and systemically suggests a poor prognosis. Couples with apparently intractable immuno-infertility can be effectively treated using intracytoplasmic sperm injection (ICSI) [19].

10.5 Postfertilization Effects of ASA on Fertility

Definitive studies in various animal models have shown an association between ASA and pre- or postimplantation embryonic degeneration [20]. In one study on rabbits, reproductive tract secretions containing ASA were found to cross-react with rabbit morulae and blastocysts, resulting in embryotoxic effects during in vitro culture [21]. In a number of tightly controlled experiments, this group demonstrated that only secretory IgA (sIgA) from the uterine fluid of semen-immunized does was embryotoxic during in vitro culture. In contrast, blood sera with high levels of ASA were not embryotoxic, nor were IgG fractions isolated from the immune uterine fluid (IUF). Absorption of IUF with either sperm or anti-sIgA removed the embryotoxicity, thereby providing evidence of specificity. Other experiments indicated that the sperm antigen stimulating the sIgA embryotoxic antibody in IUF was distinct from the antigen stimulating IgG and IgA class ASA with the ability to inhibit fertilization. In unpublished observations, absorption of the IUF with paternal lymphocytes did not remove the embryotoxicity, indicating that transplantation antigens were unlikely to be involved. Additional investigations suggested that the antigen responsible for the sIgA-associated embryotoxicity was a subsurface component. Thus, immunization of does with isolated sperm membrane fractions resulted in reduced fertilization, whereas immunization with submembrane fractions caused only the postfertilization effects on embryos.

Why should ASA react with embryos? Firstly, the sperm membrane is integrated as a mosaic into the zygote membrane during the process of fertilization, so that sperm antigens are incorporated, although at relatively low densities, into the developing embryo [22]. Secondly, embryonic gene expression commencing from the four to eight cell stage results in the synthesis of various developmental antigens which can cross-react with sperm antigens (for review, see Menge and Naz [23]). Consequently, during embryo development and perhaps particularly around the time of blastocyst hatching, there is a chance for the ASA to bind to cross-reacting embryonic antigens and potentially cause embryo degeneration or possibly prevent implantation.

There is also some evidence for postfertilization effects associated with ASA in humans. Concerning negative effects, Warren Jones [24] reported that around 50 % of pregnancies conceived in women with ASA subsequently ended in first trimester spontaneous miscarriages. Similar observations have been reported by other groups

[11, 25]. In the latter study, it was found that 7/16 (44 %) of women who miscarried were positive for ASA in their serum, compared with only 2/17 (12 %) of women who had successful ongoing pregnancies. Examination of the immunoglobulin classes of the antibodies revealed that IgA was significantly ($p < 0.01$) more common in those women who miscarried. The IgA class antibodies in serum may be a marker for local secretory IgA in the female reproductive tract. However, despite the strong evidence in rabbits, it is still not known whether sIgA class ASA in humans are embryotoxic. In another clinical study [26], it was found that of 173 women referred for a history of three or more consecutive spontaneous miscarriages, there was a significantly higher incidence of sperm immobilizing antibodies when compared with the infertile group. Interestingly, they also observed a higher incidence of ASA in the group of women shown to have an immunological basis for their recurrent miscarriages (for example, couples sharing at least three HLA determinants, or couples with the female showing a relatively low response to her partner's lymphocytes in mixed lymphocyte culture). Other groups have reported a significant association between ASA and some autoantibodies such as antiphospholipids, which may be involved in deleterious effects on the fetus. In contrast to the studies cited above which have reported an association between ASA and recurrent miscarriage, others have not seen a statistically significant association [27, 28]. Further investigations in this area would be useful, particularly focusing on the possible involvement of subsurface sperm antigens which react with IgA class ASA. It is important to note that sperm antibodies specific for subsurface antigens are unlikely to be detected by assays such as the immunobead test (IBT) or the MAR which are designed to measure reactivity with membrane antigens on motile sperm. It could be very informative to conduct a clinical investigation of IVF patients with repeated implantation failure or early spontaneous miscarriages, using a new generation of highly specific ELISA and immunofluorescence assays in conjunction with the MAR (unfortunately immunobeads are no longer available so the IBT has become obsolete).

With respect to positive effects of sperm immunity, there is some evidence from analysis of IVF data, suggesting that some ASA may be associated with increased implantation rates [29, 30]. If confirmed, this could add an interesting new dimension to our analysis and understanding of sperm immunity. It also underlines the potential importance of efforts to develop routine assays, which can identify sperm antibodies reacting with defined antigens.

10.6 Origins of ASA in Females

It is obvious that normal fertile women do not usually mount strong immune reactions to sperm, resulting in high titers of ASA capable of blocking sperm function and reducing fertility. Although it is still uncertain what exact mechanism is acting to suppress the female immune response to sperm antigen after sexual intercourse, there are several possible ways in which this could occur. Firstly, experimental evidence indicates that seminal plasma contains potent immunosuppressive factors.

Some sperm antigens may carry suppressor epitopes, which could inhibit an effective B-cell response and ensuing sperm antibody production. The potential relevance of asymmetric immunoglobulin in modulating sperm immunity also requires thorough evaluation [31]. If the initial immunosuppressive mechanisms fail to prevent the initiation of sperm antibody production, then it is also possible that anti-idiotype antibodies, if produced in sufficient quantities, could inhibit production of the related idiotype (anti-idiotypes are discussed in more detail below). Despite these hypothesized safeguards, a small proportion of women do develop significant levels of ASA in their blood and reproductive tract.

What information is currently available regarding the development of or predisposing factors for sperm immunity in females? Observations of potential relevance to understanding the underlying causes of ASA in women include evidence that they are more likely to have detectable sperm antibodies if their male partner also has ASA in his semen [32]. Another important observation was that in about one-third of cases women apparently react only to their partner's sperm antigens, rather than to sperm-specific antigens [33]. Several hypotheses have been proposed in order to explain the origins of female sperm immunity and the observed association between male and female sperm immunity in a proportion of couples.

The first hypothesis is based on observations that human spermatozoa have antigens which cross-react immunologically with certain microbial antigens. Thus, Sarkar [34] reported that antibodies with specificity for certain yeast mannan molecular configurations cross-reacted with sperm membrane antigens. For example, 75 % of sera from men with ASA were found to react with the one, six yeast mannan specificity. In addition, some patients reacted with the one, three mannan specificity or with chemotype C1 from Salmonella paratyphi C. In another investigation, Blum et al. [35] observed a strong association between Chlamydia antibodies and ASA in young women using oral contraceptives. Similarly, Cunningham et al. [36] reported that 56 % of women with primary pelvic inflammatory disease (PID) had ASA detectable by the indirect mixed agglutination reaction. Sera from these patients uniformly reacted with a 69 Kd band by western blotting. Because both partners would be likely to be exposed to the same microbes during unprotected sexual intercourse, they would also be expected to have an increased chance of concurrently developing ASA. In summary, although several clinics have reported significant associations between genital tract infections and ASA [35, 36], a more recent and very thorough study did not confirm such an association [37]. More research in this fascinating area should be encouraged.

A second interesting hypothesis was based on the observation by Steven Witkin [38] that antibody-coated sperm stimulated in vitro interferon gamma (IFN-γ) synthesis by lymphocytes from female donors. In contrast, antibody-free sperm did not cause IFN-γ production. Given the evidence that IFN-γ induces macrophages to express Ia antigen (MHC class II marker) on the cell surface, the resulting juxtaposition of sperm antigen and Ia on the macrophage cell surface would be expected to facilitate the recruitment of T-helper cells and subsequent initiation of ASA production by B lymphocytes. These observations are consistent with the finding that women are more likely to develop sperm antibodies if their partner has sperm

autoimmunity. Of significant relevance here is a recent investigation [39] which demonstrated the complexity of the in vitro cytokine response when peripheral blood mononuclear cells (PBMCs) from infertile women were incubated with sperm antigens for up to 5 days. Crucially, this study found that sperm antigens induce differential cytokine response patterns in PBMCs from infertile women with ASA, versus those without ASA, or fertile controls. Specifically, the study observed a marked increase in IL-2 and IL-4 in the former group. The authors concluded that if these changes also occur in vivo, then the modulated cytokine environment could facilitate potentiation of the Th2-type response with heightened ASA production. Logically, the ASA could be of either the female partner or from her male partner if he had sperm autoimmunity.

A third tentative hypothesis has recently been postulated [40] based on the likelihood that if a male had ASA in his semen, then during repeated acts of sexual intercourse his female partner would be expected to develop a range of anti-idiotype antibodies which could potentially facilitate an immune response to his sperm. A summary of the background to this hypothesis is presented below.

Jerne [41] proposed that antibodies should be antigenic to the individual's own immune system, resulting in the production of autoantibodies directed against the unique (idiotypic) parts of the antibody which comprise the antigen-binding site. The result is a network of idiotype/anti-idiotype interactions which are involved in regulation and modulation of the immune system. The antigen-binding site of the anti-idiotype mimics the original antigenic structure which was recognized by the individuals' immune system (Fig. 10.1). Consequently, immunization against a particular antibody idiotype can potentially provide a means of stimulating an immune response directed towards the original "native" antigen. There have been numerous investigations into the application of anti-idiotypes for generating enhanced immune responses to cancer cells and infectious agents [42].

Several groups have shown that polyclonal heterologous anti-idiotype antibodies can be generated against the idiotypes on monoclonal ASA [43–45] and that the anti-idiotype could significantly inhibit the binding of the monoclonal antibody to sperm. Testing of the anti-idiotype supported the hypothesis that its' ability to inhibit the original monoclonal antibody was due to its antigen-binding site forming a similar shape to the original antigenic epitope, the so-called internal antigen image [44].

If the male partner had ASA in his semen, how would the female immune system respond to repeated exposure to these antibodies? In light of the above information about idiotype/anti-idiotype responses, it is possible that the female would produce anti-idiotype antibodies, which could ultimately potentiate an antisperm immune response. It is also important to note that the female could potentially form anti-idiotype antibodies directed against the male partner's antibodies specific for intracellular sperm components, in addition to those specific for sperm membrane antigens. The associated "parallel set" of anti-anti-idiotypes could also potentially react with some sperm surface epitopes. In other words, it is feasible that the idiotype hypothesis could potentially explain most of the observed range of female ASA activity.

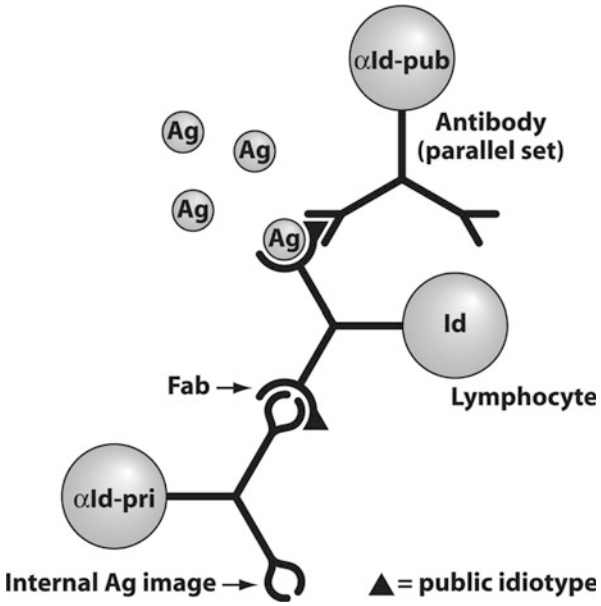

Fig. 10.1 The immune response to antigen (*Ag*) generates antibodies bearing unique idiotypic (*Id*) signatures comprising the antigen-binding site or paratope of the antibody [47]. The individual's immune system subsequently sees the unique Id as foreign and responds by forming anti-Id (*α-Id*) antibodies, some of which recognize public Ids (*Id-pub*) present on other antibodies of different Ag specificity, while some recognize internal or private (*Id-pri*) parts of the Fab (internal Ag image). The former may recruit B lymphocytes producing antibodies of various specificities (the parallel set), while the latter can potentially augment the production of antibodies reacting with the original Ag

An extremely interesting study by Naz et al. [46] demonstrated the presence of anti-idiotype antibodies in women (albeit against their own antibodies, rather than their partner's, however it provides solid evidence that women can produce anti-idiotype antibodies against sperm antibodies). These authors concluded that both fertile and infertile women form immune responses to sperm, but that sperm antibodies are usually not detected in fertile women because their reactivity in assays is blocked by high levels of anti-idiotype antibodies. They concluded that higher levels and incidence of sperm antibodies are detected in infertile women because their sera contain relatively low concentrations of the blocking anti-idiotype antibodies. However, an alternative explanation of these findings is more consistent with current knowledge about the immune response [47]. Thus, higher levels of anti-idiotype antibodies to a particular antigen lead to active suppression of the host immune response, whereas low levels can lead to a significant stimulation of production of the idiotype (i.e., sperm antibody in this case). Thus, with respect to the study by Naz et al. [46], it is probable that sperm antibodies were not detected in the fertile women because their production had been inhibited by the anti-idiotype antibodies, rather than the anti-idiotype antibodies blocking the binding of sperm antibodies during the assay. Low concentrations (nanogram range) of anti-idiotype antibodies

on the other hand can lead to enhancement of the immune response to the original antigen (ie sperm in this case). Naz et al. [46] detected anti-idiotype antibodies in only 3/23 infertile women, but the sensitivity of their assay at this concentration range may have been a factor. Further investigation of this phenomenon is vital in order to improve our understanding of female immune reactions to sperm.

With regard to the idiotype hypothesis, further research is still required in order to try to understand the relationship between anti-male idiotype antibody, which could be generated in women exposed to semen containing ASA, and anti-female anti-idiotype antibody formed when women react to their own sperm antibodies. Another consideration is whether seminal plasma contains anti-idiotype antibody in suitable amounts to have direct effects on the female immune system?

It is quite possible that the development of ASA in some women may involve one or more of the several postulated mechanisms operating in concert. For example, the stimulation by antibody-coated sperm of IFN-γ gamma synthesis in the female partner's lymphocytes could potentially augment her immunological response to antibody idiotypes in semen (cytokines such as IL-2 and IL-4 may also be involved, as discussed above). It is also feasible that some women initially respond to microbial antigens (microbes attached to the sperm surface can also stimulate IFN-γ gamma production by the female's lymphoid cells), resulting in the formation of antibodies which cross-react with sperm – this immune response could then be maintained over a longer period by her ongoing exposure and response to antisperm idiotypes in semen and/or generation of anti-idiotype antibodies against her own sperm antibodies. The relationship between the three hypothesized mechanisms requires investigation.

Conclusions

Unfortunately there has been relatively little research interest in female sperm immunity in recent years. Further understanding of the reactivity of the female immune system to semen antigenicity, including experimental investigation of the idiotype hypothesis, may help to explain immuno-infertility, but could also have significant implications for the development of immuno-contraceptive vaccines and for the wider understanding of normal pregnancy and its' associated pathology. Thus, the recognition of the male partner's antibody idiotype spectrum in semen by the female's immune system provides a potentially important means of cross talk, which could prove vital for the establishment of normal pregnancy. It would also be very interesting to explore the possible implications of idiotype responses within the seminal priming hypothesis proposed by Robertson [48].

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Sperm Immobilizing Antibody and Its Target Antigen

11

Akiko Hasegawa, Minoru Shigeta, and Hiroaki Shibahara

Abstract

Complement-dependent sperm-immobilizing antibodies (SI-Abs) are detected exclusively in the serum of infertile women. In this chapter, first we suggest a treatment protocol for these infertile patients based on antibody titers. Second, we describe the identification and characterization of an antigenic epitope for SI-Ab. A number of sperm immobilizing monoclonal antibodies were generated to examine the antigenic epitopes for SI-Abs. Among them, a human monoclonal antibody (Mab H6-3C4) was established from the peripheral B lymphocytes of a patient with strong SI-Abs. The epitope for Mab H6-3C4 was found to be the carbohydrate moieties of CD52. CD52 is known as a glycosyl-phosphatidyl inositol (GPI) anchor glycoprotein present in lymphocytes and male reproductive tracts (mrt) including mature sperm and seminal plasma. However, the immunochemical properties of mrt-CD52 are different from these of lymphocyte CD52. This article describes the biological and immunological functions of mrt-CD52 and a possible mechanism of sperm impairment by the corresponding antibody.

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11.1 Introduction

ASAs detected by sperm immobilization tests are present exclusively in unexplainedly infertile women [1, 2]. When sperm-immobilizing antibodies (SI-Abs) are detected in the serum, they are also found in the peritoneal fluid, follicular fluid and cervical mucus. Patients with a high titer of SI-Abs are found to have difficulty conceiving a child [3, 4]. We have reported that SI-Abs impair passage of sperm in female reproductive tracts from the cervix through the Fallopian tubes and also blocks binding of sperm to the zona pellucida [5, 6]. Although previous studies have shown that carbohydrate moieties of sperm and seminal plasma are major target antigens for SI-Abs [7, 8], the identification of antigenic epitopes has been difficult due to the heterogeneity of SI-Abs in patients. Elucidation of the epitopes recognized by SI-Abs is important not only for understanding the mechanism of immunological infertility but also for developing a means of treatment for infertility resulting from SI-Abs.

11.2 Clinical Treatments

ASAs are detected in unexplainedly infertile couples by several methods. ASA production in females may be a natural event, because sperm are foreign antigens for females. However, most females do not produce ASA in which impair sperm and have normal fertility. What is the ingenious mechanism by which sperm escape immune surveillance in the female reproductive tracts? This basic question is still unanswered.

In clinical observation, collapse of the escape mechanism has happened, although occurrence is not high (2–3 % in infertile females). ASAs primarily impair sperm transportation in female reproductive tracts.

There are several methods to detect ASAs. One of them termed the Sperm Immobilization Test (SIT), detects sperm motility impairment and is a feasible assay for detection of ASA, because inhibition of sperm motility is directly correlated to sperm impairment. Antibodies detected by this assay are called Sperm Immobilizing Antibodies (SI-Abs) [9]. SIT is carried out using a mixture of the patient's sera, sperm and complement. Complement-dependent sperm immobilization possibly detected the effective antibody inducing infertility. The assay method is shown in Fig. 11.1. The results are represented as a Sperm Immobilizing Value (SIV). SIV is a ratio of % sperm motility in the control to that in test sera, where a value of more than 2 is assessed positive and less than 2 is assessed negative. SIT is a semi-quantitative assay, because the SIV of positive sera possibly can show different values from 2 to infinity (∞). ∞ means all sperm examined are immotile. For precise analysis of such cases, antibody concentrations are reduced by serum dilution. Serum dilutions that recover sperm motility by up to 50 % are represented as SI_{50}.

Currently, SI-Abs in patients' sera can be examined by several clinical testing companies in Japan. They provide quantitative values (SI_{50}) as well as SIV. According

1. Test serum 10µl
   sperm suspension 1 µl
   complement 2 µl
   → 32°C, 60 min → sperm motility (T%)

2. Control serum 10µl
   sperm suspension 1 µl
   complement 2 µl
   → 32°C, 60 min → sperm motility (C%)

$$SIV = \frac{(C\%)}{(T\%)} : \begin{matrix} \geqq 2 : \text{positive} \\ < 2 : \text{negative} \end{matrix}$$

Fig. 11.1 Protocol of sperm immobilization test. The mixture of test serum, sperm suspension (4×10^7/mL, >90 % motility) and guinea-pig serum (>200 CH_{50}) as complement are incubated for 60 min at 32 °C. Sperm motility is represented as % of motile/examined sperm. Sperm immobilizing value (SIV) is calculated by dividing (T%) by (C%)

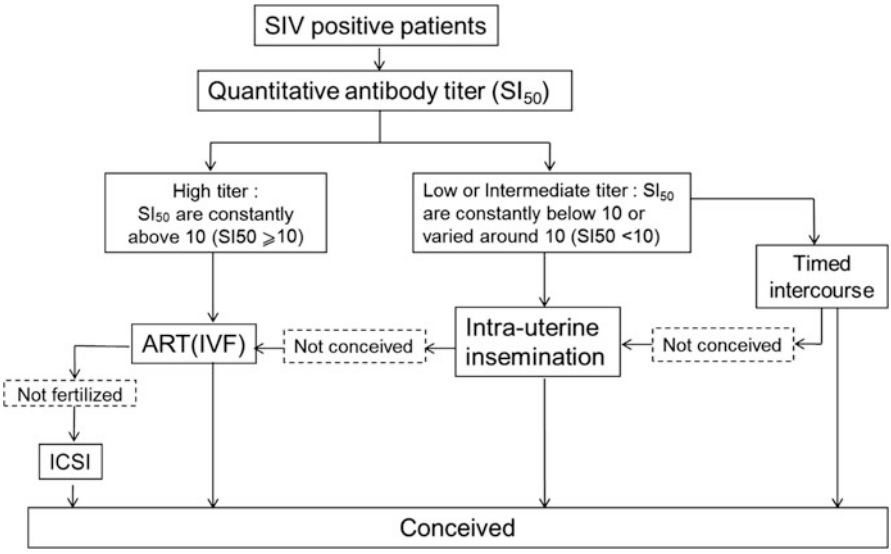

Fig. 11.2 Treatment flowchart for female patients with sperm immobilizing antibodies

to the results, the therapy process is selected as shown in the flowchart (Fig. 11.2). It is important to test in a single patient several times over 2–3 months because the antibody titers change. When keeping high titers of more than 10 ($SI_{50} \geqslant 10$), IVF-ET is suitable as a treatment choice, while for lower titers ($SI_{50} < 10$) or changing titers, intra-uterine insemination or timed intercourse are occasionally successful (Table 11.1). Our investigations indicated that SI_{50} was not correlated to the fertilization rates (%) in IVF-ET as shown in Fig. 11.3. Even cases in high titer ($SI_{50} \geqslant 100$) showed a fertilization success rate of more than 50 %. On the other hand, it is noticeable that some patients with lower titers had low fertilization rates.

Table 11.1 Correlation between quantitative antibody titer of sperm immobilizing antibodies (SI_{50}) and cumulative probabilities of conception by different[a]

Group	No. of patients conceived		
	Timed intercourse	IUI[b]	IVF-ET[c]
Higher titer[d]	0/36 (0.000)	1/34 (0.029)	29/234 (0.853)
Lower titer[e]	1/12 (0.083)	4/12 (0.333)	7/8 (0.875)

[a]Values in parentheses are cumulative probabilities of conception that was calculated at seventh treatment cycle using Kaplan Meier method
[b]Intra-Uterine Insemination
[c]In Vitro Fertilization and embryo transfer therapy
[d]SI_{50} in this group were constantly above 10
[e]SI_{50} in this group were constantly below 10 or varied around 10

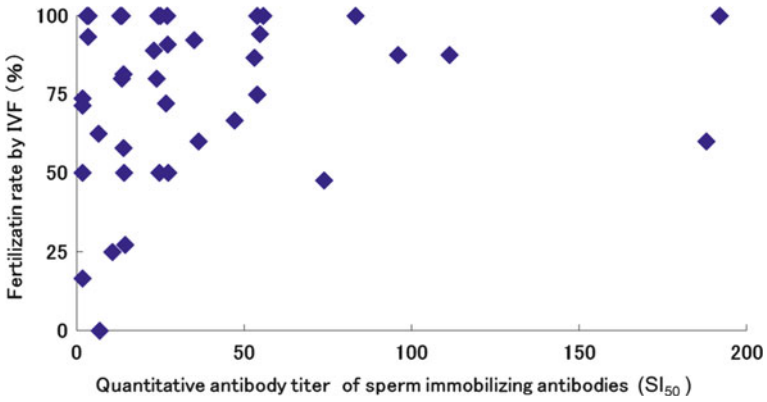

Fig. 11.3 Correlation between quantitative antibody titer of sperm immobilizing antibodies (SI_{50}) and fertilization rate by clinical treatment of IVF-ET

This suggests that complex factors including SI-Abs interfere with fertilization. In these cases ICSI may be recommended. Furthermore, attention should be paid to patients' ages. Patients at higher ages need earlier examinations so as not to consume their reproductive time.

11.3 Characterization of Sperm Immobilizing Monoclonal Antibodies

For characterization of antigen epitopes for SI-Abs, a number of human and mouse monoclonal antibodies with complement-dependent sperm-immobilizing activity were generated in our laboratory [10–12]. A human monoclonal antibody, Mab H6-3C4, with a high titer of SI activity was established using peripheral B-lymphocytes from an infertile woman [13]. A mouse monoclonal antibody, 1G12, reactive to human sperm membrane also showed a high titer of SI activity [4]. Another mouse monoclonal antibody, S19, generated by Herr's group, showed

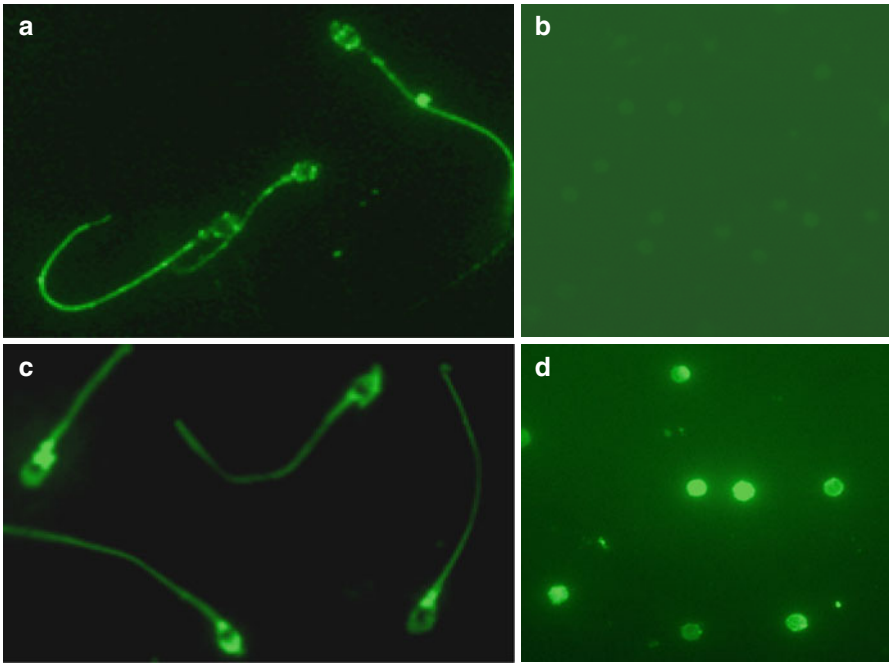

Fig. 11.4 Immunofluorescent stainings of formalin-fixed human sperm with monoclonal antibodies. Mab H6-3C4 reacts exclusively with sperm (**a**) but not with lymphocytes (**b**), while campath-1 recognizes the lymphocytes (**d**) as well as sperm (**c**)

strong sperm agglutinating and SI activities and the corresponding antigen was termed as SAGA-1 (sperm agglutination antigen-1) [14]. Campath-1 is a rat monoclonal antibody defining CD52 as an antigen [15]. It was established against human spleen cells and reacted with virtually all leucocytes. Subsequent studies showed campath-1 was cross-reactive to mature human sperm [16] with sperm agglutinating and immobilizing activities similar to monoclonal antibodies generated to sperm antigens.

Tandem mass spectrometric analysis shows that there are distinct differences in the N-linked carbohydrates between lymphocyte-CD52 and mrt-CD52 [17, 18]. Both lymphocyte- and mrt-CD52 are GPI (glycosylphosphatidylinositol) anchor glycoproteins and the molecular conformation formed by three C-terminal amino acids and the GPI anchor is recognized by campath-1 [19]. The observation that Mab H6-3C4 recognizes exclusively with sperm suggests that this monoclonal antibody reacts a sperm-specific antigen present in a carbohydrate moiety [20]. Other CD52-recognizing monoclonal antibodies such as 1G12 and campath-1 react with sperm and also with lymphocytes [4]. This suggests that the epitopes for these monoclonal antibodies are the common sites of mrt-CD52 and lymphocyte-CD52.

Figure 11.4 shows indirect immunofluorescent stainings of human sperm and lymphocytes with Mab H6-3C4 and campath-1. Both monoclonal antibodies stain

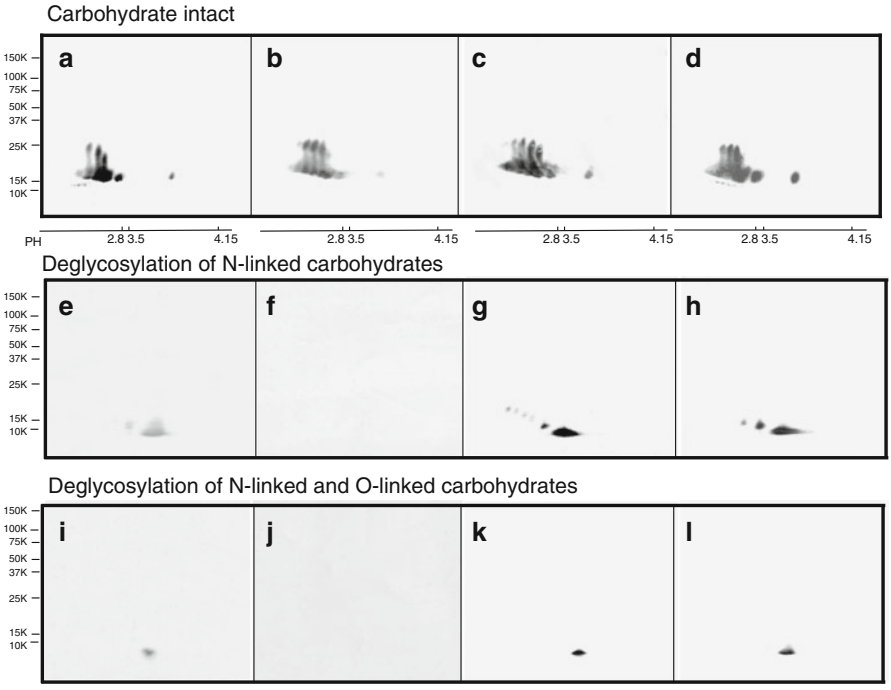

Fig. 11.5 Two-dimensional PAGE and Western blot analysis of sperm extracts with monoclonal antibodies before and after the treatment with deglycosylation. The sperm extracts were separated by two-dimensional PAGE, blotted onto a PVDF membrane and probed with antibodies. (**a**–**d**): intact mrt-CD52; (**e**–**h**): N-linked carbohydrate-deglycosylated mrt-CD52; (**i**–**l**); N- and O-linked carbohydrate-deglycosylated mrt-CD52. (**a**, **e**, **i**): anti-peptide antibody (positive control); (**b**, **f**, **j**): Mab H6-3C4; (**c**, **g**, **k**): 1G12; (**d**, **h**, **l**): Campath-1

the whole sperm surface but lymphocytes are stained with campath-1 only. It appears that the antigens recognized by these monoclonal antibodies are similarly distributed on the sperm surface. Mab H6-3C4 did not react with lymphocytes and exclusively recognizes mrt-specific antigen while campath-1 recognizes a core structure of CD52 shared by lymphocytes and mrt.

For detailed analysis of the epitopes, sperm extracts were subjected to high-resolution two-dimensional polyacrylamide gel electrophoresis with the first dimension in a pH 2–4 range and the second dimension in molecular sieving followed by Western blot analysis [20]. As positive control, anti-CD52 antibody produced to a core peptide comprising 12 amino acids was used. For carbohydrate analysis, mrt-CD52 extracted from sperm was treated with N-glycosidase F to remove the N-linked carbohydrate. The presence of O-linked carbohydrates was examined by mild alkaline treatment. Figure 11.5 shows that anti-CD52 peptide antibody reacts with intact mrt-CD52 molecules showing a heterogeneous staining pattern of PI <2.8 and MW 15–25 K (Fig. 11.5a). This heterogeneity is markedly reduced by deglycosylation of N-linked carbohydrate (Fig. 11.5e). Additional removal of O-linked carbohydrates results in staining of a single spot (Fig. 11.5i), suggesting that the O-linked carbohydrate

contributes to molecular polymorphism of mrt-C52. Recently, the existence of the O-linked carbohydrate in mrt-CD52 has been demonstrated by lectin binding assay [21] and MALDI-TOF mass spectrometry [22]. Monoclonal antibodies, Mab H6-3C4, 1G12 and campath-1, show similar polymorphic staining pattern in the region of PI <2.8 and MW 15–25 K (Fig. 11.5b–d). The patterns of staining change after removal of the N-linked carbohydrate. In the case of Mab H6-3C4, no staining is observed after the removal of the N-linked carbohydrate (Fig. 11.5f). In the case of 1G12 and campath-1, heterogeneity is reduced but still several spots remained. 1G12 shows with six spots at different pH, while campath-1 reacted with three spots, suggesting that the epitope for 1G12 is not identical to that for campath-1 (Fig. 11.5g, h). These results show that Mab H6-3C4 recognizes the N-linked carbohydrate moiety of mrt-CD52, while 1G12 and campath-1 recognize the core portion of mrt-CD52. After further removal of the O-linked carbohydrate, 1G12 and campath-1 yield single spots like the positive control (Fig. 11.5k, l). Collectively, these results confirm that Mab H6-3C4 and 1G12 recognize mrt-CD52 but the epitopes are different. The epitope for Mab H6-3C4 is present in the N-linked carbohydrate, while the epitope for 1G12 is present in the core portion of CD52. These results indicate that SI-Abs in some infertile women produced against mrt-specific carbohydrate antigens in the mrt-CD52 molecule. Indeed, it has been reported that mrt-CD52 contains specific carbohydrate chains [18].

11.4 Hypothetical Structure of CD52

Based on biochemical and immunological analyses, a hypothetical structure of mrt-CD52 is presented in Fig. 11.6. The core peptide of CD52 is composed of just 12 amino acids and common to lymphocytes and mrt. This suggests that the peptide portion is a scaffold for supporting carbohydrate moieties. The core peptide contains ^{3}Asn(N)^{4}Asp(X)^{5}Thr(T), a consensus sequence, for N-linked carbohydrate binding. It has been reported that mrt-CD52 is heavily glycosylated with heterogeneous carbohydrate chains comprising more than 50 different glycoforms which are almost completely sialylated and fucosylated in 10–15 % of total mrt-CD52 [18]. In contrast, the carbohydrate moieties in lymphocyte CD52 are much smaller and only lightly sialylated but not fucosylated [17]. Another distinct structure of the mrt-CD52 carbohydrate is [GluNAcβ1-6Man] in the N-linked carbohydrate chain. The presence of the β1-6 bond possibly allows branching of a carbohydrate chain to the backbone [23]. The carbohydrate branching via this bond has been reported to contribute to the metastatic potential of tumor [24].

The GPI anchor portion is bound to Ser residue at the carboxyl terminal through ethanolamine linked to three mannose, inositol residue and glycerolipid. Approximately 80 % of the inositol residue are acylated (mainly palmitoylate) at the 2-position as shown by ** in Fig. 11.6. The low susceptibility of mrt-CD52 to phospholipase C may be due to this acyl group anchoring into the cell membrane because this anchoring is known to be refractory to phospholipase C. Another structure difference from lymphocyte CD52 is that the glycerolipid portion of mrt-CD52 is a sn-1-alkyl-lyso-glycerol type (single-footed) in which only one fatty acid chain at the 1-position is linked as shown by * in Fig. 11.6. 1-Alkyl structure is reported to

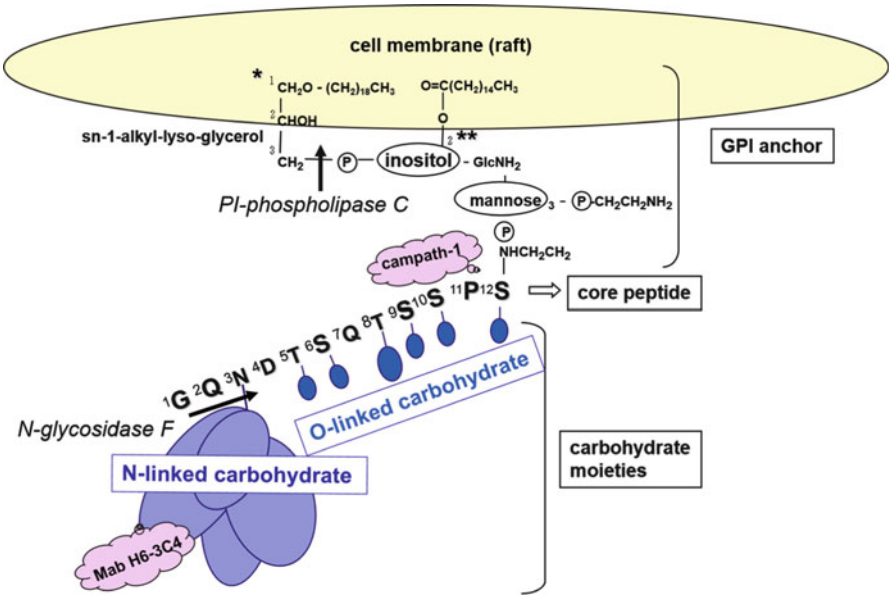

Fig. 11.6 Hypothetical structure of male reproductive tract CD52. The male reproductive tract CD52 molecule is composed of 12 amino acid residues, N-linked and O-linked carbohydrates, and a GPI anchor portion inserted in the plasma membrane. The amino acid sequence of core peptide is shown in capitals. Carbohydrate chains are shown by ovals

be synthesized by sperm as a 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine but mono-alkyl structure has not been documented in mammalian species. Phospholipase A_2, which is detected abundantly in seminal plasma, removes the acylation at the 2-position, although inhibitory factors are also detected in the seminal plasma. The lyso- (single-footed) glycerolipid anchor may play an important role in transportation of this molecule to sperm from the epithelium in the cauda epididymis or from seminal plasma.

mrt-CD52 has been reported easily to be transferred from epithelial cells to mature sperm in the epididymis [25]. Epididymosome, exosome from epithelial cells, may be a possible transporter of mrt-CD52 as reported by Sullivan et al. [26]. More recently, ACE (Angioteisin-converting enzyme) found in the epididymis and testis has been shown to exhibit GPI anchor protein-releasing activity (GPIase) [27]. Considering the crucial role of this enzyme for fertilization, GPI anchoring proteins may play important roles at different stages of reproduction.

11.5 Biological Function of CD52

CD52 is a GPI anchor glycoprotein present in lymphocytes and male reproductive tissues including mature sperm and seminal plasma [28, 29]. It has been reported that CD52 on the lymphocyte surface induces regulatory T cells with

immunosuppressive activities [28], while soluble mrt-CD52 from epididymis induces clot formation and liquefaction of human semen [30]. However, the biological significance of mrt-CD52 anchoring to the sperm membrane is not well understood.

Not only the N-linked carbohydrate but also the O-linked carbohydrate possibly contributes to the heterogeneous negative charge of mrt-CD52. These molecules may prevent sperm from auto-agglutination and non-specific tissue adherence [31].

Considering that the monoclonal antibodies targeted to mrt-CD52, Mab H6-3C4, campath-1 and 1G12, exhibit strong complement-dependent sperm-immobilizing activities, it is speculated that CD52 possesses a function to suppress complement activity. Campath-1 has also been shown to induce strong complement-dependent cytolysis of lymphocytes [32]. The female genital tracts are subject to frequent infection with various pathogens including sexually transmitted bacteria and viruses. However, its antibody-producing ability is not so strong compared to other mucosal tissues [33]. Innate immunological systems including complement activation are thought to play a major role in the host defense. Functionally active complement has been shown to be present in the female genital tract [34] and follicular fluid [35].

Complement-regulatory proteins such as C1-INH, CD55, CD46 and CD59 are present in spermatozoa and seminal plasma [36–38], and CD55 and CD59 have been shown to serve as GPI anchor proteins like CD52. CD55 and CD59 protect the cells expressing these molecules from complement-dependent cytotoxicity. CD55 is known to be a decay-accelerating factor that inhibits C3/C5 convertase formation and CD59 inhibits MAC (membrane attack complex) formation in the final stage of the complement pathway. These molecules are suggested to protect sperm from complement-dependent cytotoxicity.

Recently, Kinoshita et al. have reported an interesting clinical observation that a clonally acquired disorder in PNH (paroxysmal nocturnal hemoglobinuria) leads to intravascular hemolysis due to defect in the synthesis of GPI anchors such as CD55 and CD59 [39]. Campath-1H, humanized anti-CD52 antibody cross-reactive to CD55 and CD59 GPI anchors, has extensively been used clinically for the elimination of T-cells from bone marrow to prevent graft-versus-host disease. It has also been reported that patients treated with campath-1H develop PNH-like symptoms with hemolysis and thrombosis [40]. These patients show increased numbers of cells deficient in CD55 and CD59. The reactivity of campath-1H with GPI anchor induces a lack of CD55 and CD59 and causes PNH-like disease. These results suggest that CD52, like CD55 and CD59, has a role in complement regulation. In hemolytic assay of sensitized erythrocytes, the addition of purified mrt-CD52 significantly reduces hemolytic activity (CH50) by complement and this inhibitory effect of mrt-CD52 is neutralized by the addition of anti-CD52 antibody to the reaction mixture. These results indicate mrt-CD52 regulates the complement system. In complement pathway analysis [41], purified mrt-CD52 interfered with the classical pathway but not lectin-binding and alternative pathways [42].

Previously, we extracted CD52 from seminal plasma and sperm membrane [43]. Both materials formed immunoprecipitates with C1q and anti-CD52 antibodies

(Mab-H6-3C4 and Campath-1). This shows that naturally occurring mrt-CD52 inhibits the classical pathway interfering with C1q function in female reproductive tracts. Furthermore, detailed analysis showed the carbohydrate moiety of mrt-CD52 binds to C1q, which initiates the complement classical pathway. As the carbohydrate moiety is specific in mrt-CD52, it is possible to protect sperm during their transportation.

CD52, particularly the carbohydrate portion, has a special function different from other ordinary antigens, because it is involved in complement-regulating functions causing sperm immobilization. CD52 inhibits sperm damage by inhibiting the classical pathway if ordinary antigen-antibody complexes form on sperm membrane surface. This is the reason that many kinds of antisperm antibodies just bind to the sperm membrane and do not show sperm toxicity. ASA does not always evoke infertility.

However, once an antibody is raised to mrt-CD52, it suppresses the complement inhibitory effect of mrt-CD52 and hence even the other immune complexes induce tremendous sperm damage by classical complement pathway activation.

Conclusion

In this chapter, we have focused on SI-Abs among many kinds of ASAs. CD52 was identified as a target antigen that an infertile patient's antibodies recognized. The molecular mechanism causing sperm impairment has also been clarified in part in terms of complement suppression. However, CD52 is not the only antigen recognized by SI-Abs. A number of antigens including structures formed by complicated molecular interactions and foreign antigens may plausibly produce SI-Abs.

The reproductive system is quite different among mammalian animal species. In mice, for example, CD52 is present in male reproductive tracts but its significance is not so high compared to humans [44]. CD52 knockout mice were found to be fertile, both males and females [45]. We realized that the only way to understand the human reproductive system was to examine human clinical phenomena. Ongoing data collection and analysis are important for clarification of the physiology and pathology of human reproduction.

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Part III

The Clinical Impact of Sperm Antibodies

Male Autoimmune Infertility

12

Felice Francavilla and Arcangelo Barbonetti

Abstract

Naturally occurring antisperm-antibodies (ASA) exert an impairment to fertility, which is related to the extent of sperm autoimmunization. It determinates the degree of the interfering effect on sperm penetration through the cervical mucus independently from the antigenic specificity of ASA. Therefore, sperm-autoimmunization relevant to infertility can be diagnosed in the presence of a high proportion of ASA-covered spermatozoa, associated with a poor result of a carefully performed postcoital test. Whether or to what extent an ASA-interfering effect occurs, in each individual patient, downstream from the impairment of cervical mucus penetration, is still hard to establish. The main reason is the inability of current diagnostic tests to determine the antigenic specificity of ASA and to quantify the antibody density on the sperm surface, which are main determinants of ASA-impairment at the level of sperm/oocyte interaction. In any case, from a clinical point of view, to establish whether, or to what extent, this ASA-interfering effect occurs, in each individual patient, is not needed to diagnose ASA-related subfertility, because such impairment cannot occur in the absence of the interference at the level of mucus penetration. But, it would be relevant in choosing the more appropriate assisted reproductive treatment option.

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12.1 Introduction: Does Antisperm-Antibodies-Related Infertility Really Exist?

An etiological link between naturally occurring antisperm-antibodies (ASA) and male infertility has been claimed since Rumke [45] and Wilson [53] reported the presence of serum sperm-agglutinating activity in some infertile men in 1954. However, although the clinical significance of ASA has been extensively investigated, it is still a debated matter. On one hand, the previous assertions that any link between sperm antibody presence and impaired conception has to be considered hypothetical [49] and the routine use of current ASA testing is not justified as an essential procedure in the fertility work-up [28] were more recently reasserted in a cohort study where no independent association was observed between the occurrence of ASA and reduced pregnancy rates in subfertile couples [32]. On the other hand, intracytoplasmatic sperm injection (ICSI) has been claimed as the primary choice of treatment in the presence of sperm autoimmunization [33], and a screening test for ASA has been reconfirmed as integral part of semen analysis in the last edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen [56].

Different approaches used for the recognition of the ASA-related infertility, paucity of prospective studies on the occurrence of spontaneous pregnancies, and lack of well-designed and controlled studies on treatments effectiveness have strongly contributed to generate confusion on the clinical significance of ASA.

This chapter critically reviews current understanding of the clinical relevance of naturally occurring ASA in men.

12.2 Prevalence of Antisperm Antibodies

A variable prevalence of ASA has been reported depending on the specificity and sensitivity of the test used for their detection and on the screened population. The first assays to be utilized were indirect tests detecting biological activities of ASA in serum and seminal plasma, i.e., sperm agglutination techniques and complement-dependent sperm immobilization/cytotoxicity techniques. Subsequently, widespread acceptance has been gained for antiglobulins-based tests used to detect antibodies coated to the surface of ejaculated spermatozoa, including the mixed antiglobulin reaction (MAR) test and the immunobead binding test (IBT). They reveal the percentage of antibody-coated spermatozoa, the Ig-isotype, and grossly the regional specificity of ASA. Old multicentric comparative studies [7, 54] showed that all these tests determine in large measure the same antibody specificities for surface antigens, but with different sensitivity, which is lower for complement-dependent sperm-immobilization/cytotoxicity techniques.

In epidemiologic studies, serum sperm-agglutinating activity ranged from 8.1 to 30.3 % in unselected men with infertile marriages [13, 21, 26, 40], but at low titers it was also reported up to 10 % of control sera [26]. When stricter criteria were used (i.e., the occurrence of sperm-immobilizing activity in addition to high titers of

sperm-agglutinating activity in the serum and/or sperm-agglutinating activity in seminal plasma, indicating an excess of free antibodies in the semen), the prevalence of ASA in men with infertile marriages ranged from 4.6 to 5.7 % [21, 38]. Direct tests (MAR or IBT) gave positive results (>10 or >20 %) in 7.6–12.9 % of unselected infertile patients [11, 21, 38, 47], but highly positive results (≥50 %) were restricted to 5–6 % of patients [11, 21, 47]. In the most recent and largest multicentric survey, including 1794 consecutive subfertile couples, a positive IgG-MAR ≥10 % was detected in 7 % of men and a positive IgG-MAR ≥50 % was detected in 3 % of men [32].

Although a higher prevalence of ASA has been reported in some clinical conditions, including genital tract obstructions and infections, especially by *Chlamydia trachomatis*, testicular trauma, torsion, and surgery (see Chap. 8), only acquired genital tract obstructions represent a well-established risk factor: in vasectomized men the prevalence of ASA ranges from 33 to 74 % [5, 19, 29, 37], with their persistence in 38–60 % following successful vasovasostomy [5, 37].

12.3 Prognostic Studies

A causal link between ASA and fertility impairment, although suggested by epidemiologic studies, can only be proven by the association of the occurrence and the degree of sperm autoimmunization with a reduced pregnancy rate, independently from semen parameters and other clinical characteristics.

In retrospective studies, when the degree of sperm-autoimmunization was taken into account, it exhibited a significant inverse correlation with the incidence of spontaneous pregnancies. In an old report by Rumke et al. [46], during a 10-year follow-up of 254 infertile men with serum sperm-agglutinating activity (SAA), the titer of SAA was inversely correlated with the occurrence of spontaneous pregnancies. Notably, restricting the analysis to normozoospermic men, no pregnancy was observed with very high titer of serum SAA (≥1:1024), a low (15.8 %) pregnancy rate (PR) with titers ranging from 1:32 to 1:512, and a high PR (48.4 %) with titers <1:32. Ayvaliotis et al. [4] reported that in 108 infertile couples where the male exhibited a direct IBT >10 %, and the female was treated for other factors leading to impaired reproduction, PR was significantly higher when IBT was <50 % than when it was >50 % (43.4 % vs. 21.8 %) during a follow-up of at least 18 months. The difference in PR was ever of greater significance in a subgroup of 35 couples, where no other cause of infertility was found (15.3 % vs. 66.7 %). Abshagen et al. [1] reported that in 157 infertile couples with a direct IBT >10 %, cumulative spontaneous PR over 6 years was high (~50 %) when IBT was <50 %, lower (~30 %) when IBT was 50–90 %, and very low (~15 %) when IBT was >90 %, independently from the IgG-class (IgG and/or IgA). A significant inverse correlation between the degree of sperm autoimmunization and PR was also found in a follow-up study of 216 men after vasovasostomy by Meinertz et al. [39]. While no pregnancy was observed in a median period of ~4 years in men where all spermatozoa were antibody-coated at MAR test, in association with a high titer of serum SAA, pregnancy occurred in

64.3 % of couples with a less degree of sperm-autoimmunization. While in this study only a prevalent IgA autoimmunization was associated to a reduced fertility, a major role of IgA was not found in another study on vasovasostomized men by Matson et al. [37].

Altogether, these observations suggest that ASA represent a relative, rather than absolute, cause of infertility and the degree of fertility impairment appears to be related to the extent of sperm-autoimmunization. Accordingly, a threshold of 50 % positivity at MAR or IBT test has been established by WHO [56] for a degree of sperm-autoimmunization which might be clinically relevant.

However, the best evidence for a causal link between ASA and fertility impairment would be provided by prospective studies comparing the occurrence of natural pregnancies in men with and without ASA. Unfortunately this evidence is difficult to obtain: (1) the low incidence of sperm autoimmunization in unselected infertile couples requires multicentric studies including a large number of infertile couples (or, for the best evidence, couples without history of infertility) and a large number of observed cycles; (2) the inter-individual variability of semen parameters, not related to the presence of ASA, makes it very difficult to obtain a study- and a control-population, homogeneous for semen quality; and (3) the inter-couples variability in other clinical characteristics. Owing to these limitations, little information along with conflicting results has been produced by scanty prospective studies so far reported [13, 18, 55]. In the most recent and largest study [32], a positive IgG-MAR test ≥50 %, detected in 3 % of 1794 patients with infertile marriages, reduced, albeit not significantly, the probability of pregnancies during a 1-year follow-up. At the multivariate analysis, including semen parameters and other clinical characteristics, a positive MAR test ≥50 % did not contribute to the prediction of spontaneous pregnancy.

Therefore, the proof of a causal link between ASA and infertility has not yet been produced by the evidence-based medicine.

12.4 Mechanisms of Fertility Impairment by Antisperm-Antibodies: Clinical Relevance

Only ASA directed towards surface antigens have a physiopathological and clinical significance in the male immunological infertility, because subsuperficial antigens cannot be exposed to antibodies by living cells along the male genital tract.

12.4.1 Effect on Semen Quality

Sperm agglutination is the only well established semen alteration related to the presence of ASA [24]. However, sperm agglutination, which is a time-dependent phenomenon, only rarely involves a large proportion of motile spermatozoa soon after liquefaction, even when all ejaculated spermatozoa are antibody-coated. Therefore, sperm agglutination, although extremely suggestive of sperm-autoimmunization,

does not represent either a sensitive marker of autoimmunization or an important mechanism of the antibody-interference with fertility in most cases. Apart from sperm-agglutination, there is little evidence that suggests a cause/effect relationship between ASA and abnormality of semen parameters [24]. Actually, an effect on sperm motility/vitality should involve a complement (C)-mediated sperm injury, but it is prevented by anticomplementary activity in human seminal plasma [15, 42].

12.4.2 Interference with Cervical Mucus Penetration

The impairment of sperm penetration through the cervical mucus represents the primary, well-documented mechanism of the ASA interference with fertility. Several studies have shown a significant association between a poor postcoital test (PCT) outcome and sperm autoimmunization [6, 25, 35]. Interestingly, in the above mentioned study by Leushuis et al. [32], although the evidence for an independent association between sperm-autoimmunization and reduced pregnancy rate was not provided, a negative PCT result was significantly associated with a positive MAR test result (relative risk 2.5, 95 % CI 1.4–4.3). The degree of the impairment of sperm penetration "in vivo" through the cervical mucus was found to correlate with the proportion of antibody-covered spermatozoa [6], as well as with the titer of circulating ASA [35]. The demonstration of the actual responsibility of ASA in impairing cervical mucus penetration was provided by matching donor sperm suspensions exposed to sera containing ASA against the same sperm suspensions exposed to control sera without ASA, using the in vitro cervical mucus penetration test [3]. Although a prominent role for IgA-ASA in impairing sperm penetration of cervical mucus was reported [10, 30, 52], other findings indicate that an abnormal interaction between the Fc portion of both IgA and IgG bound to the sperm surface and constituents of the cervical mucus is responsible for the impairment of mucus penetration and the shaking pattern of sperm motility observed in "in vitro" sperm-cervical mucus contact test (SCMC) [8]. Antibodies directed against the tail-tip do not impair sperm/cervical mucus interaction [52], and therefore have no role in infertility.

12.4.3 Complement-Mediated Cytotoxicity Through the Female Genital Tract

When spermatozoa coated with complement-fixing antibodies enter the female reproductive tract, they could undergo deleterious effects of complement activation, supposing that complement components are present in a sufficient amount through the female genital tract. In an old study by Price and Boettcher [43], although the level of complement activity in cervical mucus was only 11.5 % of the serum activity, this amount of complement was enough to cause complement-dependent immobilization of 50 % of ASA-coated spermatozoa in 1 h. Higher levels of complement activity were detected in human follicular fluid (one half of that in serum), and

IgG-ASA were able to activate follicular fluid complement on human spermatozoa [16]. Due to the dilution of follicular fluid after ovulation, any sperm damage or dysfunction related "in vivo" to its complement activity is difficult to ascertain. Therefore, its clinical relevance is not proven.

12.4.4 Interference with Sperm/Egg Interaction

Although experimental "in vitro" studies have largely demonstrated that ASA can affect sperm functions involved in the sperm/egg interaction (see Chap. 3), the clinical relevance of these effects might be proven above all by the results of in vitro fertilization (IVF) as a model of study. In most reports, the overall fertilization rate was significantly lower in the presence of sperm-bound antibodies than in the case of other indications for IVF [2, 9, 12, 20, 36, 44, 50]. But, in some other reports no significant difference was found [14, 34, 48, 51]. In a meta-analysis by Zini et al. [57], including 10 studies (8 prospective and 2 retrospective), the presence of sperm-bound antibodies (with ASA cut-off value at direct tests ranging from 10 to 80 % as inclusion criterion) was not related to pregnancy rates after IVF: the combined OR for failure to achieve a pregnancy using IVF in the presence of ASA was 1.22 (95 % CI: 0.84, 1.77). However, the assessment of the actual interference of ASA on sperm fertilizing ability from the analysis of IFV results is hindered by the effect of concomitant nonimmunological sperm abnormalities and by the different degrees of sperm autoimmunization. Nevertheless, when the extent of sperm autoimmunization was taken into account, it was inversely correlated with the overall fertilization rate [17, 31, 41]. But, notably: (1) even when the percentage of fertilized oocytes was reduced in the presence of ASA, some oocytes were fertilized; (2) in some individual patients, a high fertilization rate was achieved even in the presence of a high extent of sperm autoimmunization.

This variable interfering effect emerging from the analysis of IVF results is also supported by experimental laboratory-based studies aimed to determine the level of the interference of ASA on sperm functions involved in gametes interaction [24]; see also Chap. 3]. Particularly illustrative is a study from our group where the occurrence of the ASA-interference with zona pellucida (ZP)-binding was tested in 22 patients exhibiting all ejaculated spermatozoa coated "in vivo" with antibodies against the sperm head [23]. Excluding patients with abnormal semen from the analysis, an impairment of the ZP-binding was observed in 50 % of cases, by matching patients and donor spermatozoa, labeled with different fluorochromes, for their binding ability to the same ZPs. It is worth noting that: (1) in no case the inhibition of ZP-binding was complete; and (2) a normal ZP-binding was observed even when all ejaculated spermatozoa were coated with both IgG- and IgA-ASA.

On the whole, human IVF results and experimental laboratory-based studies suggest that, at the level of the sperm/egg interaction, ASA exert a relative impairment, which, to some extent, is related to the degree of sperm autoimmunization.

However, the degree of autoimmunization does not completely explain the variability of the antibody impairment. Apparently, at the level of gamete interaction, more than at other levels (i.e., cervical mucus penetration), the interference of ASA exhibits qualitative, apart from quantitative, differences among patients. Most likely, this interference also depends on the relevance of the specific antigens, targeted by natural ASA, to the fertilization process.

12.5 Clinical Implications

Given that the only ASA-related semen alteration is sperm-agglutination, which, however, is not a sensitive indicator, a direct screening test (MAR or IBT) should be performed on all semen samples examined in the couple-infertility work-up. As IgA-antibodies, whenever they occur, are always found in association with IgG [22, 27, 38], only IgG-ASA have to be screened. In all positive samples for IgG, even at a low degree, IgA-ASA should be screened, to determine whether and at what extent they are also bound to the sperm surface.

If ASA-direct tests are negative or with a low positive rating (<50 %), an ASA-related subfertility may be excluded. On the other hand, when ≥50 % of motile spermatozoa are coated by ASA, an immunological male subfertility can be diagnosed in the case of a poor PCT outcome, especially when a shaking pattern of sperm motility is observed in an in vitro sperm-cervical mucus contact test (SCMC).

Whether, or to what extent, an ASA-interfering effect occurs, in each individual patient, downstream from the impairment of cervical mucus penetration, when all or nearly all spermatozoa are antibody-coated, is still hard to establish. The main reason is the inability of current diagnostic tests in quantifying the antibody density on the sperm surface and in defining the antigenic specificities of ASA, main determinants of the ASA-impairment at level of sperm/oocyte interaction, which, however, seems to be less effective and certain than that at level of cervical mucus penetration.

In any case, from a clinical point of view, to establish whether, or to what extent, an ASA-interfering effect occurs, in each individual patient, downstream from the impairment of cervical mucus penetration is not needed to diagnose ASA-related subfertility, because such an impairment cannot occur in the absence of the more effective interference on mucus penetration. But, it would be relevant in choosing the more appropriate assisted reproductive treatment option. Although ICSI has been claimed as the primary choice of treatment for ASA-related subfertility, because it overcomes any potential interference of ASA with sperm fertilizing ability [33], it would be better to reserve it for patients for whom achieving a pregnancy with less invasive techniques would be most unlikely. In the light of preventing inappropriate aggressive intervention, the main question is whether/when intrauterine insemination (IUI) could represent an effective first-line ART treatment, as discussed elsewhere in the book.

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Tests for Sperm Antibodies

13

Tamer M. Said and Ashok Agarwal

Abstract

Several methods have been used to identify the presence of antisperm antibodies (ASA). The clinical significance of the results derived from ASA testing methods is widely debated. The conflicting data reported by various investigators may be due to confounding factors that include the use of different ASA testing modalities, specimen preparation, and test interpretation standards. Furthermore, sperm specimens are dynamic, undergoing maturational changes including capacitation and the acrosome reaction, which results in changing ASA epitopes as the outer acrosomal membrane and its associated proteins are lost and the antigens present on the inner acrosomal membrane become exposed. Currently, there is a consensus that results of testing for ASA in seminal fluid using the mixed antiglobulin reaction and immunobead tests can be significantly associated with unexplained infertility. On the other hand, testing of seminal plasma or serum was found to be less relevant for fertility assessments.

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13.1 Introduction

The sperm antigenicity was first identified in the nineteenth century following sperm injection into a foreign species. Thereafter, it was reported that sperm can be also antigenic when injected into the same species [28]. The coating of spermatozoa with antisperm antibodies (ASA) can significantly interfere with fertility. Possible mechanisms include immobilization of the spermatozoa, impaired cervical mucus penetration, inhibition of capacitation, and disturbance in sperm-ovum interaction. Furthermore, ASA have been associated with the secretion of cytokines which impair sperm function and can eventually lead to cell death [32]. Although ASA can be detected in serum, they were not reported to impair fertility unless present within the reproductive tract or are detectable on living spermatozoa [11].

Several methods have been described for the detection of ASA. They include the tube slide agglutination test (TSAT), gelatin agglutination test (GAT), sperm immobilization test (SIT), immunobead test (IBT), and mixed antiglobulin reaction (MAR) test using sensitized erythrocytes. Despite the variety of available testing methods, the World Health Organization (WHO) Special Program of Research Development and Training in Human Reproduction has recommends only the MAR test or the IBT for the assessment of human semen [37].

There is considerable disagreement between the different testing methodologies and their clinical significance in relation to infertility. Existing data do not support the widespread use of immune testing in clinical practice. Additionally, ASA treatment options do not have confirmed benefits and may even prove to have negative effects [23]. It was previously reported that the routine use of ASA testing can have some cost saving advantage relative to the expenses encountered during in vitro fertilization (IVF) cycles [5]. Detection of ASA was considered an indication for intracytoplasmic sperm injection (ICSI) versus routine IVF since it has been shown to yield higher fertilization rates [8]. Nevertheless, it was not accepted both routine IVF and ICSI are of equal benefit in cases diagnosed with ASA.

13.2 Sites of Different Antisperm Antibody Classes

Antisperm antibodies IgM have been detected in the circulation of men; however, no traces of the IgM molecules were detected in the male genital tract. Therefore, testing for the IgM class does not appear to be of value in the context of male fertility evaluation [35]. On the other hand, 1 % of the serum IgG has been documented in the male genital tract. The presence of seminal IgG could be the result of transudation from circulation or it could be due to local antibody production [17]. As regards IgA class in human semen, it appears to be the result of local production since seminal plasma IgA is of the secretory IgA type [38].

13.3 Testing Methods

Several tests have been developed to detect and quantitate ASA, which may be categorized into groups based on the antigen source: (a) live sperm assays such as macroagglutination, microagglutination, cytotoxicity, or sperm/cervical mucus interaction tests; and (b) fixed sperm assays such as immunofluorescence, enzyme-linked immunoassays and radioimmunoassay, and mixed antiglobulin tests [4]. Currently, the mixed antiglobulin reaction test and the immunobead test are the only tests which are routinely performed by diagnostic laboratories [37].

13.3.1 Macro/Microagglutination and Immobilization

A macroscopic approach has been described to identify the presence of ASA in serum. The gelatin agglutination test (GAT) is conducted by suspending the semen from a donor known not to have ASA with the complement-inactivated serum of the suspected subfertile patient in a gelatin mix. Sperm agglutinates at the bottom of the gelatin mix can be interpreted as positive [26]. The GAT test is known to reveal false-positive results due to the presence of debris in seminal plasma. Therefore, it no longer plays a role in the diagnosis of immunological infertility especially that the clinical relevance of ASA in serum is now hugely debated. A similar test, the tube slide agglutination test (TSAT), is performed by mixing donor semen with complement-inactivated patient serum followed by detection of the sperm agglutination using a microscopic drop [16]. The TSAT is also no longer recommended as a testing modality.

The sperm immobilization test (SIT) procedure resembles the TSAT, but smaller volumes of rabbit or guinea pig serum are added as a source of complement. During microscopic assessment, the number of motile sperm is determined and the test is considered positive if more than half of counted sperm are found to be nonmotile [21]. In addition to the disadvantages noted above for the GAT and the TSAT, SIT lacks the ability to detect IgA since fixation of complement and initiation of the cascade sequence is only possible for antibodies of the IgG and IgM classes [6].

13.3.2 Tests for Cervical Mucus

The presence of ASA in cervical mucus can be assessed by using in vivo or in vitro sperm-mucus interaction tests. The in vivo postcoital test (PCT) is conducted by sampling the cervical mucus several hours after intercourse and examining it for the presence of spermatozoa. The presence of less than ten sperm/HPF could be interpreted as a poor result. Most importantly, the presence of a distinctive “shaking” pattern of sperm motility is suggestive of the presence of ASA [31]. Both IgG and IgA can be found in the cervical mucus. Although PCT shows poor results in the presence of ASA, technical problems may also be responsible; thus, caution is urged when attributing poor PCT to immunologically hostile mucus.

The in vitro sperm-cervical mucus contact (SCMC) test could be also used to evaluate the presence of ASA in cervical mucus. During the SCMC, aliquots of cervical mucus and liquefied semen are mixed and examined for the characteristic "shaking" pattern of sperm motility. The test is considered positive if more than 25 % of spermatozoa display the motility shaking pattern. In a study on 17 couples who repeatedly demonstrated unexpected poor postcoital tests, 15 of them revealed a positive SCMC test [15]. Therefore, the SCMC test can be considered as a reliable screening test for the detection of ASA among infertile couples.

13.3.3 Immunofluorescence and Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) technique has been adapted to quantitatively assess the presence of ASA. ELISA combines the specificity of the antigen-antibody reaction with the continuous degradation of chromogenic substrate by an enzyme to amplify the sensitivity of the reaction. Numerous materials and methods have been used as variations for the ELISA procedure: solid-phase materials (silicon rubber, glass), carriers (tubes, beads, disks), enzymes (alkaline phosphatase, horseradish peroxidase), substrates (p-nitrophenyl phosphate), and different wash solutions [3]. Other variables include sperm concentration, type of sperm fixation, blocking agents, serum, and seminal plasma dilutions. The complexity, instrumentation, and expense of the ELISA have prevented its widespread use in the workup of male immunological infertility.

The use of flow cytometry has been reported to detect sperm-bound antibodies and to quantitate the sperm antibody load (antibody molecules/spermatozoa). Following staining of the washed sperm samples, dead sperm are excluded with fluorescein-isothiocyanate-conjugated F(ab')2 fragments of anti-IgG and IgA antibodies by the use of calibration standards. Flow cytometry has the potential reliability and objectivity to quantitate sperm antibodies; therefore, the sperm antibody load can be used to compare different patients or to follow up the progression of the same patient [25]. Similar to ELISA, flow cytometry is not currently widely used for the detection of ASA due to its complexity, expense, and instrumentation requirement. In the same context, the agglutinin radiolabeled antibodies assay for the detection and quantitation of ASA is of limited use. This method is also limited by an inability to determine specific ASA location, expense, and reliance on highly skilled labor [18].

13.3.4 Mixed Antiglobulin Reaction (MAR) Test

The MAR test has been developed to detect surface ASA [22]. It is based on a modification of the famed Coombs test which was described in1956 by Coombs et al. [8]. The simple initial version of the assay entailed mixing of three ingredients as single drop and covering them with a cover slip. The semen sample is mixed with a suspension of group O, Rh-positive, human red cells of R_1 R_2 type, sensitized with

human IgG in addition to rabbit or goat, undiluted, monospecific anti-IgG antiserum. The reaction is then observed after 10 min of incubation. Since the red cells are coated with IgG as well as the sperm cells if they have antibodies on them, the added anti-IgG antiserum will then link together the two kinds of cells. Agglutination can be seen under a light microscope as mixed clumps of spermatozoa and red blood cells with a slow "shaky" movement.

Results of the MAR test are indicated as percentages of motile spermatozoa incorporated into the mixed agglutinates. The site of attachment could be also noted. No interpretation of the test was given unless agglutination of red blood cells and the presence of sufficient motile spermatozoa are observed. A MAR test was considered positive and of clinical significance when >50 % agglutination is seen [1]. The advantages of the MAR test are that it can be applied directly to untreated semen samples and the results can be obtained within few minutes, which renders the assay quick, simple, and repeatable. The MAR test correlates with most other sperm antibody tests, e.g., SIT and IBT [22]. Although MAR test is considered an ideal method for screening of ASA, it is not without limitations [30]. The assay cannot be used in patients with oligozoospermia, asthenozoospermia, and azoospermia. Also it must be performed on a fresh sample and can be difficult to quantitate due to the presence of debris, semen viscosity, mucus, and microbial factors.

Commercially available sperm MAR kit use an antiserum against human IgG to induce mixed agglutination between antibody-coated latex beads conjugated with human IgG [24]. The sperm MAR kit can be considered a superior alternative to erythrocyte MAR since it is time and cost effective (Fig. 13.1). One formulation of the kit contemplates the assessment of IgA as well as IgG classes. The assay can be

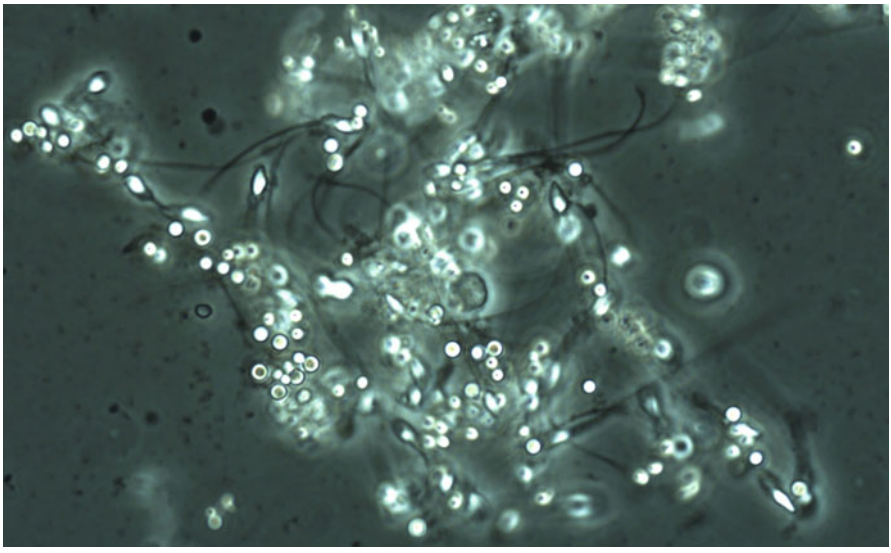

Fig. 13.1 Positive mixed antiglobulin reaction (*MAR*) test. Raw semen sample with latex beads coated with IgG seen bound to sperm surfaces mainly tails (phase contrast, 40×)

successfully used for the evaluation of male partners of infertile couples if included routinely in semen analysis [36]. An indirect MAR, i.e., using serum/seminal plasma samples and donor spermatozoa, can be considered in cases with azoospermia. However, it has been reported as difficult to interpret. Therefore, ASA should be rather detected using another approach in these cases.

13.3.5 Immunobead Test (IBT)

The IBT has been described as a relatively simple, inexpensive procedure, which takes less than 30 min to perform. Similar to the MAR test, it is very convenient, utilizing only a bench centrifuge, light microscope, and latex beads coated with anti-human IgG, IgA, and IgM [14]. IBT allows the determination of the antibody class attached to spermatozoa, the localization on the spermatozoa, and the proportions of spermatozoa coated with antibody [14]. The immunoglobulin class detected can be of clinical importance (Fig. 13.2).

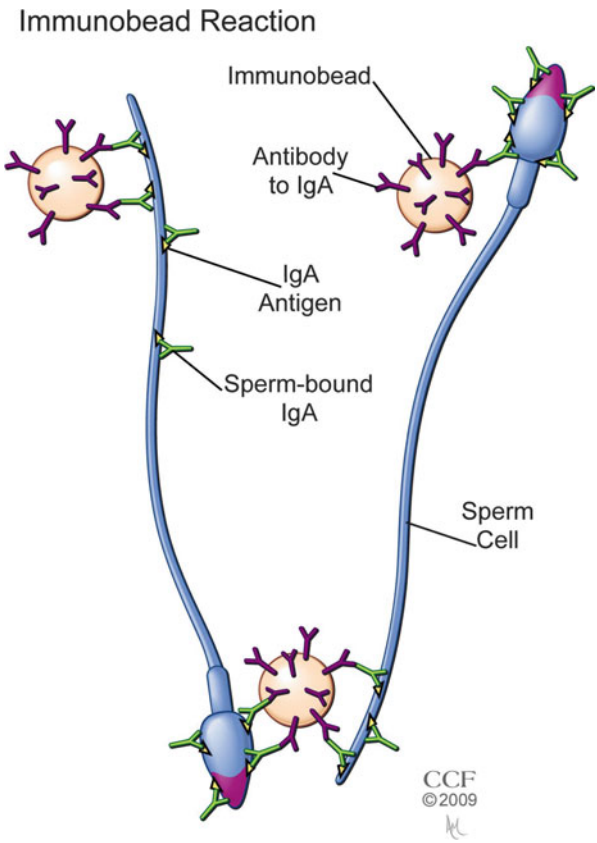

Fig. 13.2 Immunobeads are polyacrylamide spheres with covalently bound rabbit anti-human immunoglobulins. The test is considered positive if ≥20 % of motile spermatozoa have immunobead binding and is considered clinically significant when at least 50 % of the motile spermatozoa are coated with immunobeads

Prior to conducting the assay, spermatozoa must be washed to discard any free immunoglobulins, which may be in the seminal plasma and which, if present, would alter the assay results. Thereafter, sperm concentration is adjusted to 10–25 × 10^6 motile sperm/mL to optimize the microscopic assessment of sperm. These adjustments are occasionally necessary if the sample is oligozoospermic or asthenozoospermic. The IBT can also be conducted indirectly on reproductive fluids such as seminal plasma, follicular fluid, cervical mucus, as well as serum.

The intra-assay reproducibility of the indirect IBT was evaluated by testing aliquots of ASA-positive sera from two patients against the same donor sperm sample. The interassay reproducibility was also evaluated by testing a positive serum sample first with different sperm samples from the same donor and second with sperm samples from different donors. The results of those experiments showed that the indirect IBT has very low intra-assay variation and a high interassay variability [13]. Therefore, both the direct and indirect IBT can be considered as reliable, specific tests for the detection of sperm-bound antibodies and sperm antibodies in reproductive fluids and serum [24]. IBT was routinely applied in many andrology laboratories for the detection of ASA. However, a current lack in commercially available coated latex beads has led to its discontinuation.

13.4 Comparison of ASA Tests

The comparison between the MAR test and the IBT is of particular importance since both assays appear to be the most commonly used [37]. The two testing protocols are designed to detect immunoglobulins on the sperm surface; however, the MAR test is specific for the secretory IgA which is present in semen, while the IBT may cross-react with nonsecretory IgA present in serum [29]. To initiate a comparison, indirect tests for ASA using the commercially available MAR and IBT kits were applied to a panel of sera whose reactions in the TSAT, GAT, and SIT were well characterized. The results from assessments of 30 sera confirmed a significant correlation between the GAT, MAR, and IBT. When sera were titrated, the IBT proved to be slightly more sensitive than the GAT, while the MAR test was slightly more sensitive than the IBT [24].

A different comparative study between the IBT and the MAR test has also shown a high degree of agreement between both assays; however, the former was less accurate than the latter [29]. A similar study reported that the standard MAR protocol (direct test of unwashed semen) was found to be more sensitive than IBT on washed sperm. Other advantages for the MAR include not requiring washing the spermatozoa free of the seminal plasma, which makes it easier and faster than the IBT. It also uses less semen volume and could be applied to samples with a lower sperm concentration compared to the IBT [2, 34]. In contrast, when the MAR test was performed on washed sperm or with an indirect antibody transfer from serum or seminal plasma, the results gave mostly lower values for bead binding in comparison with the IBT. Therefore, the MAR test can be considered mostly

suitable in direct assays employing unwashed ejaculates making it easier to incorporate in routine semen analysis. However, it is prudent to confirm its positive results by IBT [20].

Cross-inhibition studies have revealed high specificity between positive IBT and the presence of membrane-bound immunoglobulin. Good correlations have been reported also between the IBT and other immunoassays such as PCT and sperm-cervical mucus compatibility assays [33]. Poor correlations have been reported between the IBT and sperm agglutination test, which implies that sperm agglutination may occur due to other nonimmunological factors [14]. Therefore, false-positive results may occur with agglutination tests in the absence of ASA. Finally, it is of importance to note that there are several pitfalls associated with comparisons between the different methods of ASA detection. Many methods rely on subjective determinations and variable specimen preparations. In addition, the sensitivity and specificity for each of the testing modalities vary widely [19].

13.5 Interpretation and Significance of ASA Tests

There is sufficient evidence supporting the hypothesis that ASA play a role in selected cases of infertility. The prevalence and magnitude of this role remains not well defined. In the clinical context of male infertility, the MAR test and the IBT are currently recommended for the detection of ASA. The current consensus indicates that a semen sample is to be considered immuno-compromised if more than 50 % of spermatozoa show binding in the MAR test or the IBT [1, 37].

The identification of ASA in a given sample does not necessarily indicate other inherent defects. An attempt to correlate results of MAR test with other defects in the seminal fluid revealed a significant correlation between a positive MAR test and spontaneous sperm auto-agglutination [7]. No correlations were observed, however, between test positivity and sperm concentration, motility, morphology, macroscopic features, or leukocytes concentration. Contradictory findings were identified in a different trial that correlated the results of the IgG MAR test with the semen analysis parameters of 1176 infertile males [27]. The test was only positive in 3.1 % of the cases. The positive IgG MAR test proved to correlate significantly both with the number and motility of spermatozoa. Whether the detection of ASA is associated with other deficiencies in the semen analysis or not should not infringe on the importance of the assay, which appear to be of significant value in identifying the etiology of infertility in some cases.

In a study that evaluated ASA levels in men presenting with a history of infertility, MAR test results were found to be positive in 10 % of 484 men with normal sperm counts, 23 % of 78 with low sperm motility, and 15 % of 128 with low counts. Therefore, the MAR test may be considered as a part of the routine semen analysis, since the presence of IgG antisperm antibodies can be established in about 10 % of men who might otherwise be passed as normal. In support, the evaluation of patients whose infertility remains unexplained (by routine physical and laboratory

investigations) exhibits significantly elevated ASA levels in 18 % of males compared to fertile individuals. Thus, the identification of autoimmune imbalance may help to resolve some cases of unexplained infertility [12].

Conclusions

The assessment of ASA in the context of infertility has not been devoid of controversies. It is duly noted that "neither a specific antigen (s) nor a superior antibody detection assay exists, although both are requisite to an understanding of the significance of ASA production and for the purpose of infertility reduction" [10]. The only agreed upon current indication for performing an ASA detection assay appears to be unexplained infertility. Published data support the use of either IBT or MAR as the only reliable tests capable of assessing the presence of ASA in a clinical setting. The MAR test is easier to perform and may be more sensitive but is not suitable for testing of serum or plasma using an indirect approach.

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Impact on Fertility Outcome

14

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Abstract

Autoimmune and/or isoimmune infertility can be caused also by sperm antibodies, which are able to stop in vitro and in vivo sperm progressive penetration needed for fertility. This chapter covers all aspects concerning male and female antibody and cellular immunological disorders to spermatozoa complicating human fertility, and possible ways of the treatment. Regarding the incidence of sperm antibodies in an infertile population, which is 9 % of the men and 15 % of the women, it is necessary to define significant sperm antigens for desensitization of immunological infertility and/or for development of contraceptive treatment.

14.1 Introduction

Many different factors may cause human infertility, which is a serious problem for many people (about 15–20 %) wishing to have children. Impairment of semen quality in men, anovulation, endometriosis, as well as adhesions inside and outside Fallopian tubes, sperm, zona pellucida antibodies, antiphospholipid antibodies, and other immunological factors in women are frequent causes of decreased fertility. The presence of antibodies to spermatozoa (ASA) in men as well as in women is

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also considered an immunological problem of conception. High titers of ASA in serum, seminal plasma, or in ovulatory cervical mucus are associated with fertilization failure, and markedly reduce the likelihood of natural conception.

Sperm have been known to be antigenic for more than a century. The presence of ASA has long been suggested since the antigenicity of spermatozoa was first demonstrated by Landsteiner and Metchnikoff in 1899 [1, 2]. Positive levels of human ASA were first reported in 1969 when Fjallbrant [3] demonstrated that antibodies obtained either from infertile patients or from immunized rabbits were able to produce agglutination and thereby to block in vitro penetration of human spermatozoa into female cervical mucus. The principle of sperm agglutination depends on specificity of sperm antibody to sperm antigen coating sperm head, or midpiece, tail, or tail tip.

The immune response against sperm cells (spermatozoa) is genetically determined [4]. ASA occur in both men and women, and also in homosexual men [5]. It is supposed that testicular trauma (e.g., biopsy, torsion, accident during sports activity), varicocele, testicular cancer, infection such as orchitis during mumps, and vasectomy are associated with autoimmunity to spermatozoa in up to 70 % of men [6]. The majority of cases of sperm autoimmunity is spontaneous and idiopathic. Rational access for the origin of autoimmune ASA in men is the disturbance of hematotesticular barrier. In infertile women, ascendent and frequent isoimmunization during long lasting, unprotected coital experience is suspected.

Seminal plasma, as a natural medium for spermatozoa has many immunological properties influencing the fertilization capacity of reproductive cells. In the past, clinical significance of ASA in human infertility was supported by many investigators and authors [7–16].

14.2 Influences of ASA on Infertility Prognosis

The most valuable sign for infertility prognosis appears to be the local ASA activity (in seminal plasma in men, in cervical ovulatory mucus, endometrial, peritoneal, and follicular fluids in women). Our daily experience [17] shows that infertile women are able to produce local immune response to spermatozoa more often and more earlier than in serum. Their systemic reaction as ASA activity is proven later and, from that point, ASA are detected in both ovulatory cervical mucus and serum.

Generally, high titers of ASA are able to block initial stages of the reproductive process. Infertile patients with immunological cause are less likely to conceive because in some the ASA not only interfere with sperm migration, but also inhibit the fertilization process at various stages, and exhibit negative effect on the early embryo development. ASA can affect the mechanisms of transport of spermatozoa within the female genital tract, may alter sperm capacitation or acrosome reaction, can interfere with egg fertilization, or have postfertilization effects on the zygote and preimplantation embryo [18–26].

The reason for the investigation of ASA is to determine clinically significant sperm antibody titers and to estimate their role in iso-/autoimmune reactions

leading to infertility. The levels of ASA also depend on character of spermiogrammes. We noticed the relationship between sperm antibodies in seminal plasma and with proteins of acute phase of inflammation [7]. Sperm-agglutinating antibodies are much more frequent in IgG and IgA class in seminal plasma and in IgG, as well as in IgM and IgE in sera. Interindividual findings of seminal levels of lactoferrin, albumin, C3, and C4 were reported [6, 7]. Secretory immunity plays important role in male infertility. Infertile men have significantly higher levels of lysosyme, C3, alpha-1-antitrypsin, alpha-2-macroglobulin, and beta-2-microglobulin in their seminal plasma. Immunological changes in seminal plasma caused by local inflammatory reactions and characterized especially by the presence of ASA or pathological levels of seminal proteins in acute phase of inflammation decrease sperm function. Contemporary trends in andrology also clarify pathology of acrosomal functions [14].

As reported earlier [15], ASA effectively block fertilization. Some mechanisms, such as the inhibition of sperm binding because of sperm agglutination, sperm immobilization or cytotoxic reaction, the inhibition of sperm penetration through the zona pellucida, and the fusion of the sperm plasma membrane with the vitelline membrane of the oocyte, have been described. ASA may block implantation or inhibit the development of early embryos [27–33].

14.3 Recent Research of the Cellular Reactivity of Peripheral Blood Mononuclear Cells (PBMCs) of Infertile Women, to Sperm Cells or Sperm Cell Lysate

We compared the cytokine response of peripheral blood mononuclear cells (PBMCs) from infertile female patients with or without ASA and healthy fertile women and teenage virgins (virgo intacta) [34]. We screened the supernatants for 40 cytokines by antibody array after cultivation of the PBMCs together with sperm antigens (whole cells or cell lysate). When stimulated with whole sperm cells, the PBMCs from patients with ASA produce less IL-3, IL-11, IL-13, ICAM-1, and GCSF and more IL-2, IL-4, and IL-12p70 as compared to healthy women. PBMCs from patients with ASA produce typically less IL-13, IL-7, IL-17, and MIG, and more MIP-1β and IL-8, compared to PBMCs from patients without ASA. In response to sperm cell lysate, PBMCs from infertile women without ASA respond initially by increase in production of growth factors (GCSF, GM-CSF, and PDGF-BB) followed by increase in chemokines (e.g., IL-8, MCP-1, and MIP-1β).

As compared to antigen-specific humoral immune response, the specific cellular immune responses to sperm cells or sperm antigens in humans is only rarely investigated [34, 35]. The interaction between T, B, and natural killer cells determine if the embryo is accepted or rejected [36]. The result of immune response to the sperm antigens is greatly influenced by cytokine environment at the time when immunocompetent cell encounters the antigen. Besides this crucial immunomodulatory function, cytokines are very important in implantation and embryo development. The disturbance in cytokine environment could thus play a significant role in

mechanisms of the immune-mediated infertility either by disturbing the balance of immune system regulation or by directly interfering with fertilization process and early embryo development. Understanding the cytokine function during reproduction is also complicated by the pleiotropy and redundancy of cytokine action and by the fact that overall cytokine environment mediates the effects of individual cytokines.

We found that sperm antigens influence the cytokine response in infertile women with ASA differently, as compared to fertile women, or to infertile women without ASA. These changes were in cytokines characteristic for distinct functional T cell subsets, including typical Th1-type (IL-2), Th2- or NK-T-type (IL-4 and IL-13), and Th17-type (IL-17) cytokines. Moreover, we found changes in pro-inflammatory cytokines and chemokines (e.g., eotaxins, MIPs, IL-8, IL-12p70, and TNF-α), and in growth factors (e.g., IL-7, GCSF, and GM-CSF). These pronounced changes suggest quite extensive dysregulation of immune response to sperm in infertile women involving several types of cells, and indicate the involvement of several pathogenic mechanisms including enhancement of the immune reactivity with slight shift towards Th2-type of response and lag of the embryo implantation and its growth.

The increase in IL-2 and IL-4 production by whole sperm cells-stimulated PBMCs of infertile patients with ASA, as compared to controls, could create the environment that leads to generation of Th2-type of response resulting in antibody production. This change could be the basis of the main pathogenic feature – ASA. PBMCs from infertile women with ASA produce less IL-11 early upon stimulation with sperm cells as compared to healthy women. IL-11 is a pleiotropic cytokine from the IL-6 family that stimulates hematopoiesis and has an important role in embryo implantation [36, 37].

Decreased production of ICAM-1 by PBMCs of infertile women with ASA as compared to healthy controls is of particular interest, suggesting more complex interplay of factors involved in diapedesis. ICAM-1 is a glycoprotein expressed on the surface of several cell types, including leukocytes and endothelial cells, that binds to the lymphocyte function-associated antigen-1 (LFA-1), expressed on leukocytes, thus mediating leukocyte extravasation and interaction. High levels of soluble (s) ICAM-1, a shedding form of ICAM-1, can disturb the adhesions that occur between immune cells and their targets and thereby prevent an immunological reaction. Therefore, its low production by PBMCs from patients with ASA could result in higher ability of reactive leukocyte to exit the blood stream and initiate the immune response in tissues, resulting in shift towards inflammation.

PBMCs from our patients with ASA produce less IL-13 and more IL-12 after their cultivation with sperm cells, as compared to healthy controls. As a key inducer of Th1-type inflammatory responses, IL-12 is involved in autoimmune tissue destruction. IL-13, on the other hand, is described as an anti-inflammatory cytokine, which inhibits the production of inflammatory cytokines by LPS-activated macrophages [38]. This change in patients with ASA could result in deregulated inflammation with subsequent infertility. Moreover, decrease in IL-13 could be also lead to impairment in angiogenesis.

There is a striking difference in cytokine response to sperm cell by PBMCs from infertile patients with ASA as compared to those from patients without ASA. When we compared cytokine spectra between both groups of infertile patients, we found that, e.g., IL-13, IL-7, IL-17, and MIG are higher in patients without ASA, and IL-8 and MIP-1β are higher in patients with ASA. This suggests that these forms of infertility differ in antigen-specific immune response to sperm cells not only on humoral but also on cellular level.

PBMCs from infertile patients without ASA produce significantly higher amounts of IL-7 after their cultivation with whole sperm cells-antigen, as compared to either unstimulated or sperm cell lysate-stimulated PBMCs. The role of IL-7 in the physiological reproduction is not well documented; it believes that due to its general growth promoting activities it stimulates folliculogenesis during in vitro fertilization [35–37]. Its high production by PBMCs from infertile patients without ASA could lead to more aggressive cellular response in these patients, which can compensate for the lack of above-mentioned pathogenetic role of ASA in these women. We found the response to whole sperm cells is generally less diverse than response to sperm cell lysate. These antigens induce high production of IL-8, MCP-1, TNF-α, and MIP-1β, but not IL-7, as compared to either nonstimulated or whole sperm-stimulated PBMCs, which corresponds to protective immune response. These differences also point out the differences between the response to extracellular and to intracellular antigens.

There are several mechanisms involved in immune response to sperm cells in infertile women with ASA, and the presence of ASA is only one of them. We described PBMCs from infertile women with ASA producing different cytokines when encountering sperm cells, as compared to healthy controls. This type of cytokine response could contribute to proinflammatory environment, Th2 favorable conditions, or impairment of angiogenesis leading to infertility. Some of these cytokines could influence the cell proliferation and recruitment or participate in the sperm cell-specific immune response. We showed that cellular response to sperm cells is different in infertility with and without ASA, suggesting that cellular response differs between these two types of infertility. The dysregulation of the immune response with shift towards Th2-type of response suggest how the initial disturbance in cellular immune response could result in ASA.

14.4 Recent Research of Female Patient's Immunoglobulin Reaction to Seminal Antigens

Semen is defined as a complex fluid containing sperm cells, cellular vesicles, enzymes, ions, proteins, peptides, hormones, cytokines, and other cells migrating leucocytes or spermatogenic cells [6, 7, 38, 39]. Each component could immunize the female genital tract.

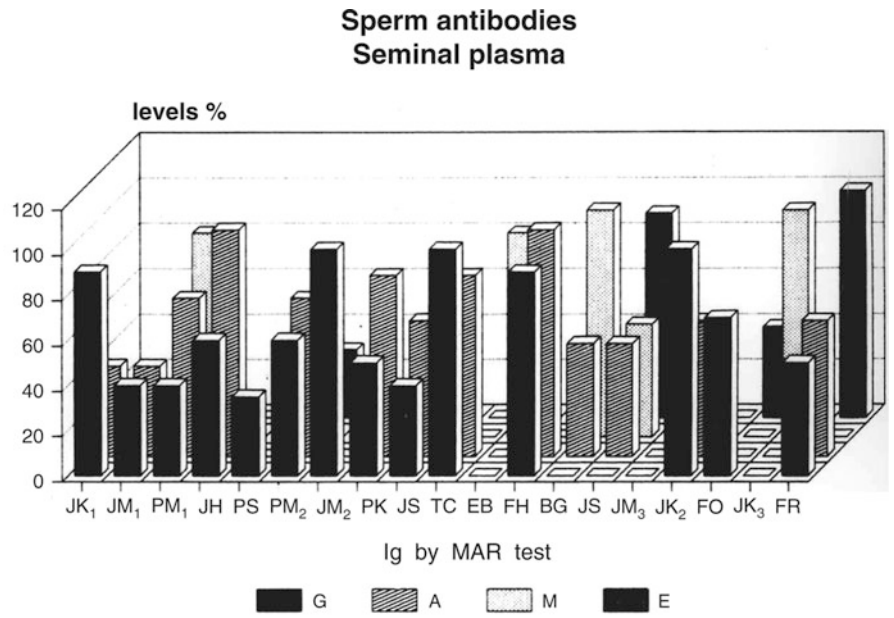
We focused on the characterization of seminal proteins to illustrate the IgG, IgA, and IgE immune responses of 31 infertile women. The biochemical characterization was performed by one-dimensional sodium dodecyl sulphate polyacrylamide gel

electrophoresis and isoelectric focusing, both of which were followed by immunoblotting analyses. IgG mainly recognized the antigens with relative molecular masses (Mr) 95 and 183 kDa and isoelectric points ranging from 6.9 to 7.0. The immunodominant antigens recognized by IgA had 35 kDa and isoelectric points ranging from 6.2 to 7.2. The reactivity of IgE was not confirmed within our group of patients. The seminal IgG- and IgA-binding patterns were analyzed immunochemically to determine the characteristics of possible seminal proteins associated with female immune infertility [39, 40].

14.5 Coitus Condomatus and Steroid Treatment of Patients with ASA

Recent evidence and simple field experience suggest that immunocompetent cells in immunological anti-sperm mechanisms could be influenced by condom blocking effect, by oral corticosteroids [17, 41–49], by local hydrocortisone application to ectocervix [17], or by plasmapheresis [49]. One of our earlier studies [49] showed good therapeutic effect of oral prednisone in infertile men with ASA in seminal plasma. It succeeded to reduce sperm-agglutinating levels in seminal plasma. Treatment scheme of oral prednisone adopted in 19 selected patients with high titers of ASA in seminal plasma only was as follows: prednisone at dose 20 mg (in the morning) – 20 mg (at noon) – 20 mg (in the evening) daily for the first week, 20-10-10 mg for the second week, 15-10-5 mg for the third week, 10-5-5 mg for a fourth and fifth weeks, 5-5-5 mg for the sixth and seventh weeks, 5-5-0 mg for the 8th–12th weeks. The levels of sperm-agglutinating antibodies estimated by direct MAR-test (mixed anti-immunoglobulin reaction test) in IgG, sIgA, IgM, and IgE before the prednisone treatment, and after 2, 4, and 6 weeks during the above-mentioned scheme of therapy are shown in Fig. 14.1. Individual decrease of ASA level is well recognizable after 6 weeks of the treatment. Patients JK1, JH, PK, JK3, and FR became fathers. Reduction in activity and levels of ASA was observed especially in immunoglobulin class A and G. The dynamics of seminal ASA as mean values of MAR tests can be also seen in Fig. 14.1. After 6 weeks of prednisone treatment, individual sensibility to corticosteroids was observed. In 10 patients, ASA were not detected any more in any of the patients, the wives of patients JK1, JH, JK3 became pregnant by the end of their husband's treatment, and the wives of PK and FR became pregnant within 6 months. Impact of immunosuppressive treatment of immunocompetent cells creating ASA is evident from the fertility outcome.

Increased pregnancy rate after various schemes of prednisone dosages is referred between 11 and 56 % [41–49]. At every consultation of our patients, blood pressure was measured and anamnestic data were screened. No unwanted systemic effects were registered during the oral prednisone treatment, although some nonspecific side effects such as aseptic necrosis hip, exacerbation of incipient duodenal ulcers, or cardiovascular effects were described [43, 44].



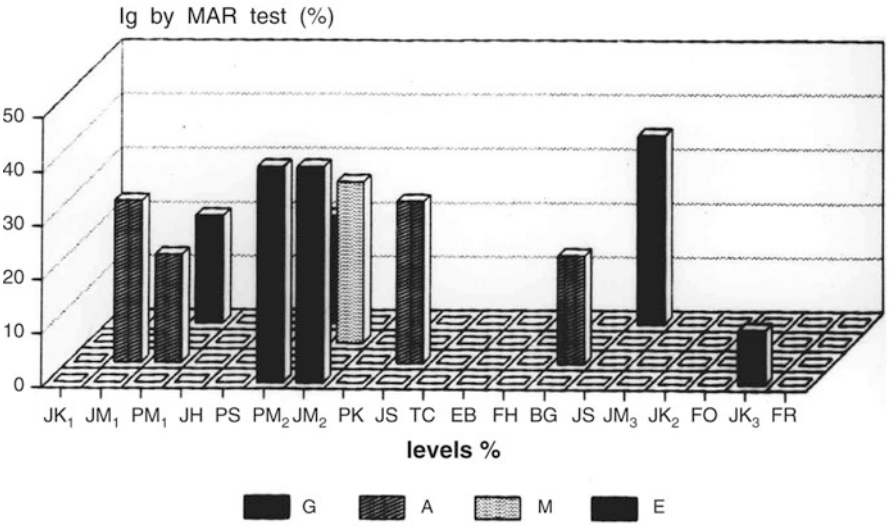


Fig. 14.1 The effect of prednisone on immunocompetent cells in men with positive sperm-agglutinating antibodies in seminal plasma

Highly individualized sensitivity and mechanisms responsible for the influence of immunocompetent cells producing ASA are supposed to exist. In treated patients, we monitored the ASA in seminal plasma three times during the corticosteroid therapy. When pregnancy is not observed in time of the husband's treatment, in vitro fertilization (IVF) is planned, as a logical way in long lasting "unexplained" infertility, in three stimulated ovulations of their wives. Our experience shows that in some men better treatment is the parenteral administration of corticosteroids timed 24 or 12 h (depending on the levels of ASA) before the collection of semen for IVF.

As we know, ASA impair sperm transport within the female genital tract. Sperm penetration and progressive motility through cervical mucus are stopped or decreased [6, 8, 12, 50]. When all sperm cells are coated with antibodies, the cervical ovulatory mucus has no motile spermatozoa [24, 48]. ASA are detected not only in semen, but also in ovulatory cervical mucus owing to ascendent female immunization by sperm antigens. IgG and/or IgA have been found in the cervix uteri [15, 17, 24, 33]. Sperm head directed antibodies, mainly those in IgA class, and/or IgA and IgG class antibodies directed against antigens on the principal piece of the sperm tail severely impair the ability of spermatozoa to penetrate mucus [24, 48]. In the second mechanism of sperm transport impairment, complement-mediated IgG and/or IgM antibodies are able to activate the complement cascade, which results in target cell lysis [8]. Complement-mediated sperm immobilization within cervical mucus does not act immediately after sperm penetration into the cervical mucus, but requires 4–8 h. High decrease of sperm immobilization was observed in IgG. Practically all antibodies that are formed against sperm are designed to immobilize them.

The next mechanism of interference with fertilization by ASA acts via antibody dependent cell-mediated cytotoxicity [22, 26], which impairs the process of capacitation (calcium dependent biochemical and structural changes, namely acrosomal reaction – exposure of enzymes as acrosin, trypsin like proteinase, and hyaluronidase allowing contact with egg plasma membrane) [14, 41]. Sperm-egg interaction could be also influenced by the attack of ASA, which can alter fertilization by affecting the ability of spermatozoa to bind to the zona pellucida as well as to the egg plasma membrane. The evidence suggests that ASA may interfere with sperm recognition of the zona pellucida in humans (see also [4]. The isotypes of ASA bound to the head may be important in determining the degree of impairment of sperm-zona pellucida binding. It was found [26] that IgA sperm antibodies were more inhibitory than IgG. Mahoney et al. [27] found that antibodies directed against the sperm head can affect zona binding, but not in every case. This suggests that impairment of zona pellucida binding depends on the antigens against which antibodies are directed, that is, whether they are the functional epitopes of a zona receptor ligand.

14.6 ASA Influences on Pregnancy Rate

The presence of ASA in the IVF culture medium results in an impairment of fertilization. Immunoglobulins of the IgA class appeared to be more effective in impairing fertilization than IgG. More critical examination of the results of IVF in women

with isoimmunity to sperm revealed both diminished fertilization rate and diminished embryonic cleavage rate, and reduced chance of pregnancy as shown by the study of Vasquez-Levin [25]. On the other hand, significant pregnancy rates can be achieved despite the presence of ASA. These antibodies cannot be eliminated from the egg by washing. Pagidas et al. [31] claimed that IVF is not significantly affected by the presence of ASA in female sera used to supplement the culture media or by antibodies bound to inseminated sperm. Inhibition of IVF fertilization may be caused by a synergistic effect of IgG and/or IgA classes of ASA. In IVF trials, other authors did not find any differences between antibody positivity and antibody negativity in the outcome of the IVF embryo transfer attempt [28].

Menge and Naz [9] suggested three mechanisms by which ASA can affect embryo survival. The first mechanism consists of the possibility that sperm surface antigens are incorporated into the zygotic membrane at fertilization. Fertilization involves the possibility of sperm made oolemma plasma membranes moving to a mixture of antigens. The second mechanism proposed is that similar epitopes are present on spermatozoa and embryos. Several common antigens have been found. The last proposed mechanism to account for postfertilization reproductive loss mediated by ASA is via an indirect effect of antibodies on embryo development.

A woman whose partner has ASA following a vasectomy, inflammation process, or testicular tumors often produces also ASA [10, 16, 29]. In humans, clinical evidence of an association between early pregnancy loss and immunity to sperm is not definitively clear. Approximately 15 % of women produce antibodies to sperm in the cervical mucus and in the blood. Over 50 % of men with low sperm motility have been found to carry these antibodies in semen plasma and in serum. Sperm cells have unique surface antigens that elicit an immune response.

Beer also speculated [10] about the rising incidence of ASA in women. It may be due to delaying pregnancy until late in life, by which time they will probably have had several sexual relationships. This increases the risk of immune sensitization. If ASA in woman are present that agglutinate and/or immobilize partner's sperm and those from any sperm donor, we consider non partner-specific ASA. Beer studied [10] ASA levels in over 100 prostitutes and compared them with those of 40 age-matched women. More than 40 % of prostitutes had ASA compared with just 5 % among the control group. Over 60 % of these women who had never used any form of contraception became infertile within 9 years.

The same author also observed [10] that ASA can be associated with antibodies to phospholipids. They are strong indication that the woman will have anti-DNA antibodies in addition to elevated levels of circulating natural killer cells and CD19+/5+ B-cells that produce the ASA. Such a problem can manifest itself as repeated IVF failure where embryo is implanted, but later it leads to very early miscarriage. Immunotherapy is an effective way of treating women with antibodies to sperm who experience this problem. A number of autoimmune and/or isoimmune conditions of the reproductive system are associated with poor fertility. Several topics [13, 23] have described the relationship between ASA and pregnancy prognosis. Also allergies to heavy metals can negatively influence reproduction, because in

sensitive persons they are able to alter the immune reactions including production of autoantibodies. The altered immune reaction can then cause infertility. In patients with metal intolerance diagnosed by the MELISA test, the release of metal ions from dental materials can be one of the stimulating factors which may adversely affect fertility [11]. An Italian group [50] studied the presence of ASA in 190 patients with testicular cancer 1 month after orchiectomy and before radiotherapy or chemotherapy. The results support the hypothesis that testicular cancer is not a cause of ASA and infertility. Marconi et al. [29] did not find any relationship between ASA detected by mixed agglutination reaction or immunobead test and chronic inflammation and infection of the seminal tract.

14.7 ASA and Pathology Such as Azoospermia and Severe Oligospermia

Presence of sperm antibodies connected or not connected with pathology in semen such as azoospermia and severe oligospermia (oligoasthenospermia) is a reason to make examination of genetic causes of male infertility. Our genetic laboratory investigates karyotype, on an exclusion of Y chromosome microdeletions in AZF area, and on exclusion of mutations in the CFTR gene. If chromosomal aberrations and DNA mutations in our patients are found, their offsprings will be at an increased risk of transmitting genetic alterations. We recommend to perform preimplantation genetic diagnosis (PGD) to select embryos without genetic changes. Nowadays, the most commonly used method PGD/PGS (preimplantation genetic screening) includes the examination of cells of trophectoderm of early embryo, which is taken on the fourth to fifth days of the development stage of blastocyst in vitro. DNA isolation from trophectoderm follows. The VeriSeq PGS Kit offers a highly sensitive screening of all 24 chromosomes for selection of euploid embryos. We exclude by the VeriSEq PGS method both numerous and extensive structural aberrations, but also to detect microdeletions and microduplications. If genetic cause in male patient exists we use sperm cells from healthy and anonymous donor without ASA. The examination of ASA in donors is required.

In men with severe obstructive oligospermia and/or azoospermia, we use the surgical sperm retrieval option such as microsurgical epididymal sperm aspiration (MESA), testicular sperm aspiration (TESA), or percutaneous sperm aspiration (PESA).

Conclusion

In earlier reports, an incidence of ASA in an infertile population is 9 % of the men and 15 % of the women was published [41]. ASA play an important role in the etiology of immune infertility. Circulating and local ASA may be markers for disorders of the reactivity of the immune system and may be involved in iso- and autoimmune process. ASA testing is important in cases with explained or unexplained infertility, but without pregnancy success in repeated procedures of IVF. Today effort is necessary to define sperm antigens with significance for

fertility. The association of the ASA with infertility demands their detection and couples with these immunological findings have to be treated appropriately. As these antibodies can induce infertility, they have the potential to induce the development for contraceptive purposes in humans [49].

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Sperm Antibodies and Assisted Reproduction

15

Jerome H. Check and Jasmine Aly

Abstract

The presence of antisperm antibodies (ASA) coating sperm can be a cause of infertility. However, their presence is not an invariable cause of infertility. This lack of consistency may be related to the percentage of sperm that are coated with the ASA or the concentration of ASA on each sperm. Also sometimes the explanation for achieving a pregnancy despite the presence of ASA may be related to the ASA directed against inert antigens. Based on studies showing very poor pregnancy rates following intrauterine insemination (IUI) of sperm where 100 % are coated with ASA, one can make a reasonable assumption that the majority of males with a high percentage of their sperm coated with ASA will be a factor causing a couple's infertility. Another reasonable assumption is that the majority of ASA must effect the fertilization process as the negative effects of immobilizing ASA would be circumvented by IUI. One of the best treatment options for overcoming ASA is to perform in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI). Though conventional insemination of oocytes may be more effective than IUI, the ASA can sometimes lead to failure of sperm to bind to the zona pellucida leading to low fertilization rates. Unfortunately, IVF with ICSI is very expensive. A less expensive option is to try to neutralize the adverse effects of the ASA before IUI. There is evidence that this can be accomplished by treatment of the sperm prior to IUI with a protein digestive enzyme, e.g., chymotrypsin.

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15.1 Introduction

A very recent manuscript was published by a group well known to the andrology field entitled "Functional defect of sperm and fertility impairment in men with antisperm antibodies" by Bozbedomov et al. [4]. In their introduction, they mention that "antisperm antibodies (ASA) have been detected in 8–21 % of infertile men; however the presence of ASA has also been detected in 12–19 % of fertile men" [4]. They quote references from Krause et al., Francavilla and Barbonetti, Vasquez-Leven et al. [4, 24, 30, 46].

The study by Bozbedomov et al. discusses the results of the largest study to date, which evaluated 1794 infertile couples by Leushuis et al. [4, 33]. The conclusion from this study states "that the MAR test is not able to predict spontaneous pregnancy chances." Their conclusion from Leushuis et al. about measuring ASA was that "It's routine use in the basic fertility workup for identification of couples with low spontaneous pregnancy chances is not justified when a threshold of >50 % is used" [33].

The results of these studies suggested no higher frequency of significant ASA levels (>50 %) in fertile vs. subfertile males and no evidence of a significant impairment of fertility even when ASA are present. This has led to the majority of present day infertility specialists abolishing the measuring of ASA in their routine practice.

The objective of this manuscript is to explore whether it is useful to measure ASA routinely or in certain specific circumstances. In addition, this manuscript will also examine if their presence should influence treatment by assisted reproductive technology, i.e., intrauterine insemination (IUI) or in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI). If routine measurement of ASA is not recommended, an objective of this article is to carefully review the literature to determine if there are any specific circumstances that would warrant ASA testing. Another objective is to consider whether the seemingly lack of correlation between ASA and fertility outcome or lack of increased frequency of ASA in infertile couples may actually be related to the parameters set for a positive ASA level. This study will address if the cutoff of >50 % is too low, i.e., will levels >80 % or levels restricted to 100 % show better correlation. Finally, the intent of this manuscript is to explore the relative value of treatment options in infertile cases that seem to be related in part, or in whole, to ASA, i.e., is IVF with ICSI the best option? How do other treatment options, e.g., intrauterine insemination (IUI) compare? Are there other methods to improve the success of IUI when ASA are present?

15.1.1 Mechanism by Which ASA May Impair Fecundity

1. Interference with sperm progression through cervical mucus and thus preventing sperm access into the uterine cavity.
   It has been known for over two decades that when sperm are laden with ASA, this can impair sperm progression through the cervical mucus, even if there does

not appear to be any detriment to sperm motility upon evaluating the semen analysis or not finding sperm agglutination [19, 22, 36].

Probably the best way to detect if there is the presence of immobilizing antibodies is to find the absence of sperm progressing through the cervical mucus on postcoital testing [9]. However, recently the Practice Committee from the American Society of Reproductive Medicine considered the postcoital test to be an archaic test and recommended against its routine use [42]. Nevertheless, they did state that in the hands of some clinicians the test has value [42]. Part of the problem with interpreting the value of the postcoital test is the lack of standardization, e.g., how long after intercourse to perform the test, how many sperm should be present per high powered field to consider the test normal, and the need for precision of the timing of the test. The author's preference is to perform the postcoital test in natural cycles, 8–12 h after intercourse when the serum estradiol exceeds 200 pg/mL with the serum progesterone level <1.5 ng/mL but preferably <1 ng/mL, and before the luteinizing hormone (LH) surge [7].

The reason why antisperm antibodies do not immobilize the sperm in the ejaculate is because of the absence of complement in the seminal plasma [19]. The cervical mucus, however, does contain complement, and thus the ASA-complement reaction causes sperm immobilization, thus impairing sperm penetration through the cervical mucus [19]. However, a certain amount of time is needed for this ASA-complement reaction to occur and that is why one should wait perhaps 8 h from intercourse to perform the postcoital test when screening for ASA immobilization antibodies.

If ASA causing immobilization is the main type of problem, theoretically simple IUI should overcome the problem. There has been a tendency for most infertility specialists to give the infertile couple the option of an IUI or an IVF cycle no matter what seems to be the etiologic factor. Thus, based on performing IUI as a routine, if indeed the majority of those with ASA have exclusive immobilizing antibodies, and IUI overcomes the problems, the tendency for routinely performing IUI could justify not measuring for ASA when performing semen analysis and also not doing a postcoital test. In contrast, if a postcoital test is performed correctly, and the results showed a poor postcoital test despite the presence of normal appearing cervical mucus quality and normal standard semen parameters, this would otherwise alert the physician to measure ASA [9]. This would be especially important for physicians who do not routinely treat every woman with IUI.

The peak quality cervical mucus may be 36–40 h before ovulation. The theoretical advantage of performing an IUI is to allow sperm to enter the uterine cavity at the time of ovulation when the cervical mucus quality generally wanes, and thus prohibits sperm movement [7]. However, IUI can considerably increase the cost of conceiving (either to the patient or to the insurance company). There are data suggesting that IUI does not improve pregnancy rates in infertile couples where semen parameters are normal and postcoital tests are adequate [16]. Related to a tendency for infertility specialists to favor expensive IVF proce-

dures, for economical reasons there has become a trend for the obstetrician gynecologist (OB/GYN) to initially investigate and treat infertility before referring to an infertility specialist. Because the OB/GYN's are generally not equipped to perform IUI, they must rely on the postcoital test. The OB/GYN generalist may be the most likely physician to order sperm ASA if faced with normal sperm and a poor postcoital test despite normal mucus quality and a normal semen analysis [8].

2. Evidence that ASA may impair conception in other ways than inhibiting cervical mucus penetration:
There are various sites of potential ASA action. There is evidence that ASA can affect sperm-zona binding and/or zona-induced acrosome reaction [2, 23, 34, 45, 48]. Antibodies to the sperm antigen FA-1, which is found in the sperm membrane, can interfere with zona binding [28, 38, 39]. Another protein, IZUMO, is found in the acrosomal region of intact sperm and the equatorial segment of reacted sperm [26]. This protein is involved in sperm-oolemma fusion. The YLP-12 antigen is located in the acrosome and is functional in both the acrosome reaction and zona pellucida binding [40]. Of course there is the possibility that ASA can sometimes be directed to inert proteins not involved in the conception process.

Is there any evidence that ASA other than immobilizing antibodies when present play a significant role in infertility? Suggestive evidence that ASA effecting zona binding, acrosome reaction, or other negative effects on fecundity was provided by Francavilla et al., who found no live pregnancies following 119 IUI cycles when ASA was present in 100 % of the sperm [25]. If immobilizing ASA was the only immunoglobulin involved, IUI should have been successful. The study by Francavilla et al. suggests that if ASA may be directed against inert sperm proteins, exclusive ASA related to antigens not impairing fertility are not common [25].

If ASA other than immobilizing antibodies, e.g., ones that interfere with sperm binding to the zona pellucida, play a significant role in causing infertility, one would expect reduced fertilization rates and lower pregnancy rates following conventional oocyte insemination. Indeed the first publication exploring the effect of ASA claimed that ASA adversely affected fertilization rates [27]. However, subsequently, other studies did not support these findings [44, 47]. These studies failing to support ASA as a negative factor for conventional insemination suffered from inadequate power and a generous range of what they considered positive for ASA [44, 47]. For example, the study by Vujisic et al. only evaluated 14 patients, they used a cutoff of only 20 % for positive ASA, and there were only four men with ASA over 75 % [47]. However, more studies, supporting the original premise of ASA reducing fertilization, were published [1, 5, 21, 31, 35, 43]. Again the percentage of couples used whose male partner had 100 % of the sperm coated by ASA vs. 50 % (some even call positive ASA at 20 %) could explain the differences in outcome.

Some of the studies finding no significant differences in frequency of sperm with ASA present in fertile vs. nonfertile couples may be related to too low of a

threshold for a positive test. Perhaps to be able to ensure a likelihood of an adverse effect of ASA, one should consider a positive test as >80 %. Using an 80 % cutoff, we found the presence of ASA in 3.54 % of 6551 male partners of couples with infertility and it was 4.22 % using a 50 % cutoff and 1.8 with a 100 % cutoff (unpublished data).

15.1.2 Treating Sperm Bound with ASA with Chymotrypsin Galactose Prior to IUI

A study was performed involving 16 couples where all infertility factors were corrected except a poor properly timed postcoital test (no sperm with progressive linear motion) despite what appeared to be appropriate quality cervical mucus [3]. Furthermore, the male partner was found by immunobead testing to have >50 % of sperm with ASA. Intrauterine insemination was performed with all the males ejaculating into 5 ml of equal parts of modified human tubal fluid buffered with HEPES solution and 7.5 % bovine serum albumin in an attempt to dilute the sperm to theoretically negate the attachment of antibodies at the time of ejaculation. Another group ejaculated into media with the protein digestive enzyme chymotrypsin with galactose in order to cleave part of the ASA immunoglobulin molecule to neutralize its function prior to sperm washing. The 16 couples were randomly assigned one of the two sperm preparations, and if no pregnancy was achieved, the other preparation was used for the second cycle of treatment [3]. With each failure, they were switched to the other protocol for the next treatment [3]. There were 65 treatment cycles – 32 with chymotrypsin/galactose and 33 with albumin. Pregnancies were achieved in 8 of 32 (25 %) cycles following IUI with chymotrypsin/galactose vs. only one of 33 (3 %) performed with sperm ejaculated into albumin fortified media [3]. When 100 % of sperm was found to be coated by ASA, Francavilla et al. found no live pregnancies following 119 IUI cycles [25]. In contrast, a pregnancy rate of 25 % per cycle was found following IUI with chymotrypsin-treated sperm when evaluating the subset of couples whose male partner had 100 % of the sperm coated with ASA [3].

Interestingly the pregnancy rate for female partners of males with 100 % ASA was 75 % with three treatment cycles when treated with chymotrypsin/galactose. This suggests that the antibodies that may inhibit the fertilization process rather than the antibodies that impede sperm from reaching the oocyte play a significant role in infertility and they need to be neutralized prior to insemination. Though IUI is less expensive than IVF, nevertheless, it still adds expense and missed time at work. Though significant levels may only be present in <5 % of the couples, theoretically, it makes sense to measure this potential infertility factor prior to initiating any therapy. There do not appear to be any subsequent studies refuting or corroborating the beneficial effects of treating sperm with chymotrypsin/galactose prior to IUI.

15.1.3 The Use of In Vitro Fertilization (IVF) with Intracytoplasmic Sperm Injection (ICSI) for Treating ASA Related Infertility

The patient's choice of IUI or IVF with ICSI is frequently determined by economics and rules of the insurance companies. Most insurances that cover IVF-ET and ICSI will approve this procedure if a high percentage of the sperm are laden with ASA. Nevertheless those without sufficient funds or insurance coverage may elect to try IUI first. Based on the poor pregnancy rates demonstrated by Francavilla et al., and the good success rates demonstrated by Bollendorf et al. with sperm first pretreated with chymotrypsin/galactose, if IUI is planned, it makes more sense to first treat with the aforementioned protein digestive enzyme [3, 25].

However, experience dictates that most treating physicians are not aware that enzymatic pretreatment before IUI is an option. Even if they read about the procedure, they may be reluctant to use it because of lack of experience. Thus, if ASA is detected on the sperm, and if the decision is made to try IUI first, many infertility specialists will perform the procedure without any pretreatment of the sperm hoping that either the antibodies are directed to inert antigens or merely to immobilizing antibodies, and therefore, in theory, by bypassing the cervical mucus, IUI would overcome the problem.

Infertility specialists who are confronted with couples who have failed to conceive in other infertility practices, and where the female partner has failed to conceive with IUI either with or without the knowledge of the existence of ASA being present, the option of chymotrypsin pretreatment of sperm prior to IUI or IVF-ET with or without ICSI should be considered.

Based on the very poor results of IUI without enzymatic pretreatment for couples whose male partners have 100 % of the sperm coated with ASA, one must assume that the ASA must be directed in a high frequency to antigens involved in the fertilization process not only involved in immobilization. Thus, conventional IVF, where sperm is merely added to the oocyte, could result in low fertilization rates with subsequent poor pregnancy rates if a high percentage of the time the ASA is directed toward antigens involved in the fertilization process. The use of ICSI would negate sperm-zona pellucida binding problems and would overcome the first part of oocyte activation if that was a problem by acting as a pseudo-trigger [17].

15.1.4 IVF with Conventional Insemination with Sperm Pretreated with Chymotrypsin

As described in the previous section, before ICSI, chymotrypsin was found to improve pregnancy rates following IUI. Theoretically, if there was a high percentage of sperm coated by ASA, but not 100 %, there may be enough sperm without ASA to fertilize the oocyte in view of the large number of sperm used for conventional insemination. A study was performed comparing fertilization rates and subsequent pregnancy rates following conventional insemination with sperm with 100 %

ASA coating, which were either pretreated with chymotrypsin or just ejaculated into culture medium (to allow dilution of ASA in case, a significant amount is added to the sperm at the time of ejaculation) [29]. The study did show a low fertilization rate of 27 % using conventional oocyte insemination without enzymatic treatment (21 cycles in 11 patients). The fertilization rate was significantly higher in those using enzymatic pretreatment (47 % – 38 cycles in 25 patients). The clinical pregnancy rates were twice as high with chymotrypsin pretreatment 21 % vs. 9.5 % (not statistically significant) [29]. The study was published in the early days of IVF-ET (1995) and thus at a time when pregnancy rates with IVF-ET were much lower than today.

In the aforementioned study, failed fertilization occurred in 13 of 59 (22 %) of cycles and 11 of these occurred in cycles not using enzymatic pretreatment [29]. Interestingly a requirement for the study was failure to conceive after a minimum of 6 IUI cycles (without chymotrypsin therapy) thus hopefully excluding cases of ASA directed against inert antigens or antigens only effecting immobilization [29].

15.1.5 The Use of ICSI for Sperm Coated with ASA

Unfortunately there are no studies comparing enzymatic pretreatment of sperm using conventional oocyte insemination vs. ICSI. However, there is a study comparing the efficacy of chymotrypsin-treated sperm followed by IUI vs. IVF with ICSI [14]. Again males with 100 % of the sperm coated for ASA were selected for comparison. There were 17 women inseminated with chymotrypsin-pretreated sperm in 47 IUI cycles vs. 25 women having IVF with ICSI in 38 IVF-ET cycles. There was a significantly higher pregnancy rates per cycle with IVF with ICSI (28.9 %) vs. IUI with enzymatically treated sperm 10.6 % [14]. The pregnancy rate per patient was 29.4 % with IUI (avg. 7.7 cycles) vs. 44 % for IVF with ICSI (44 %). Considering the marked difference in price, chymotrypsin pretreatment with IUI seems to be a legitimate alternative treatment [14]. Though this study was performed later than the aforementioned study of chymotrypsin pretreatment before conventional therapy, the study was published in 2004 at a time when there was not as much experience with ICSI [14].

The first studies showing the efficacy of IVF with ICSI were published in 1995 by Lahteenmaki et al. and by Nagy et al. [32, 37]. These studies were confirmed by two subsequent studies in 1997 and 2000 [18, 20].

There has been some question as to whether ASA on sperm could lead to some postfertilization issues that could result in clinical pregnancies but result in higher miscarriage rates. Lahteenmaki et al. found a miscarriage rate in 38 % (5/13) in those with ASA vs. zero % for controls [32]. Check et al. found a 14 % miscarriage rate in those with ASA <50 % vs. 25 % for those with >80 % [18]. In contrast, Clarke et al. found no increase in miscarriage rates [20]. This issue has not been resolved to date.

15.1.6 The Future and Historical Perspective

The ultimate study to determine the influence of the presence of ASA on sperm and the efficacy of various treatment options would enlist a multicenter randomized controlled trial with various treatment options in couples with all female factors corrected. The couples would be randomized to one of four groups: 3 cycles of intercourse only, 3 cycles of IUI with sperm not treated with protein digestive enzymes, conventional oocyte insemination, and the last treatment group, IVF with ICSI. Ideally, inert antigens could be determined and those with exclusive ASA to inert antigens would be excluded. The data would then be stratified according to the percentage of sperm coated with ASA.

It is highly unlikely that such a study could ever be performed because of the relatively low percentage of males with ASA, especially with a high percentage of sperm coated with ASA. Furthermore as mentioned, there is much less interest from infertility specialists even measuring ASA.

Besides the low prevalence, other factors contribute to disinterest. In the United States, one of the problems is that CLIA (Clinical Laboratory Improvement Amendments) requires certain criteria for ASA testing. CLIA requires positive and negative controls performed each day of testing. Immunobeads IgG were sold by Bio-Rad; however, the company did not sell controls. Fertility Solutions, Inc. introduced positive and negative controls for indirect testing.

However, Bio-Rad has discontinued making this product. Therefore, ASA testing is only available today using the sperm MAR assay. Controls are available for indirect testing. However, to perform indirect testing, one needs ASA-negative sperm. One way of obtaining this is to use patient's own sperm and hope he is negative. Another option is to use a known ASA-negative donor and reimburse him. A final option is to freeze an antibody-negative donor's sperm in aliquots using a freezing medium without human serum albumin (HAS) since HAS will interfere with the sperm MAR assay. This could be used as a control for direct testing. However, HAS is a common protein used in most commercially available cryopreservation medium.

This dilemma has caused a large majority of infertility centers to discontinue direct antisperm testing. Complicating this is the fact that despite the high costs involved just in controls, there is generally a low insurance reimbursement (sometimes as low as $4.00).

As mentioned earlier, the authors have evaluated ASA using the direct immunobead test in over 6000 males and found positive ASA in 263 using a 50 % cutoff (less with males that are positive with higher cutoff levels). The authors hope to answer some of the questions regarding the influence of ASA in the modern IVF era by evaluating the subset of couples with positive ASA (50 % or higher) undergoing IUI with chymotrypsin pretreatment vs. IVF with ICSI. These couples could be retrospectively matched to the next couple having IVF with ICSI but with sperm negative for ASA.

Such a study unfortunately would not be able to ascertain if in the modern era conventional oocyte inseminated with sperm laden with ASA results in better fertilization rates and lower failed fertilization rates than in the earlier era because the policy of our infertility center is to perform ICSI if ASA is >50 %. However, such a

study could still compare fertilization rates with ICSI according to percentage of sperm coated with ASA to sperm without such a coating.

Failed fertilization with conventional insemination is rare when sperm have normal parameters and are negative for ASA (0.1 % in 12,448 IVF cycles evaluated) [11]. A study of the subset of couples where semen parameters are normal and ASA is positive could help determine if ASA can still cause fertilization failure related to defects in phase 2 of oocyte activation. Though only 12 cases of failed fertilization were detected in 12,448 cycles, half were related to sperm not attaching to the zona pellucida (which would be overcome by ICSI) but half would not be corrected [11]. If a fair percentage of low or failed fertilization is found in males with a high percentage of sperm positive for ASA, perhaps some oocytes should be fertilized with the addition of calcium ionophore [17, 41].

The proposed study with greater power than the previous aforementioned ones could possibly help solve the debate as to whether the presence of ASA leads to a higher miscarriage rate even if IVF with ICSI is performed.

One could argue that perhaps ICSI should be performed on everyone rather than using conventional oocyte insemination? First of all, ICSI lowers pregnancy rates following embryo transfer [12, 13, 15]. Furthermore, ICSI adds extra expense to an already expensive procedure and tremendously increases embryologists work load.

The authors suggest that the frequency of ASA in the population be made known to the couple. A discussion with the patient should then follow, offering the option of measuring ASA either before IUI or IVF to prevent the wrong method being used. Perhaps if either the couple or IVF center does not want to test for ASA in all cases, one could consider a properly timed postcoital test to help determine which specific subset of patients may benefit from measurement of ASA [9], as it has been known for over two decades that when sperm are laden with ASA, this can impair sperm progression through the cervical mucus [19, 22, 36].

It is our policy that all couples with positive ASA have IUI with chymotrypsin/galactose pretreatment. Thus, a matched controlled study cannot be performed with our data to determine, in the modern era, whether we can corroborate previous data, suggesting enzymatic pretreatment improves pregnancy rates compared to other methods [3]. Some infertility specialists would argue that IUI is used routinely in most couples, and if ASA was present, the IUI would overcome the problem. However, until proven otherwise, the use of IUI does not seem to overcome the problems of ASA empirically when present in a high percentage of the sperm [25]. As an aside, there is no evidence that IUI improves pregnancy rates in infertile couples where semen parameters are normal and postcoital tests are normal [16]. Perhaps the problems of performing ASA testing, and the apparent severe detriment to the infertile couple if not detected when ASA is present, could reinstitute the inexpensive properly timed postcoital test as a screening test for evaluating the presence or absence of ASA [6, 7, 42].

Finally, the possible presence of ASA in cervical mucus should be mentioned although the frequency is far less common than ASA on sperm [10]. Furthermore, IUI should overcome this problem. Thus, where IUI is performed routinely, or performed for a poor postcoital test, the problem of ASA in the cervical mucus should be corrected assuming ASA is absent in uterine and tubal fluids.

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Treatment Modalities for Antisperm Antibodies-Mediated Immune Infertility

16

Rajesh K. Naz

Abstract

Immune infertility due to antisperm antibodies is an important cause of infertility in humans. The incidence of ASA in infertile couples is 9–36 % depending on the reporting center. ASA directed against the fertilization-related antigens are more relevant to infertility than the immunoglobulin binding to sperm antigens that do not play a role in fertility. Several methods have been reported for treatment of immune infertility. These include immunosuppressive therapies using corticosteroids or cyclosporine; assisted reproductive technologies such as intrauterine insemination, gamete intrafallopian transfer, in vitro fertilization, and intracytoplasmic sperm injection; and laboratory techniques such as sperm washing, immunomagnetic sperm separation, proteolytic enzyme treatment, and use of immunobeads. Some of these available techniques have side effects, and others are invasive and expensive, with low efficacy, and provide conflicting results. Presently, antisperm antibodies-mediated immune infertility is primarily treated in the clinics using the assisted reproductive technologies. Recent findings on delineating sperm antigens that have a role in fertilization/fertility may provide novel modalities for treatment which will be less invasive and expensive.

16.1 Introduction

Antisperm antibodies (ASA) can cause infertility. Incidents of antisperm immunity in infertile couples are 9–36 %, depending on the reporting center [7, 15, 40, 54]. ASA reactive with sperm antigens that are involved in fertilization and expressed on

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the surface for antibody binding are more relevant to infertility. Also, these antibodies have to be present in the genital tract secretions of a female partner or bound on the sperm surface in a male partner in sufficient amount to cause infertility effects. The kinetics, valency, and class/subclass of antibodies play an important role in defining the significance of ASA in infertility.

Although there are several articles written on various aspects of immune infertility, there are only a few covering the therapeutic treatment modalities for male and female immune infertility. The aim of this chapter is to review the conventional treatment methods for immune infertility, discuss their relative merits and limitations, and describe the recent novel perspectives that are being investigated. The focus of the chapter is on antisperm antibodies-mediated immune infertility and not on pregnancy loss due to immune dysfunction. A PubMed search (1971–2015) was performed using keywords: "immune infertility," "antisperm antibodies," and "treatment of immune infertility." All of the articles were read, and also the articles referenced in these publications were thoroughly examined.

16.2 Discussion

Although the understanding of etiology of ASA has increased, the therapeutic measures have not made the same strides [46]. Various treatment methods available at present can be divided broadly into four categories: immunosuppressive therapies, assisted reproductive technologies (ART), laboratory techniques, and novel recent perspectives using defined sperm antigens.

16.2.1 Immunosuppressive Therapies

The method of immunosuppression that has been most commonly used is corticosteroid therapy. Pregnancy rates of 6–50 % have been reported after corticosteroid therapy [56]. However, almost all studies reported in the literature for the effect of steroid treatment on immunosuppression of ASA titers lack appropriate placebo controls, have employed different doses and regimens of various immunosuppressive drugs, and have used different laboratory techniques to monitor the ASA titers to examine the effect of drug treatment. These factors make it difficult to compare and conclude whether or not immunosuppression is indeed effective in the treatment of immune infertility.

Two of the clinical trials had appropriate placebo controls and are worth describing here. One study conducted a 6-month randomized trial using high dose of prednisolone given through cycle days 1–10 of the female partner, which was then tapered rapidly for the next 2 days [27]. The steroid treatment group resulted in a pregnancy rate of 31 % compared with 9 % in the placebo group. Another prospective, double-blind, placebo-controlled study included 43 men that had ASA bound to sperm [25]. Of these, 24 were given methylprednisolone and 19 received placebo for three cycles. There was a statistically significant decrease in sperm-associated

IgG, but not IgA, in the steroid treatment group and not in the placebo group. However, in spite of decrease in the antibody titer, there was no statistically significant difference in pregnancy outcome between the two groups.

The efficacy of steroid treatment, if any, must be judged against the potential adverse side effects. The steroid therapy could cause several side effects [55]. The potential adverse effects and lack of effectiveness in many cases have decreased the enthusiasm for use of steroids for treatment of immunologic infertility. As an alternative, cyclosporine was tested in a cohort of men with ASA. After treatment, a pregnancy rate of 33 % was observed [9]. Since this study did not have placebo controls, no definite conclusions can be drawn.

16.2.2 Assisted Reproductive Technologies (ART)

Recently ART have been used to treat ASA. Several studies have examined the use of intrauterine insemination (IUI), gamete intrafallopian transfer (GIFT), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) procedures for the treatment of immune infertility in men and women as discussed below.

16.2.2.1 IUI Procedure

IUI has been found to be useful for treatment of ASA-positive infertile men and women. Theoretically, it should circumvent problems related to sperm transport in the female genital tract especially sperm passage through the cervical canal/mucus. However, in women having ASA in the cervical mucus, pregnancy rates after IUI were identical to women who did not have ASA, if the male partner did not have ASA or male factor infertility [13]. In two other studies of female sperm immunity, IUI treatment did not increase the pregnancy rates per couple or per cycle [23, 37]. However, the pregnancy outcome significantly improved after including the ovarian hyperstimulation treatment along with IUI.

IUI also has been found to enhance pregnancy rates in some cases of ASA-positive infertile men. A 56 % pregnancy rate has been reported after IUI procedure in ASA-positive infertile men, who had a poor postcoital test, compared with an 83 % pregnancy rate in ASA-negative infertile men, who also had a poor postcoital test [12]. In another study, after IUI, the pregnancy outcomes in 19 couples having male immune infertility were compared with 86 couples having other diagnoses. No pregnancy was seen in 110 IUIs in the ASA-positive group (0 %) versus a 26 % pregnancy rate per couple and 5.6 % cycle fecundity in the control group [21]. From the Cleveland Clinic Foundation, Agarwal compared 42 ASA-positive couples with 117 ASA-negative infertile couples who were treated with sperm washing and IUI over a 2-year period [5]. There were 15 pregnancies in the ASA-positive group compared with 37 for the entire group.

Another study compared IUI with oral steroid therapy [34]. This study included 46 couples in which the male partner had ASA. The immune infertile men either received 20 mg/day of prednisolone for days 1–10 followed by 5 mg/day for days 11 and 12 of the cycle and timed intercourse or underwent IUI with no steroid

treatment for three cycles. The couple was switched to the other group if not pregnant. The pregnancy rate before switching for the IUI group was 16.7 % and for the steroid group was 0 %. After switching, one more pregnancy occurred in the IUI group and one in the steroid group. This study concluded that IUI is better than low-dose steroid therapy for treating male immune infertility.

It is not clear, theoretically speaking, how IUI can circumvent male immune infertility. Washing the sperm in the incubation medium should not elute the antibodies bound to the sperm surface proteins, unless: (a) these antibodies are directed against the adsorbed seminal plasma proteins that are shed off during capacitation/acrosome reaction, (b) the antibodies are of low binding affinity, which does not seem to be the case in immune infertility, and/or (c) the swim-up sperm used for IUI are not coated with antibodies like non-swim-up sperm, which also seems highly unlikely.

There are mixed reports on simple sperm washing on ASA elution from various laboratories. Adeghe [2] found that washing decreased IgG bound on the sperm surface. Another group [58] did not find the similar positive effects, nor did Haas and associates [26], even after subjecting the sperm to multiple washings. Antibodies were also not reduced by passing sperm through percoll gradient [6].

16.2.2.2 GIFT Procedure

In GIFT procedure, sperm and eggs are mixed in vitro and then transferred to the fallopian tubes for fertilization. Theoretically speaking, there is not a strong rationale to how it will help either the ASA-positive infertile men or women. Nevertheless, in one study, GIFT was performed in 16 immune infertile couples. This group achieved pregnancy rates of 43 % per couple and 24 % per cycle [57]. This study did not include any control group, and the pregnancy rates are comparable to those that are reported after GIFT in patients having other etiologies.

16.2.2.3 IVF Procedure

Several studies have shown decreased rates of oocyte fertilization in IVF in immune infertile patients [30]. An inverse relationship between ASA titers and fertilization rates has been reported [19]. In a study, 33 ASA-positive infertile couples were subjected to 47 IVF cycles [32]. The couples with high ASA titers had lower fertilization rates than those with lower ASA titers. In contrast, there are also studies that found fertilization to be identical in ASA-positive and ASA-negative population [17]. Interestingly, there are also studies reporting increased rates of IVF outcome including implantation and pregnancy rates in ASA-positive infertile women compared to women with tubal factor infertility [16]. In IVF procedure, generally albumin instead of female partner's serum is used as a protein source in the insemination medium that circumvents the antibodies if present in the female partner. Thus, theoretically speaking, IVF can take care of female but not male immune infertility. Indeed, fertilization and pregnancies have been achieved using oocytes from ASA-positive infertile women where the men had normal semen analysis and were free of ASA [1, 62]. In ASA-positive infertile men, both the class/subclass specificity and subcellular localization of the antibodies on sperm

have been correlated with various degrees of fertilization failure rates in IVF [54]. ASA that bind to the sperm head may decrease fertilization more than ASA bound to midpiece or tail regions of the sperm cell. In the IVF procedures involving 21 immune infertile couples, it was found that the couples that had ASA bound to the head region of the sperm cell showed more fertilization failure than those having ASA bound to the tail region [62]. Yeh and associates [59] reported that IgA significantly reduces fertilization rates in IVF procedure only when it was associated with IgM and was present on the sperm head. Equality of embryo obtained after IVF using sperm from ASA-positive men is generally poor compared to sperm from ASA-negative men [33, 36].

16.2.2.4 ICSI Procedure

IVF with ICSI have become a routine and widely acceptable procedure in the clinics. In ICSI procedure, a single sperm is injected into the cytoplasm of the oocyte. ICSI has been tried using sperm of ASA-positive infertile men. Two of these studies are worth mentioning here. One study subjected 29 infertile ASA-positive couples to ICSI; 22 of them were tested before in IVF procedure and had poor fertilization rate (6 %) [33]. After ICSI, the fertilization (79 %) and cleavage (89 %) rates in the ASA-positive group were similar to those (68 % and 93 %, respectively) in the ASA-negative group. Surprisingly, 46 % of the pregnancies in the ASA-positive group ended in spontaneous pregnancy loss compared with none in the ASA-negative group. In contrast, another study did not demonstrate any difference in pregnancy rates (30 %) between the ASA-positive and ASA-negative group undergoing ICSI procedure [42].

Recently, meta-analysis was performed to obtain an odds ratio (OR) for the effect of ASA on pregnancy rates using IVF or ICSI [61]. This study analyzed 16 studies (10 IVF and 6 ICSI). The meta-analysis revealed that the combined OR for failure to achieve a pregnancy using IVF or ICSI in the presence of positive semen ASA was 1.22 (95 % Cl: 0.84, 1.77) and 1.00 (95 % Cl: 0.72, 1.38), respectively. The overall (IVF and ICSI) combined OR was 1.08 (95 % Cl: 0.85, 1.38). The meta-analysis indicated that semen ASA are not related to pregnancy rates after IVF or ICSI. However, all these studies ASA were sperm-reactive immunoglobulins, rather than fertility antigens-related antibodies [14, 53].

16.2.2.5 Postfertilization Effects of ASA

Some antisperm antibodies can have deleterious postfertilization effects on developing preimplantation embryos [3, 4, 38, 44]. ASA can affect early embryonic development if: (a) an oocyte is fertilized with a sperm cell, which carries these specific antibodies into the ooplasm, and/or (b) these antibodies are cross-reactive with the antigens present on the developing embryos. Some of these antigens and antibodies have been characterized, and the cDNA encoding for a few of these antigens has also been cloned and sequenced [29]. Using the ICSI procedure in immunoinfertile men, one can achieve higher fertilization rates than using the IVF procedure; however, the fertilized zygotes show higher degeneration and mortality and decreased embryonic development.

16.2.3 Laboratory Techniques

Several innovative laboratory techniques have been investigated and can broadly be classified into two categories: (1) methods that prevent binding of ASA to sperm or elute the bound ASA from sperm surface and (2) methods that separate ASA-free sperm from ASA-coated sperm. Although these methods have been explored extensively, due to conflicting findings, these techniques have not been accepted as the methods for treatment in the clinics. Some of these reports and their findings are discussed below.

It was erroneously thought that ASA bind to sperm during and/or just after ejaculation and the antibodies are mostly present in the secretions of prostate and seminal vesicles. Based upon this notion, the antibodies and the sperm are present and ejaculated in different fractions of the semen. To avoid binding of antibodies to sperm, splitting the semen into various fractions during ejaculation was attempted in various laboratories. However, it has been proven ineffective in decreasing ASA binding to sperm [35]. Collection of semen into insemination medium containing high concentrations of fetal cord/maternal serum has also been investigated to examine if it would decrease the antibody binding to sperm. Two studies [11, 18] observed that semen collection into serum-supplemented medium results in increased fertilization rates in IVF procedure, and one of these studies also showed an increase in pregnancy rates. We conducted a study to investigate at which site of the male genital tract the antibodies percolate from serum to bind to sperm [49]. I^{125} labeled antibodies to sperm-specific FA-1 antigen were injected intravenously into male mice. The results indicate that the antibodies preferentially transude into epididymis (especially corpus or caudal regions) and vas deferens to bind to sperm cells and not into testes. These findings indicate that in men ASA bind to sperm before ejaculation via transudation through epididymis, vas deferens, and probably rete-testis.

The immunomagnetic separation technique has been tried to separate the antibodies bound on the sperm surface [20]. The sperm with antibodies are tagged with anti-immunoglobulin antibodies coupled to magnetic microspheres, and then magnetic field is applied. However, limited success in isolating sufficient number of ASA-free sperm of good motility makes this procedure theoretically interesting but clinically an unacceptable procedure.

Bronson suggested that protease treatment may be utilized to destroy antibodies on the sperm surface [10]. Kutteh and associates reported that IgA1 protease treatment was effective in reducing IgA on sperm [31]. In another study, incubation of sperm with chymotrypsin before IUI resulted in a 25 % cycle fecundity versus 3 % in controls [8]. However, this needs to be examined whether or not the treatment with proteolytic enzymes affect proteins especially the oocyte binding receptors present on the sperm surface [28].

The use of immunobeads has been suggested as a treatment to remove the sperm-bound antibodies. It has been reported that simple incubation of ASA-positive sperm from immune infertile men with immunobeads results in a time-dependent

decrease in antibody concentration on sperm surface [22] and even enhanced pregnancies [24]. The explanation that the antibodies are removed from the sperm surface after incubation with immunobeads is not widely accepted, and it is generally believed that the immunobeads just select ASA-positive sperm, leaving ASA-free sperm. Theoretically, there is not a strong rationale why and how incubation with immunobeads should elute antibodies from sperm surface.

16.2.4 Novel Recent Perspectives Using Defined Sperm Antigens

Several sperm antigens have been defined from various laboratories that may be involved in fertilization and fertility [45]. Our laboratory showed that one of these, namely, fertilization antigen-1 (FA-1), is an exciting molecule because it is involved in human immune infertility and thus will be discussed here.

16.2.4.1 Immunoelution of Antibodies with FA-1 Antigen

FA-1 antigen is a well-defined novel sperm-specific surface molecule that is evolutionarily conserved on sperm of various mammalian species including humans [50]. Antibodies to FA-1 antigen inhibit human sperm-zona interaction and also block human sperm capacitation/acrosome reaction by inhibiting tyrosine phosphorylation [47, 52]. The cDNA encoding for mouse FA-1 and human FA-1 have been cloned and sequenced [49, 60], and vaccination of female mice with recombinant FA-1 antigen causes a long-term reversible contraception by raising sperm-specific immune response [48].

FA-1 antigen is involved in human immune infertility in both men and women. The antibodies are found in sera as circulating antibodies and also locally in genital tract secretions, such as seminal plasma of men and cervical mucus and vaginal secretions of women [39, 43]. The lymphocytes from immunoinfertile but not fertile men and women are sensitized against FA-1 antigen and proliferate on incubation with the antigen in vitro [51]. The presence of these antibodies inhibits fertilization in IVF procedure. The involvement of FA-1 antigen in human involuntary immune infertility has been confirmed in several laboratories by leading investigators working in the field of antisperm antibodies. Based upon these findings, a clinical trial was conducted at the University of Michigan Medical School to determine whether immunoadsorption with the human sperm FA-1 antigen would remove autoantibodies from the surface of sperm cells of immunoinfertile men and thus increasing their fertilizing capacity [41]. Adsorption with FA-1 antigen increased immunobead-free swimming sperm on an average of 50 % and 76 % for IgA and IgG antisperm antibodies, respectively. The acrosome reaction rates increased significantly and showed improvement in 78 % of the sperm samples after FA-1 adsorption. The IUI of FA-1-treated antibody-free sperm resulted in normal pregnancies and healthy babies, indicating that the antigen treatment does not have a deleterious effect on implantation, and embryonic and fetal development. This study needs to be extended to a

larger number of ASA-positive infertile men and constitutes an exciting therapeutic modality using well-defined sperm antigens.

Conclusion

In conclusion, although various methods have been tried, none have provided a satisfactory means of treating immune infertility. Almost all methods have yielded contradictory results, with the findings of one clinic reporting a positive outcome and another contradicting it. This may be due to how immune infertility was defined in one clinic versus another. As discussed earlier, any immunoglobulin that binds to sperm should not be defined as "antisperm" antibody unless it has a functional significance. This, along with the kinetics, valency, titer, class/subclass, and circulating/local nature of the immunoglobulins, plays an important role in defining the significance of antisperm antibodies in the immune infertility. The antisperm antibodies present in a female partner may be bypassed by assisted reproductive technologies such as IVF/ICSI procedures. However, embryos obtained after IVF are of poor quality, and there are more spontaneous abortions/miscarriages after ICSI using sperm from ASA-positive men in several studies. The sperm-bound antibodies present in a male partner are difficult to remove even by using invasive and expensive reproductive technologies. The antibodies have to be of low affinity to elute them from the sperm surface by simple washing techniques. The developing knowledge of local immunity and sperm antigens that have a role in fertility will help to better define immune infertility and develop better methods for treatment. The animal models immunized with defined sperm antigens will help in elucidating the mechanisms involved in physiology and pathophysiology of immune infertility and may assist to solve the controversy and confusion regarding the significance of antisperm antibodies in infertility and in the development of novel treatment modalities.

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Part IV

Immune Contraception

Antisperm Contraceptive Vaccine

17

Rajesh K. Naz

Abstract

Contraceptive vaccines (CV) may provide a viable and valuable alternative that could fulfill most, if not all, of the properties of an ideal contraceptive. The molecules for CV development either target gamete production [luteinizing hormone-releasing hormone (LHRH)/GnRH, FSH], gamete function [sperm antigens and oocytes zona pellucida (ZP)], or gamete outcome (LIF and HCG). Sperm cell is an exciting target. Several antigens including Izumo, which are specifically expressed in sperm cell, have been/are actively investigated for CV development. Vaccines based on native/recombinant sperm proteins, synthetic peptides, and naked DNA have shown reversible contraceptive effects without any serious side effects in various species of animals. Presently, no sperm vaccine has undergone clinical trial in humans. As with any vaccine, the progress has been restricted due to variability of the immune response after active immunization to attain sufficiently high antibody titers, especially in the genital tract in all the vaccinated individuals, and uncertainty regarding how long the bioeffective antibodies will remain in circulation. It is envisaged that these concerns may be obliterated by the passive immunization approach using the performed antibodies. The antibody therapies are successful against various infectious diseases, both in animals and humans. Phage display technology has been widely used to obtain a variety of engineered antibodies, including single chain variable fragment (scFv) antibodies. Using this technology, recently our laboratory has isolated, produced, and characterized fully functional human scFv antibodies against specific human sperm antigens. These human antibodies are being examined for their utility as novel immunocontraceptives.

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17.1 Introduction

There are several methods available for contraception [18, 52, 53, 75, 84]. In spite of the availability of these contraceptive methods, the world population has exceeded 7.28 billion and will grow by one billion every ~11 years at the current rate [88]. Also, unintended pregnancies continue to impose a major public health issue. In the USA alone, half the pregnancies are unintended which result in over one million elective abortions each year [16, 20]. In over half of these unintended pregnancies, the women were using some method of contraception. An estimated 80 million women have unintended/unwanted pregnancies worldwide annually, of which 45 million are electively aborted [87]. Thus, there is an urgent need for a better method of contraception that is reversible, nonsteroidal, nonbarrier, and intercourse-independent. An ideal contraceptive method should be safe, inexpensive, have a prolonged duration of action, be rapidly reversible, require infrequent administration, and be easily accessible [52–54]. A contraceptive vaccine (CV) can fulfill most of these properties of an ideal contraceptive [55]. The development of a vaccine for contraception is an exciting proposition because the developed and most of the developing nations have an infrastructure for vaccination.

Various targets have been explored for the development of a CV. These can broadly fall in three categories: vaccines inhibiting gamete production [gonadotropin releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone, (LH)], gamete function [zona pellucida (ZP) proteins and sperm antigens] or gamete outcome [leukemia inhibitory factor (LIF) and human chorionic gonadotropin (hCG)] [30, 31, 38, 59]. GnRH based vaccines are effective in several species and can be used for both males and females. However, they are not acceptable for human use because they affect sex steroids causing impotency. They have been taken over by pharmaceutical companies for fertility control in domestic pets, farm and wild animals, and for noncontraceptive purposes such as prostatic hypertrophy and carcinoma [14, 52, 76]. FSH-based vaccines have shown to inhibit spermatogenesis in males of several species and can potentially provide a male contraceptive. However, they cause oligospermia rather than azoospermia [37]. LH/LH receptor-based vaccines are effective in both males and females [71, 81]. Conversely, they affect sex steroids, so they are not acceptable for human use. Thus, disadvantages of CVs targeting gamete production are: they affect sex steroids and/or show only a partial effect in reducing fertility. CVs targeting gamete functions are better choices. Vaccines based on ZP proteins are quite efficacious in producing contraceptive effects [1, 54]. However, they induce oophoritis which affects sex steroids [83]. They have been successfully used for controlling wild and zoo animals such as deer, horses, elephants, and dogs [26, 54]. Sperm antigens constitute a promising and exciting target for CVs and at the present time no disadvantages are known. Vaccines targeting gamete outcome primarily focus on the LIF and hCG molecules. The vaccination of female mice with LIF causes contraceptive effect [30, 31]. The hCG-based vaccines have undergone phase I and II clinical trials in women and demonstrated efficacy and lack of immunopathology [79]. However, there is variability of immune response among vaccinated women. The present entry will focus on the development of contraceptive vaccines based on sperm

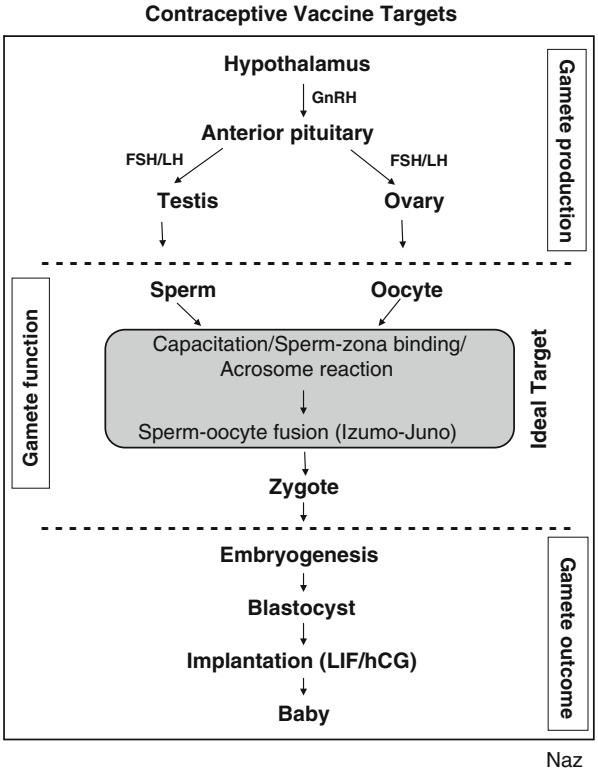

Fig. 17.1 Schematic diagram showing various targets which are being investigated for contraceptive vaccine development. These can be divided broadly into three categories: targeting Gamete Production, Gamete Function, and Gamete Outcome. *FSH* Follicle stimulating hormone, *GnRH* Gonadotropin releasing hormone, *hCG* Human chorionic gonadotropin, *LH* Luteinizing hormone, *LIF* Leukemia inhibitory factor

antigens. A PubMed search (1971–2015) was performed using keywords: "contraceptive vaccines," "sperm antigens," "antisperm antibodies," and "immunocontraception." All of the articles were read and also the articles referenced in these publications were thoroughly examined.

17.2 Discussion

There is a strong rationale for the development of sperm based vaccine. Sperm are immunogenic in both males and females. Immunization of several species of animals and humans with sperm/testis preparations develop antisperm antibodies (ASA) leading to infertility [2, 33]. In 1932, Baskin injected 20 fertile women, who had at least one prior pregnancy, with their husband's sperm [6]. These women developed ASA and no conception was reported for up to 1 year of observation. A US patent

was issued for this spermatoxic vaccine in 1937 (US patent number 2103240). Over 70% of men develop ASA after vasectomy [32] and there is a limited success in the regain of fertility even after successful surgical re-anastomosis in vasovasostomy attributed to the presence of ASA [73]. Up to 2–30% cases of infertility may be associated with the presence of ASA in the male or female partner of an infertile couple [63]. These ASA are causative factors of infertility since their disappearance causes regain of fertility [8]. These findings provide evidence that spermatozoa can generate an immune response in both men and women that can lead to a contraceptive state. However, the whole sperm cannot be used for the development of contraceptive vaccine because there are numerous antigens present on the surface and internally in sperm that are shared with somatic cells. Thus, the immunization with the whole sperm can cause immunopathological consequences in other tissues and organs. The utility of a sperm antigen is contingent upon sperm-specific expression, surface expression accessible to antibody binding, and its role in fertilization/fertility. Also, the sperm antigen, alone or after conjugation with appropriate carrier protein, should be able to raise high titer and long lasting antibody response in circulation and locally in the genital tract. If it is also involved in human immune infertility then it is an especially attractive candidate. An ideal sperm antigen for immunocontraception should have tissue-specific expression on the sperm surface and be involved in sperm-zona pellucida binding and human immune infertility (Fig. 17.1).

17.2.1 Sperm Antigens

Various methodologies of genomics and proteomics have been used to delineate sperm antigens that have a role in fertilization/fertility and can be used for the contraceptive vaccine development. Recently, using gene knockout technology, >100 novel testis/sperm genes/proteins have been identified that have a crucial role in various aspects of fertility [50, 51]. Some of these gene knockouts cause a defect in testis development and endocrine milieu, some in spermatogenesis, some in mating behavior, some in sperm structure/function/motility, and others in fertilization. The majority of these knockouts also demonstrated an effect on nonreproductive organs concomitant with an effect on fertility. We did an extensive database analysis of these genes/proteins to examine how many of these have the characteristics required for the contraceptive vaccine development as discussed above. The knockouts of only a few genes/proteins induced a specific effect on fertility without a serious side effect. The majority of them are not expressed on the sperm surface, and thus not amenable to antibody binding. Although these can provide ideal targets for pharmacological inhibition for contraception, they are not suitable for contraceptive vaccine development. The gene knockout technology is a powerful approach to identify suitable novel targets and the list of gene knockout mice is ever growing.

17.2.1.1 Molecules Involved in Sperm-Oocyte Membrane Fusion

The molecules involved in sperm-oocyte membrane fusion have been actively examined for some time. Various candidates have been proposed that include DE, CD46, equatorin Sperad, and $SAMP_{32}$ [78]. CD46 gene knockout mice do not show a defective sperm-oocyte fusion [23]. ADAM family proteins have drawn considerable attention because they have a putative fusion peptide ($ADAM_1$) and disintegrin domains ($ADAM_2$ and $ADAM_3$) [60]. However, $ADAM_1$, $ADAM_2$, and $ADAM_3$ gene knockout mice did not show a defect in sperm-oocyte membrane fusion, but show impairment in sperm-zona binding [10] CD9 present on the oocyte plasma membrane seems to be essential for fusion with the sperm cell [29]. It was thought that integrins α6 and β_1 present on sperm are involved in binding to oocyte CD_9 for sperm-oocyte fusion [3]. However, gene knockout of these molecules did not inhibit fertility [19].

Recently, a gene knockout was reported that is very interesting. The gene knockout mice of a sperm gene, designated as *Izumo1*, are healthy but all males are sterile [23, 24]. *Izumo1* is named after a Japanese shrine dedicated to marriage. Male mice produce normal looking sperm that bind to and penetrate the zona pellucida but are incapable of fusing with the oocyte membrane. Izumo1 protein is a type-1, 377 amino acid long, transmembrane protein that belongs to the immunoglobulin super family (IgSF) and contains one extracellular immunoglobulin domain and one N-terminal domain. Izumo1 contains a glycosylation site that protects Izumo1 from fragmentation in the epididymis. This site, however, is not essential for normal function [25]. Mouse Izumo1 has a molecular identity of 56 kD and human Izumo1 is of 37 kD.

More recently, paralogues of Izumo1, the Izumo2, 3, and 4, have also been discovered in mammals [13]. Izumo2 and 3, along with 1, are testes-specific transmembrane proteins, while 4 is expressed in the testes and other nonreproductive tissues. They all have significant homology in the N-terminal domain [13]. All four Izumo1 genes have eight conserved cysteine residues within 144 amino acids with four alpha helices that exist between the residues. All *Izumo1* genes, with the exception of Izumo3, are conserved across humans, mice, rats, bulls, dogs, and some species of monkeys [80]. Izumo1 protein can only be detected after the acrosome reaction. Recently, it was found that Izumo1 binds to Folr4, a folate receptor, on oocytes, designated as Juno [7].

Our laboratory was first to demonstrate that immunization with Izumo1 peptides causes long-term and reversible contraceptive effect in female mice [42]. We further demonstrated for the first time ever that immune infertile women and men have antibodies to Izumo1 [11, 44]. Subsequently, at least four studies have been published demonstrating the contraceptive effect after vaccination with Izumo1 [4, 73, 85, 86]. Izumo1 is an excellent candidate for antisperm contraceptive vaccine development.

17.2.1.2 Molecules Involved in Sperm-Zona Pellucida Interaction and Function

Several sperm genes/antigens have been delineated, cloned, and sequenced. Although, the antibodies to some of these antigens affect sperm function/fertilization in vitro, the immunization with only a few of them have shown to cause a contraceptive effect in vivo in any animal model. Notable among these are lactate dehydrogenase-C_4 (LDH-C_4) [15], PH-20 [67], SP-17 [28], SP-10 [21], FA-1 [34, 55, 56, 94], and YLP_{12} [45, 58]. Most of these active immunization studies, except related to PH-20 antigen, were carried out in the mouse model. At the present time, no sperm antigen has undergone Phase I/II clinical trial in humans. Two studies have examined the effect of sperm antigen vaccination in nonhuman primate model. One study reported reduced fertility of female baboons after immunization with LDH-C_4 [62]. However, a study by another group found no effect on fertility in female monkeys after vaccination with LDH-C_4 [82]. The reason for this discrepancy is not clear at the present time. Male monkeys were immunized with an epididymal protein, designated as epididymal protein inhibitor (Eppin) [64]. After immunization, 78 % monkeys that developed high anti-Eppin antibody titers became infertile, and 71 % of them recovered fertility after immunization was stopped. To maintain high antibody titers, booster injections with Freund's adjuvant have to be given every 3 weeks for almost the whole duration of study of 691 days. The potential immunopathological effects of immunization were not examined. This interesting study indicates that antisperm CV can also be developed for men.

Antibodies to several sperm antigens inhibit sperm-oocyte interaction/fertilization in vitro. However, the active immunization with many of these molecules does not inhibit fertility in vivo. Also, the gene knockouts of many of these molecules do not inhibit fertility. For example, although antibodies to fertilin/PH-30 inhibit fertilization in vitro [78], active immunization with fertilin/PH-30 does not affect fertility in vivo [17]. Similarly, although antibodies to sperm integrins $\alpha 6$ and β_1 inhibit sperm-oocyte fusion in vitro [3], the gene knockouts of these molecules do not affect fertility in vivo [19]. These differences in in vitro and in vivo effects may be because: (a) the class/subclass, valency, affinity, and kinetics of the antibodies generated in vivo and in vitro vary; (b) antibodies have to be present in time and space to bind to the appropriate molecules; and/or (c) there may be redundancy of some of these molecules.

Another problem that the sperm vaccinologists are facing at the present time is to find an appropriate animal model to examine the efficacy of a sperm antigen. The most used animal model is the mouse. However, up until now, no one has reported 100 % block in fertility after immunization with any single antigen in the mouse model. Even immunizations with the whole sperm or their solubilized preparations do not cause a total block in fertility in mice, male or female. The maximum reduction in fertility after immunization with any antigen/sperm preparation is up to 70–75 %. Very few, if any, knockout of a single gene has made mice totally infertile. The recently reported *Izumo* gene knockout did make the male mice almost totally infertile [23]. It needs to be seen whether or not the 70–75 % reduction in fertility in the mouse model translates to 100 % reduction in humans. The female mouse

ovulates several eggs every cycle and a woman ovulates mostly one egg every cycle. So there are differences between the man and mouse. Over 70–75 % reduction in fertility in the mouse model may translate to 100 % block in humans. Maybe that is the inherent nature of the *mouse* model in which it is difficult to make mice completely infertile. However, after active immunization or deleting a single gene, one does find a few mice that are totally infertile. Vaccination with multiple sperm epitopes (peptide and DNA) enhances the efficacy but still does not cause 100 % contraceptive effect [40–42].

The phage display technology is a novel and innovative tool for delineating specific binding peptide sequences to various ligands and antibodies. It was first reported by George Smith in 1985 [77]. This technology is being widely used in several laboratories at the present time. The peptide sequences are presented on the surface of filamentous phage to examine their interaction with specific ligands/antibodies. The DNA encoding any peptide sequence gets incorporated into genome of the phage capsid protein and the encoded peptide is expressed and displayed on phage surface as fusion protein. We used this technology and the 2-D gel electrophoresis/matrix-assisted laser desorption mass spectrometry (MALDI MS) to delineate the peptide sequences that are involved in human immunoinfertility [5, 39, 66, 72] and the peptide sequences present in human sperm cell that are involved in binding to human zona pellucida [58].

Besides antibodies, various cytokines can also affect sperm function and fertility either positively or negatively. For example, interferon-gamma and tumor necrosis factor-alpha can negatively affect sperm motility and function [47] and interleukin-6 can enhance sperm capacitation and acrosome reaction [46]. A sperm cell has receptors for many of these cytokines such as interferon-gamma and interferon-alpha [57]. These factors are present in the seminal plasma and the levels are modulated to various degrees in infertility. Immunization with the whole sperm preparation or specific sperm antigens can raise many cytokines besides antibodies that can affect sperm function [48].

17.2.2 Passive Immunization and scFv Antibodies

The progress in the development of contraceptive vaccines against various targets including sperm has been hampered by the following facts: (1) Delineating the appropriate fertility-related antigen(s), (2) variability of the immune response among the vaccinated individuals, (3) attainment and maintenance of high titer of antibodies for bioefficacy, (4) time lag to achieve reasonably good antibody titers after the first injection, and (5) uncertainty regarding how long the antibody titer will remain in the circulation to exercise the contraceptive effects. The last four concerns are associated with the active immunization studies involving contraceptive vaccines. It is envisaged that these four concerns may be taken care of using the passive immunization approach [49]. The passive immunization approach has been successful for protection against various immunological and infectious diseases [9, 91].

Several of these antibodies have become treatment modalities in the clinics [9, 12, 69]. Phage display technology has been widely used to obtain a variety of engineered antibodies, including single chain variable fragments (scFv) antibodies against several antigens [65, 68, 89, 92, 93]. ScFv is an antibody fragment that plays a major role in the antigen-binding activity, and is composed of variable heavy (VH) and variable light (VL) chains connected by a peptide linker. The most widely used peptide linker is a repeat of a 15-residue sequence of glycine and serine $(Gly_4Ser)3$. The affinity and stability of the scFv antibodies produced in bacteria are comparable with those of the native antibodies and are maintained by a strong disulfide bond. ScFv antibodies can be produced on a large scale using specially modified bacterial hosts and have an advantage over the whole immunoglobulin (Ig) molecule. ScFv antibodies lack the Fc portion that eliminates unwanted secondary effects associated with Fc, and due to its small size can be easily absorbed into tissues and gene manipulated [90]. The mouse monoclonal antibody can elicit strong anti-mouse antibody reaction, chimeric antibody can cause antichimeric response, and xenogenic complementarity-determining regions (CDRs) of humanized antibodies can also evoke an anti-idiotypic response, when injected into humans [27, 36, 74]. Antibodies must be of human origin to be used in humans. The potential poor immunogenicity and toxicity of an antigen, and ethical issues, limit immunizing humans to obtain human antibodies. However, the phage display technology can be used to obtain these antibodies against target antigens if they exist involuntarily in humans, such as ASA in immunoinfertile men and women, and vasectomized men.

We recently did a study to obtain fertility-related scFv human antibodies that can be used for CV immunoinfertility. Peripheral blood leukocytes (PBL) were obtained from antisperm antibody-positive immunoinfertile and vasectomized men, activated with human sperm antigens in vitro, and cDNA was prepared from their RNA and PCR-amplified using several primers based on all the available variable regions of VH and VL chains [44, 70]. The amplified VH and VL chains were ligated and the scFv repertoire was cloned into pCANTAB5E vector to create a human scFv antibody library. Panning of the library against specific antigens yielded several clones, and the four strongest reactive (designated as AFA-1, FAB-7, YLP20, and AS16) were selected for further analysis. These clones were shown to have novel sequences with unique complementarity determining regions (CDRs) when a search was performed in the immunogenetic database. ScFv antibodies were expressed, purified, and analyzed for human sperm reactivity and effects on human sperm function. AFA-1 and FAB-7 scFv antibodies, having IgG3 heavy and IgK3 light chains, recognized human sperm FA-1 antigen, which is involved in human sperm function and fertilization. The third, YLP20 scFv antibody, reacted with a sperm protein of 48 ± 5 kD, which contains the dodecamer sequence, YLPVGGLRIGG. The fourth antibody, AS16, reacted with a 18 kD sperm protein (major band) and was found to be a human homolog of the mouse monoclonal recombinant antisperm antibody (RASA) [61]. These antibodies inhibited human sperm capacitation/acrosome reaction in a concentration-dependent manner. This is the first study to report the use of phage display technology to obtain human

antisperm scFv antibodies of defined antigen specificities from immune infertile/vasectomized men. These antibodies will find clinical applications in the development of novel immunocontraceptives and specific diagnostics for immunoinfertility in humans. The contraceptive effect of these antibodies in vivo is currently being investigated.

Conclusion

In conclusion, development of CV targeting sperm is an exciting proposition, and may provide a valuable alternative to the presently available methods. As limitation with other vaccines, the progress in CV development has been delayed due to variability of immune response after vaccination. The multi-epitope vaccines may enhance the efficacy and obliterate the concern of the interindividual variability of the response. Also, this concern may be addressed by the passive immunization approach using preformed human antibodies. Several antibodies are being tried as therapeutic agents. At the present time, >100 antibodies are in clinical trials and ~20 FDA-approved monoclonal antibodies are available in the market for various clinical conditions, including cancer and infectious diseases. Over 80 % of these antibodies are genetically engineered [22, 35]. The scFv antibodies that we have synthesized in vitro using cDNAs from antisperm antibody-positive immune infertile and vasectomized men may provide useful, once-a-month immunocontraceptive. These human antibodies are sperm-specific and inhibit sperm function in vitro. Their immunocontraceptive potential in vivo is presently being investigated. In a WHO meeting on contraception in Geneva, Switzerland, November 13–14, 2012, development of CVs was enlisted as one of the highest priorities in the contraceptive field. An International Task Force has been set for CV development [43]. This has heightened the interest in immunocontraception.

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Immune Contraception in Wildlife Animals

18

Katarina Jewgenow

Abstract

Fertility control by immune contraception is suggested to offer a long-term, effective and humane approach for reproduction control in captive animals as well as to reduce free-ranging wildlife populations. The chapter presents a summary of target species for reproduction control by immunization, the available vaccines for wildlife animals and prospective delivery methods. Based on published literature, health issues as well as behavioural changes and population level effects of contraception in wildlife animals are discussed.

18.1 Immune Contraception in Wildlife Species

18.1.1 Captive Population

Not only is there a need of reproduction control in captive animals, but also exotic animals in captivity serve as models for the establishment of contraceptive approaches for wildlife.

Because improved animal husbandry and veterinary care has led to a low adult mortality and an increase in longevity, especially in large ungulates and carnivores and even species managed in breeding programs, high quality enclosures are consequently overcrowded. This results in the demand for population control. Prevention of offspring (sterilization or contraception) is an alternative to the elimination of captive animals (euthanasia or transfer to other institutions) [1].

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In addition, zoos usually do not share the same limitations as wildlife managers, thus making it possible to apply a wider variety of contraceptive techniques, or even allow research on contraceptive approaches which would not be possible on wildlife animals. Thus, not surprisingly the first anti-fertility vaccinations were applied to captive wildlife animals [2, 3].

Twenty-five years after first attempts on immunocontraception in captive wildlife animals [4], a huge number of animals and species has been treated with a variety of contraception measures including contraceptive vaccines. Nevertheless, knowledge on efficacy and treatment protocols remains patchy. To meet this challenge, in North America, and also in Europe, a centralized database has been compiled to produce guidelines for a variety of species (www.egzac.org). The recommendation for captive animals (www.stlzoo.org/animals/scienceresearch/contraceptioncenter) lists two options of anti-fertility immunocontraception: vaccines against porcine zona pellucida proteins (PZP) and against the GnRH [5]. Both vaccines still require repeated injections and are limited to captive or small populations of free-ranging wild animals.

18.1.2 Wildlife Population

Reproductive control is important for the management of certain wildlife, and ironically also endangered species, particularly in the increasingly common situation in which the size and the nature of habitat is restricted by man's encroachment [6]. Additionally, some wildlife species have adapted successfully to changing environmental conditions and concentrated in large populations significantly impacting on their habitats or their prey species and served as reservoirs for infectious diseases, making them "pest species".

The challenge is to develop acceptable and sustainable methods to reduce and maintain populations of these animals and maintain them at levels that minimize their impact on environment. There is also a major need to control population sizes in non-indigenous animals and/or imported species such as the raccoon and American mink in Europe, fox in Australia. At the population management level with free-ranging species, the primary focus has been on wild horses, urban deer, bison, and African elephants [3].

18.1.2.1 Pest Species

Pest mammals have severe economic, environmental and social impacts throughout the world [7]. Methods of immune contraception are being investigated for small rodents, in particular for rabbits and house mice, and for overabundant marsupial species, like brushtail possum in New Zealand [8]. Most small rodent species are characterized by high reproductive rate. Therefore, fertility control need to be adapted to their reproduction strategy (r-strategy) which is characterized by a high rate of reproduction, high juvenile mortality, and strongly fluctuating population sizes within fluctuating habitats. Population models which were developed to predict possible outcomes of fertility control show that at least 80 % of females will need to be infertile and that this infertility will need to be permanent [9].

Virally vectored immunocontraception (VVIC) has been proposed as an economic way to achieve this [10, 11]. Although VVIC may have the advantage of self-regulating depending on the density of the target species [12], biological safety and regulatory concerns must be overcome in future before VVIC can be applied for field testing [7, 13, 14]. Therefore, specific bait-delivered fertility control is more likely to be used in the near future [15].

18.1.2.2 Alien Species

Alien species are any species that have spread beyond their natural range into new locations as a result of human activity. Invasive alien species are species that have some advantage over native species. These advantages are often enhanced when aliens move into ecological niches and thrive because outside their natural environments they are not held back by natural predators, parasites, disease, or competition in the way that native species are. Therefore, there is a major need to control population sizes in non-indigenous animals and/or imported species such as the grey squirrel [16] and several deer species in Europe [17], and fox in Australia [18]. In case of some alien species, a full eradication from their new habitats might be considered. The IUCN Invasive Species Specialist Group (www.issg.org) provides support for the control of invasive species through the Global Invasive Species Programme (GISP) (www.gisp.org). The expertise about eradication and control varies widely, with a wealth of experience available in a few areas, such as on islands (for review [19].

18.1.2.3 Overabundant Wildlife

Overabundance of wildlife often has nothing to do with biological carrying capacity, when population reduces the growth rate at which food resources are replenished. Wildlife is considered to be overabundant (ecological overabundance) when a population is so dense that it threatens the persistence of other species or when a population is so dense that it becomes unacceptable to humans (societal overabundance). In this respect, a number of wildlife species have become overabundant on a local or regional scale throughout the world. Traditionally overabundant wildlife is controlled by hunting and trapping, but this may be restricted or infeasible in parks and suburban areas. Application of wildlife fertility control is suggested for use in urban or suburban areas (e.g. deer species, wild boars) and in situations where immigration is limited and lethal control is restricted (e.g. elephants in national parks).

18.1.2.4 Human Wildlife Conflicts

Population control of wildlife is also considered in case of "human wildlife conflicts", which reflect any conflict in interaction between wild animals and people. It occurs when growing human populations overlap with established wildlife territory, creating reduction of resources or life to people and/or animals. The conflict takes many forms ranging from loss of life or injury to humans, and animals both wild and domesticated, to competition for scarce resources to loss and degradation of habitat. Ethical considerations regarding humane treatment of animals are shaping public attitudes toward acceptable methods of mitigating human-wildlife

conflicts [20]. Conflict management strategies usually compromise lethal control and translocation [21]. Recent management approaches also consider fertility control to reduce human-wildlife interactions [22].

18.1.2.5 Transmitters of Zoonoses

Zoonoses are infectious diseases that can be transmitted between humans and animals, both wild and domestic. Approximately 75 % of recently emerging infectious diseases that affect humans are diseases of animal origin, and approximately 60 % of all human pathogens are zoonotic. During the last 40 years, new epidemiological patterns have emerged as free-ranging wildlife have become progressively more involved in the epidemiology of both common and emerging infectious diseases of humans and domestic animals (for review [23]. This has been seen in rabies, bovine tuberculosis and more recently in wild-boar classical swine fever [24] or brucellosis [25].

Offensive lethal control, however, failed to control animals, like badgers, wild boar and foxes for tuberculosis, classical swine fever and rabies, respectively. Culling these species reduced their populations to a logarithmic part of their growth curve, such that any losses due to culling were very quickly replaced by individuals that normally would have died due to population density pressures. Consequently, immune contraception in combination with disease vaccination is being discussed as an alternative strategy to lethal control.

In particular, brucellosis in elks and bisons is an excellent example of an infectious disease present in wild populations that could potentially be managed through immune contraception because it is transmitted venereally or at parturition [25–27], thus directly connected to fertility.

18.2 Development of IC Vaccines for Wildlife Species

The challenges in the development and application of vaccine-based wildlife contraceptives are diverse and include differences in efficacy across species. Specific requirements for wildlife, both free-ranging and captive animal populations are defined by side-effects. For instance, a reduction of sexual/aggressive behaviour might be advantageous in zoo settings but not for free-ranging animals when the specific sexual behaviour, like herd hierarchy, should be retained. For wildlife animals, remote delivery is important to avoid stress of capturing and restraining animals for hand injection [5]. In addition, the uncertain reproductive status at treatment should also guarantee safety of vaccines during pregnancy. Beyond the constraints imposed by the public and a host of regulatory concerns, there exists a real limitation for funding of well-designed programs that apply this type of fertility control [3].

18.2.1 PZP

The most widely tested immune contraceptive vaccine for wildlife species is based on developing antibodies to zona pellucida (ZP), which surrounds the mammalian

egg. The PZP antigens are isolated from porcine ovaries obtained from slaughter house material. When PZP is injected into females others than pigs, the target species will produce antibodies against the antigen. These antibodies attach to the zona pellucida of ovulated eggs and cause steric hindrance which then blocks fertilization. Long-term application or hyperactive immune response, however, can cause ovarian failure and permanent sterilization.

The initial PZP vaccine was based with multiple-shot boostering [28]. Since then several technologies have been developed to achieve efficiency with a single immunization [29, 30].

PZP vaccines and species-specific ZP (native/recombinant, whole/individual ZP) components have been investigated in various animal species (wild, zoo, farm, and domestic) (for review: [5]). PZP has been shown to be effective in a wide variety of ungulates [4, 31–33], equines [34, 35], elephants [6, 36–38] and some carnivores [32, 39, 40]. Recently it was also investigated in marsupials [41, 42]. It was shown that that the fertility of grey kangaroo, brushtail possum and koalas can be compromised by immunization against ZP antigens, but immunization with bacterial recombinant brushtail possum ZP3 did not reduce fertility in the koala.

Despite a high individual variability in immune response observed [43], the biological efficacy of PZP depends on species-specific antigenicity and immunogenicity of porcine zona pellucida [44–46], and therefore must be validated before it can be applied for contraceptive population control in a particular species.

18.2.2 GnRH Vaccine

Vaccination against gonadotropin-releasing hormone (GnRH) entails the administration of a modified form of the GnRH hormone in order to stimulate the production of anti-GnRH antibodies, which bind and inactivate the endogenous GnRH. The hormone (GnRH) stimulates the pituitary to secret the gonadotropins LH and FSH. Thus, immunization against GnRH disrupts the reproductive axis by depression of gonadotropin production in order to inhibit follicle growth, ovulation or spermatogenesis. In addition, vaccination against GnRH is the most promising alternative to castration for reducing male aggressive behaviour in captive adult animals [47]. The molecular structure of GnRH is conserved between mammalian species. Therefore anti-GnRH vaccines are applied in a wide range of animals.

Studies on the use of GnRH vaccination for suppression of fertility, aggression and sexual behaviour show promising results in several domestic species, such as sheep [48], pigs [49], cattle [50] and horses [51]. In particular, for population control of feral cats [52] and street dogs [53], GnRH is discussed as an ideal contraceptive target because it regulates pituitary and gonadal hormone responses in both males and females, thus suppressing nuisance behaviours associated with sex hormones in addition to preventing pregnancy.

GnRH vaccination has also been suggested to be applied in fertility control of overabundant wildlife, such as wild boar [54], white-tailed [55] or black-tailed deer [56], white Alpine sheep [57], prairie dog [58], and ground [59], fox [60] and

tree [61] squirrels. Also for captive wildlife animals it is recommended and applied [62–64], in particular in connection to aggression and sexual behaviour suppression in males.

Suppression of steroid production by GnRH vaccines, however, is accompanied by effects which are characteristically for castration: changes in secondary sex characteristics, like antler growth in deer or the mane in lions, and therefore should be taken into account. In addition, safety trials should be performed to assure that non-reproductive function of the hypothalamus and pituitary gland are retained in treated animals.

18.2.3 Other Antigens for Immune Contraception

Most currently available targets for immune contraception interact at some point in the sequence of hormone synthesis or are involved in essential reproductive events, like ovulation, spermatogenesis, sperm or egg transport, or implantation [65]. New molecular technologies are applied to generate vast peptides libraries that are screened for their potential impact on fertility.

A variety of proteins derived from sperm and oocytes have been experimentally assessed for their contraceptive potential in wildlife species [18, 66]. In particular, sperm antigens are suggested to act as species-specific immunogens, an essential prerequisite for oral vaccines applied in free-ranging wildlife. In this respect, a promising strategy appears to be the construction of immunogens that include repeated peptides from proteins involved in fertilization [67–69]. Multi-epitope contraceptive vaccines and preformed engineered antibodies of defined specificity may eliminate concern related to inter-individual variability of the immune response [70].

Fertilization-related antigens were isolated from germ cell plasma membranes [71] or are identified from cDNA libraries of oocytes or sperm cells [72]. Also the identification epitope peptide by phage display was introduced to contraceptive vaccine development [73, 74].

Some of the sperm proteins already proved to be promising antigens for contraceptive vaccine include lactate dehydrogenase, protein hyaluronidase, Eppin or Catsper [75]. After identification of the specific sperm fusion protein Izumo [76] it is discussed as a potential contraceptive antigen as well [77].

Despite sperm-egg receptors, many other biological active components of reproduction are considered for contraceptive vaccines [78, 79], but none of them had been used yet for contraception in exotic species.

18.3 Delivery Methods

Fertility management is not yet a practical reality in wildlife management. Before it can be implemented it must meet the demands on risk assessment for health and behavioural effects in the target species, as well as any potential effects on non-target species [80]. Immune contraception in wildlife requires the acceptable and

safe application (e.g. humane use, environmental safety, target specificity) and knowledge of how to apply it strategically (e.g. where, when, how often, how intensively) [81].

The species-specific application can either be achieved through vaccine compound itself or the delivery method. Vaccine-developing strategy includes the identification of an anti-fertile antigen and the development of an effective vaccine composition including acceptable carriers, adjuvants and delivering systems [82]. In free-ranging wildlife population, a large-enough proportion of the population must be reached to suppress growth [83] still ensuring a safe, effective and efficient delivery.

Delivery can be oral, by implant or hand injection after capture, or by dart in unrestrained populations. The following delivery methods had been used or suggested for wildlife species.

18.3.1 Parenteral Immunization

Attempts to use anti-fertile vaccines to stop breeding in wildlife animals date back to late 1980. Initial studies showed that pregnancies could be prevented by multiple-shot vaccines [34] containing PZP and Freund's adjuvants. These were followed by studies showing that these vaccines could be remotely delivered effectively to free-ranging animals (horses, deer and elephants), but the requirement for repeated initial shots and annual boosters limited management application. During the following years the formulation of vaccines was improved steadily. Now vaccines are effective for several years with a single treatment [84–87].

Multiple booster treatment were replaced by microsphere particles or polymer-based controlled-released pellets [30, 88, 89] containing the antigen and the adjuvant. Immunogenicity of antigens was increased by tagging them to another protein, and implementation of different adjuvants [30, 90, 91]. The replacement of Freund's adjuvant was aimed to prevent local and systemic side effects described in several wildlife animals [92].

Beside the progress made in delivery procedure, the parenteral immunization requires an individual approach to the animal and is therefore limited to captive or small populations of free-ranging wild animals. In this respect, an individual-based rotational vaccination was suggested in long-lived, social species (e.g. wild elephant) to stretch the inter-calving interval for each individual, preventing exposing females to unlimited long-term immune contraception use [93]. It is also discussed to combine parenteral vaccination campaigns (e.g. for elimination of canine rabies) with immune contraception [94, 95] for population control.

18.3.2 Oral Delivery via Baits

Alternative contraceptive vaccines are actively being developed to enable large numbers of wild animals to be targeted by oral delivery of baits, when single

animal's treatment will be not effective or possible (pest or alien species). The distribution of baits and collecting remaining bait could be handled similarly to rodendicide baits [7]. It is proposed that vaccines which will be delivered orally by baits will stimulate a mucosal immune response to the foreign antigen(s). Such a vaccine requires a detailed understanding of reproductive-tract mucosal immunity in target species. Oral contraceptive vaccines under consideration include viral or bacterial vectors and microencapsulated antigens [18]. Although baits are increasingly used in wildlife management to deliver vaccines for disease control, a contraceptive oral vaccine is not available yet. In addition, safety requirements concern the transmissibility of the antigen in case of viral or bacterial vectors, the reversibility of the intervention within an individual animal and in animal populations, as well as the species specificity of the antigen used [96].

In particular for wild boar, bait-delivered contraceptive vaccines are increasingly advocated to assist the population management [97]. Ferretti et al. evaluated a Boar-Operated-System to deliver baits to wild boar in areas with a large community of potential non-target species [98].

18.3.3 Nasal Inoculation

Vaccination based nasal inoculation resulted in a high antibody response in mucosal tissues, including genital tracts [99], and were suggested to avoid a pathogenic T cell activation [100], a side effect causing autoimmune destruction of gonads. Thus, the intranasal co-delivery may present a safe strategy for the development of contraceptive vaccine. Vehicles for potential nasal delivery of vaccines include bacterial ghost, virus-like-particles [101, 102] or nanoparticles [103].

18.3.4 Plant Based Contraceptives

An alternative approach to protein production using bacteria or virus presents the plant-based immune contraception especially for herbivore animals. Female possums vaccinated with immunocontraceptive antigens showed reduced fertility, and possums fed with potato-expressed heat labile toxin-B (LT-B) expressed mucosal and systemic immune responses to the antigen. This demonstrated that immune contraception was effective in possums and that oral delivery in edible plant material might be possible. Prior to attempts at large scale production, more effective antigen-adjuvant formulations are probably required before plant-based immunocontraception can become a major tool for population control of overabundant vertebrate pests [104].

18.3.5 Virally-Vectored Immunocontraception (VVIC)

One approach to deliver immunocontraceptive vaccines are self-disseminating agents as a viruses. This approach employs live genetically modified viruses to

deliver immunocontraceptives and has proved successful under laboratory conditions. Under field condition, a virus may have the advantage of self-regulating depending on density of the target species [12] and can be species specific if the viral vector is species specific [66, 105]. However, despite a large number of studies on VVIC [14, 66], so far no product has been developed for field testing. The ability of an immunocontraceptive virus to control populations is not only compromised by several factors like sufficient transmission rate, competition with field strains of virus or its ability to induce infertility in the presence of field strains [106], there are also safety and regulatory concerns about maintaining the species specificity of the viral vector and other potential unexpected changes in such genetically modified virus. Once released, a vector cannot be recalled and may spread to regions where the original target species is not a pest [13]. These constraints indicate that it is very unlikely that VVIC will be applied in near future [105].

18.4 Problems Connected with IC

18.4.1 Animal Welfare and Health Issues

Potential adverse effects of contraception may include harmful effects on pregnant animals, inhibition of parturition or dystocia, changes in ovarian structure or function, changes in sex ratio, changes in lactation or mammary glands, impact on fertility of young, changes in testicular structure or function, changes in secondary sex characteristics, changes in bodyweight, changes in behaviour, changes in annual breeding season, and other physiologic and pathologic changes [107]. Reactions at injection sites and in lymph nodes are typical responses to injection of vaccines formulated as water-in-oil emulsions, especially those that contain mycobacteria [60, 108, 109].

The impact of contraception on animal welfare is intensively monitored in captive animals [110], but is difficult to study in wildlife population. Here long-term effects because of stress and disturbance associated with administration procedures, in particular when repeated administration is required [7], are of interest. Each time an animal was treated, it became more difficult to re-treat because of an increased wariness [111].

Contraceptives that disrupt endocrine function have the potential to disturb metabolic homeostasis and thereby cause disease. Antibodies that target sperm or egg proteins have the potential to incite immune-mediated damage in organs producing gametes. Longevity animals that never had a chance to reproduce may express tumour or cyst development within the uterus, as described for non-reproducing captive elephants and rhinoceros [112]. All potential risks need to be assessed and then weighted against benefits of contraception, but unfortunately the number of studies on animal behaviour and physiology are still limited.

Most data exist on long-term application of contraceptive vaccines in horses, deer and elephants. Effects of PZP treatment on feral horses appear to be limited

primarily to reproduction with differences in body condition [113]. In elephants, no detectable behavioural or social consequences were found after long-term PZP treatment [114]. Also for wild boars, no differences in bodyweight, haematology, biochemistry and no obvious sign of injection site was reported [115].

The use of PZP in seasonal breeders may result in delayed breeding and birth of offspring outside optimal breeding season [116]. In addition, PZP does not suppress the ovarian activity, thus treated animals may express recurrent or persistent oestrus. Specially in carnivores, the permanent exposure to endogenous steroids might cause the same pathological effects shown for exogenous steroids [117].

The GnRH vaccination in male deer causes delayed antler growth and retention of velvet [118]. Active immunization against GnRH in pigs also caused damage to cells in the hypothalamus other than those producing GnRH [119], but similar safety studies have not been conducted in wildlife animals. Treatment of domestic pigs with GnRH vaccine was also associated with higher food consumption and higher deposition of subcutaneous fat [120]; a similar increase in body weight was observed in wild boars [54] whereas male lambs immunized against GnRH decreased feeding [121]. Treatment with GnRH vaccine suppresses but did not completely block the production of steroid hormones [54], thus the availability of these hormones after treatment had positive implications for the welfare, since lack of steroids can have potentially wide-ranging effects on animal health.

18.4.2 Behavioural Changes

Immune contraception used in free-ranging wildlife should not disrupt species-typical behaviour patterns, like an extension of the breeding season. The presence of treated females could disrupt social interactions in a population. Although several wildlife population have been treated with immune contraception for a prolonged time, data on behavioural changes are still limited.

Male deer coexisting with non-treated females spent more time exhibiting aggressive behaviour during rut than males living with PZP-treated females, but males did not differ in time spent in mating behaviour [122]. An extension of breeding season or increased movements or ranges were not observed [123].

In captive elk, however, a prolongation of male precopulatory behaviour rates was observed toward GnRH-vaccinated females [124].

In feral wild horses, observed behavioural effects mostly appeared to be related to missing reproduction [113], although a higher risk of chronic stress was suggested due to disturbance by repeated vaccinations. Mares contracepted with PZP make approximately 10 times as many group changes as do untreated mares [125]. Thus, PZP immunocontraception has a significant effect on harem stability [126]. Interestingly, disturbances in mares were also found in the post-treatment years. Parturition was observed substantially later, resulting in asynchrony with peak forage availability [127], influencing harem-tending, reproductive, and agonistic behaviour as well [128].

The social rank of treated wild boars [54] and brushtail possums [129] did not change during treatment with GnRH, and no significant effects of vaccination on behaviour in squirrels [130] was observed.

Another potential behavioural change is the risks of prey-switching of predators feeding on immunocontracepted target species. This ecological effect of prey-switching needs to be considered before an immunocontraceptive strategy can be adopted [81].

18.4.3 Population Level Effects of Fertility Control

Immune contraception which relies on a healthy immune system to achieve contraception may not be effective in animals with compromised immune function. Thus, in wildlife population this could have a negative effect on natural selection for population fitness. Use of immune contraception could create genetic changes in the target population that would influence disease resistance. Selection for failure to respond to the vaccination will occur, and will change immune function in general. Poor scientific description of ecosystem complexity makes it difficult to predict the consequences of immune contraception on wildlife populations [131].

Also of concern is the changing age structure of populations and the potential deleterious effects associated with increasing inbreeding levels in smaller population. Diminished number of females in the breeding pool increases theoretically the size of a healthy population which is large enough to maintain the lowest levels of inbreeding. In this context, the increasing number of older females, which will not reproduce after prolonged contraception, has to be considered. Contracepted animals live significantly longer than the non-contracepted [116, 132]. Thus, the improvement in survivorship diminishes the effectiveness of contraception in reducing population size [29].

Population models have been developed to guide management decisions for many wildlife species. These models use basic information on a species' life history to predict the effect of various management actions, including contraception [133–135]. Collateral consequences of contraception can produce unexpected changes in birth rates, survival, immigration and emigration. The magnitude and frequency of such effects vary with species-specific social and reproductive systems, as well as connectivity of populations [136].

Conclusions

Traditional methods to control wildlife, such as culling and poisoning, often turn out to be ineffective, environmentally hazardous and uneconomic and may have significant welfare costs. Public concerns about lethal control, animal welfare and restrictions on biocides place increasing constraints on wildlife management options and require identifying alternative methods. One of these alternatives is population control by immune contraception. Over the last three decades, significant progress has been made in developing immunocontraceptive vaccines for

wildlife control. Fertility control by immune contraception is suggested to offer a long-term, effective and humane approach to reducing wildlife population. Contraceptive treatment, however, may alter the health and behaviour of wildlife populations and therefore must be monitored closely.

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Immuno-interception of Fertility: Current Status of Vaccines Developed Against hCG and LHRH

19

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Abstract

Three vaccines against hCG have undergone successfully Phase I clinical trial but only one HSD-hCG vaccine went through Phase II efficacy trials demonstrating its ability to prevent pregnancy without impairment of ovulation and derangement of menstrual cycles and bleeding profiles. A semisynthetic vaccine against LHRH has had Phase I/II clinical trials in India and Austria in 28 patients of carcinoma prostate. Highlights of these vaccines are presented. Issues related to clinical trials are discussed.

19.1 Introduction and Prologue

Only four vaccines have progressed to the stage of clinical evaluation so far. This stage is reached after successful experimental studies and after conducting preclinical toxicology studies in two species, whereby the safety and reversibility of the vaccine are established. The vaccine against hCG is intended as a birth control vaccine for use by women, whereas LHRH vaccine is usable in both humans and

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animals, being given that the structure of this decapeptide is largely conserved in mammals. Furthermore, LHRH is common to both males and females and is thereby a unisex molecule controlling both male and female fertility, as well as the production of sex steroids.

Clinical trials are conducted phasewise after obtaining the approval of the Drugs Regulatory Authorities and Institutional Ethics Committees. Written informed consent of the subject opting for clinical trial is also taken. Phase I trial has the objective of assessing the safety of the vaccine in humans and its ability to induce antibody response against the target hormone. The number of subjects enrolled in Phase I trial is between 10 and 20 in one or more centers. The study is intensive and carefully conducted by not only thorough clinical observations but also a variety of laboratory investigations. After establishing the safety, immunogenicity, and reversibility of the procedure, Phase II studies are undertaken to assess the efficacy and extended safety of the candidate vaccine. The number of subjects on which the Phase II trials are conducted varies from 50 to 200. Only one vaccine directed against hCG has so far completed the Phase II efficacy trial in women.

Vaccine(s) against LHRH have been employed not only to control the fertility of animals but also for therapy of hormone-dependent prostatic cancers and have the potential of blocking estrogens in breast cancer patients. LHRH regulates the secretion of FSH and LH from the pituitary which in turn act on gonads to generate sperm and testosterone in males and egg plus sex steroid hormones in females. The rationale in employing anti-LHRH vaccine in prostate carcinoma patients is to cut off the androgen support to the prostatic cancer cells. One vaccine against LHRH has undergone Phase I/Phase II trials in prostate carcinoma patients in India and Austria.

This chapter reviews the vision of making vaccines against hCG and LHRH, the achievements, and the current status of these two unique vaccines.

19.2 Counter hCG Vaccine

hCG is made and secreted by early embryo. It is present in the culture medium of the eggs fertilized in vitro [1]. It is critical for implantation; marmoset embryos exposed to antibodies against hCGβ do not implant, whereas their incubation with normal immunoglobulins has no such effect [2]. Interception of implantation by circulating anti-hCG antibodies is thus deducible in humans. Women carrying antibodies above 50 ng/ml do not become pregnant nor experience lengthening of the luteal phase [3, 4]. HCG has not only a crucial role in implantation of the embryo, it is necessary for sustenance of pregnancy by production of progesterone from corpus luteum, for the first 7 weeks. Anti-hCG antibodies given passively at this stage abort early pregnancy. They block progesterone synthesized by the cytotrophoblasts under the influence of hCG [5].

It is logical to see that a vaccine against hCG is an ideal mode of contraception. It does not impair ovulation. Women continue making their normal sex steroids and have no derangement of menstrual regularity and bleeding profiles [3, 4].

19.2.1 Diversity of hCG Vaccines

hCG is a heterodimer, alpha subunit is common to TSH, FSH, and LH, and the beta subunit imparts hormonal identity. hCGβ is a glycosylated peptide of 145 amino acids. It has high homology with hLHβ, but the carboxy terminal 30–35 amino acids are unique to hCGβ. It is for this reason that Vernon Stevens and WHO Task Force decided to make a vaccine based on the carboxy terminal peptide (CTP) of hCGβ. Our experience with CTPs was not very encouraging. These were very poor immunogens and the antibodies were elicited, only by employing strong adjuvants. The antibodies were of low bio-efficacy. Lengthening of the CTP to 45 and 53 terminal amino acids improved immunogenicity [6, 7]. However, the antibodies were still inferior in their affinity for hCG and low bio-efficacy than those generated by employing the entire hCGβ subunit.

The potential risk of anti-hCG antibodies generated by using the entire β-subunit was the cross-reaction with hLH. This was not found to be the case. The gonadotropins and the steroid hormone profiles of women immunized with hCGβ-TT vaccine were similar to their preimmunization profiles as determined by Tapani Luukkainen in Finland, Elsimar Coutinho in Brazil, Elof Johansson in Sweden, and Horacio Croxatto in Chile [8]. Even by hyperimmunization of the monkeys with ovine LH (oLH) generating antibodies frankly cross-reactive with monkey LH had no discernable side effects nor immunopathology [9, 10]. A possible explanation is that hLH surge taking place once in a month has enough surplus beyond the amount required to induce ovulation. The antibodies generated by hCGβ and the heterospecies dimer (HSD) consisting of hCGβ associated with alpha subunit of ovine LH, linked to carriers, generate primarily conformation reading antibodies with Ka of 10^{10} M^{-1} or more. Over 80 % of antibodies generated by HSD vaccine are directed to an epitope competed by a monoclonal antibody of high specificity with less than 5 % cross-reaction with hLH [11]. On the other hand, much to the surprise of all, the antibodies generated by CTP vaccine were cross-reactive with pancreatic cells, whereas anti-hCGβ-TT did not have such cross-reaction [12]. The CTP-based vaccine of Stevens promoted by WHO Task Force did undergo Phase I trials in Australia [13]. It was however abandoned in view of the unacceptable side effects it provoked in the first seven women immunized with it in Sweden.

19.2.2 Necessity of Employing a Carrier

Although nonpregnant women do not make hCG (the rationale for assaying hCG in pregnancy detection kits), the fact that the fetus (male or female) is exposed to high amounts of hCG during pregnancy renders them immunologically tolerant to hCG. Hence, there is a need of a carrier to mobilize T helper cells. We employed tetanus toxoid (TT) in the very first vaccine conceived for hCG [14]. The reasons were (i) it was an approved low-cost vaccine readily

available in unlimited quantities. (ii) It would prevent, in addition, deaths due to tetanus which occurred in large numbers in developing countries following delivery taking place at homes in aseptic conditions. hCGβ-TT vaccine generated both anti-hCG and anti-tetanus antibodies. The response was reversible. hCG alone did not act as a booster, ruling out the fear of auto-immunization. Repeated immunization with the vaccine containing TT as carrier however led to carrier-induced suppression of response to hCGβ. This could be overcome by employing alternate carriers such as diphtheria toxoid (DT) or cholera toxin B subunit (CTB) [15].

19.2.3 Phase II Efficacy Trials on HSD-hCG Vaccine

This was the first ever trial conducted with any birth control vaccine [3]; 148 women of proven fertility with two living children and one or more medical termination of early pregnancy (MTP) were enrolled in the trial. While all women generated antibodies to hCG, 119 (80 %) had titers above 50 ng/ml bioneutralization capacity. However, only 60 % women maintained antibody titers above 50 ng/ml threshold for 3 months or longer. All women continued to have regular menstrual cycles and had luteal progesterone indicative of normal ovulation. No pregnancy took place in women at and above 50 ng/ml titers. The antibody titers declined with time, but booster injections raised the levels. Eight women completed more than 30 cycles by voluntary intake of boosters without becoming pregnant. Nine completed 24–29 cycles, 12 completed 18–23 cycles, 15 completed 12–17 cycles, and 21 subjects completed 6–11 cycles. Women kept on ovulating normally and had regular menstrual cycles (Fig. 19.1). Figure 19.2 is a representation of a woman, who was protected for 12 cycles, while she was receiving boosters as and when titers were tending to go <50 ng/ml. After 12 months, she decided to have another child. She became pregnant in the very next cycle when her titers were <30 ng/ml.

The efficacy of the vaccine to prevent pregnancy was very high with only one pregnancy recorded in 1224 cycles in women having antibody titers above 50 ng/ml [3, 4]. The vaccine was well tolerated. In fact, when we were asked to close the trial for analysis of data, many women wanted to continue and offered to get the booster at their own expense. These women were hyperfertile and had had more than one MTP. On interrogation, it was brought home to us that other contraceptives did not suit them. IUD caused extra bleeding and pelvic pain, steroids were unacceptable due to either weight gain or irregularity of menstrual profile and spotting, and they were not ready to undergo tubectomy in view of the uncertainty of survival of their children. Thus, a contraceptive vaccine against hCG would be their choice, as it did not impair their ovulation nor cause derangement of menstrual regularity and hormonal profiles, while keeping them protected from becoming pregnant in spite of frequent sexual intercourses.

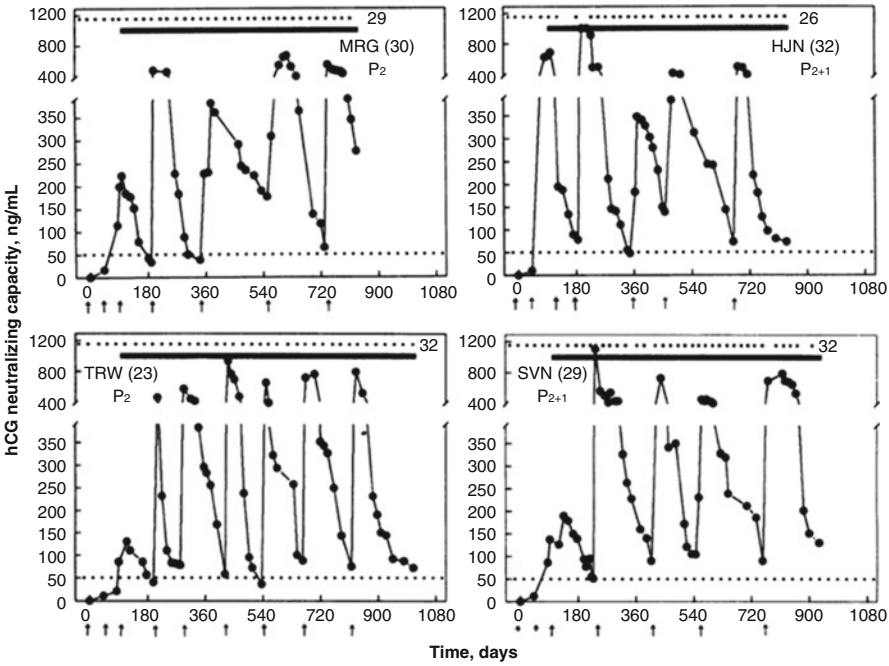

Fig. 19.1 Anti-hCG response to the HSD vaccine in four sexually active women of proven fertility. MRG 30 year old and TRW 23 year old had 2 children each; HJN 32 year and SVN 29 year old had two children each and one elective termination of pregnancy. All of them remained protected from becoming pregnant over 26–32 cycles. *Dotted lines* at top edge represent the menstrual events which remained regular, and *solid lines* denote the period over which they were exposed to pregnancy. Booster injections were given to keep antibody titers above 50 ng/ml (Adapted from Talwar et al. [3])

19.2.4 What Was the Short Coming of the HSD Vaccine?

The HSD vaccine was given with alum as adjuvant. The dose and the adjuvant, at which it was given, generated above protective threshold of antibodies in 60–80 % of recipients. This order of efficacy may be acceptable for vaccines against infectious diseases but as other contraceptives protecting up to 98–99 % of recipients are available, this order of efficacy is not sufficient to make it eligible as an option in the family planning basket.

19.2.5 Revival of the hCG Vaccine

Research on hCG vaccine was revived under an Indo-US program in 2006. The previous HSD vaccine had hormonal subunits purified from natural sources

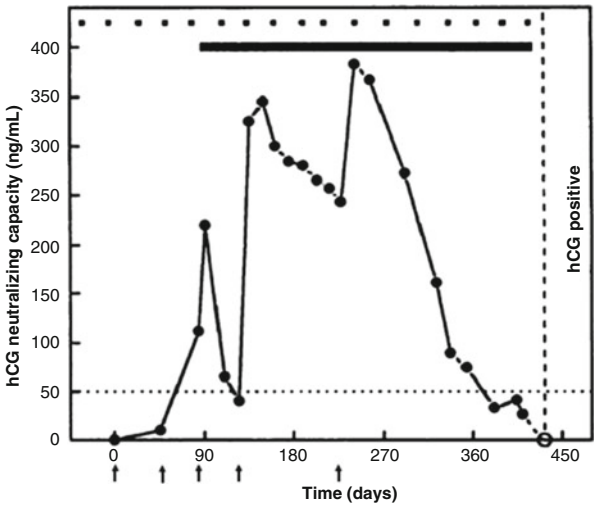

Fig. 19.2 Regain of fertility on decline of antibodies. STS 30-year with 2 children and 1 termination remained protected from becoming pregnant over 12 cycles. She conceived in the cycle when titers were below 20 ng/ml (Adapted from Talwar et al. [3])

(hence costly), linked chemically to carrier (DT/TT). The efficiency of conjugation, besides the uncertainties of the numbers and the position at which carrier was conjugated, lowered the yield and created nonhomogeneity of the product from batch to batch. To overcome these limitations, we decided to make a fully recombinant vaccine. hCGβ was cloned and expressed in *Pichia pastoris* to obtain a glycosylated hormonal subunit linked in a defined position to LTB, the B subunit of heat labile enterotoxin of *E. coli* (Fig. 19.3). The yield was good and the procedure amenable to industrial production. This recombinant conjugate has been tested for immunogenicity in Balb/C mice, and every mouse immunized so far with this vaccine given on alum with SPLPS (sodium pthylylated derivative of lipopolysaccharide of *Salmonella typhi*) in the first injection has generated far above 50 ng/ml antibody titers [16]. We also extended the studies to five inbred strains of mice of different genetic background, encompassing haplotypes H-2^d, H-2^k, H-2^b, H-2^s, H-2^q, in order to ascertain the genetic restriction, if any, of antibody response to this vaccine. Mice of all strains generated antibodies in response to this vaccine [17].

DNA vaccines are not only cheaper to make but also thermostable without requiring cold chain. Therefore, recombinant hCGb-LTB vaccine as DNA, in addition to proteinic form of the vaccine expressed in yeast *Pichia pastoris*, was made. Priming with the DNA form of the recombinant hCGβ-LTB vaccine twice at fortnightly interval followed by boosting with the proteinic form of the vaccine induced distinctly higher antibody response, showing synergy in the actions of DNA and protein versions of recombinant hCGβ-LTB vaccine [18].

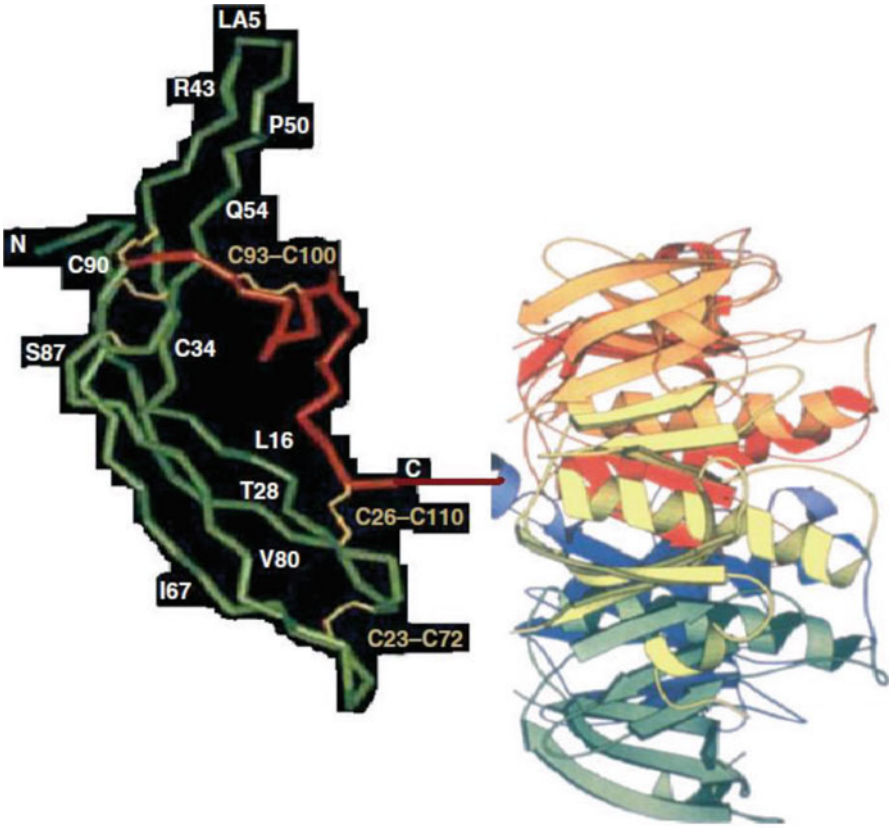

Fig. 19.3 Conceptualized structure of hCGβ-LTB vaccine. The carrier B chain of heat labile enterotoxin of *E. coli* (LTB) is fused at c-terminal glutamine of hCGβ

19.2.6 Preclinical Toxicology and Safety Studies on Recombinant hCGβ-LTB Vaccine

Preclinical toxicology, safety, and efficacy studies were carried out in a subhuman primate species, the marmosets at the National Institute for Research in Reproductive Health, Mumbai. Notable enhancement of immunogenicity of the vaccine was observed, when the first two doses of primary immunization were given with the hCGβ-LTB DNA vaccine followed by the 3rd injection given with the proteinic form of the vaccine. Normal cycling adult female marmosets were immunized intramuscularly twice with DNA version of the vaccine along with autoclaved MiP (*Mycobacterium Indicus Pranii*) as adjuvant at 2 weeks interval. DNA injected animals were distributed in groups of three and immunized with either 20 μg, 40 μg, or 80 μg of the recombinant hCGβ-LTB protein along with MiP as an adjuvant. Immunization with DNA and recombinant protein of hCGβ-LTB vaccine did not have any adverse effect on the

body weight and on the general alertness of the immunized animals. Their hematogical and biochemical parameters continued to remain normal.

All immunized and control animals were then cohabitated with adult fertile male marmosets for 2 weeks per cycle and their fertility was tested for 6 months. Except for one animal, in 80 μg dose group, none of the immunized animals became pregnant, whereas all animals in the control group conceived. After 6 months, with no booster injections given, all immunized animals regained fertility following the decline of antibody titers, indicating thereby that the circulating anti-hCG antibodies were indeed responsible for preventing them from becoming pregnant.

Preclinical toxicology studies were also conducted in rodents as per the regulatory requirements before heading for the clinical trials in humans. These studies were carried out by M/s Bioneeds at their GLP Facility in Bangalore, India, based on biosafety issues related to Genetically Modified Organisms, Schedule "Y" guidelines on Drugs and Cosmetics, and guidelines of Institutional Animal Ethics Committees (IAEC).

It was observed that both DNA and protein vaccines were devoid of sensitizing the skin of guinea pigs. The two forms of hCGβ-LTB vaccine were nonmutagenic at the highest concentration tested both in Bacterial Reverse Mutation and Mammalian Chromosome Aberration Tests. Similar nonmutagenicity observations were made in vivo Mammalian Erythrocyte Micronucleus Test conducted in mice. Single-dose acute toxicity study conducted in Sprague Dawley rats demonstrated the safety of the vaccine. Segment II studies conducted in rats showed that vaccines did not affect the embryo-fetal development, body weight, and food consumption.

Thus, extensive toxicology studies on the hCGβ-LTB vaccine in two species of rodents and a subhuman primate species the marmosets have shown the total safety of the recombinant hCGβ-LTB vaccine. These are now ready to go to clinical trials. In fact, the technology has been transferred to Bharat Biotech, a company in Hyderabad, which will make available the vaccine produced under GMP conditions for the clinical trial. A clinical trial protocol has been developed, and the Indian Council of Medical research is awaiting approval from the Regulatory Authorities to initiate the clinical trials.

19.2.7 Additional Benefits of the hCG Vaccine Against Advanced Stage Cancers

Immunization against hCG has no doubt applications for preventing pregnancy. A number of recent papers report ectopic expression of hCG or subunits in a variety of cancers: lung [19], bladder [20], colon [21], gastric [22], pancreatic [23], breast [24], cervical [25], oral [26], head and neck [27], vulva/vaginal [28, 29], prostate [30], and renal cancers [31]. It has been further observed that patients with cancers expressing hCGβ ectopically have poor prognosis and adverse survival [20, 31]. It follows that a vaccine against hCG or recombinant antibodies against hCG may have additional applications for therapy of such cancers.

We engineered a recombinant chimeric antibody of high affinity and specificity [32, 33]. This antibody, cPiPP, bound to T-lymphoblastic leukemia MOLT-4 cells

expressing hCG, whereas it had no binding with peripheral blood lymphocytes (PBLs) of normal healthy subjects [34]. Vyas et al. [35] observed that this antibody linked to curcumin, a safe anticancerous compound, killed 100 % of MOLT-4 cells as well as histocytic lymphomas U937 cells, both expressing ectopically hCG. On the other hand, the immuno-conjugate had no deleterious effect on PBLs of healthy subjects.

19.3 LHRH Vaccine

Besides hCG, the only other vaccine which has undergone clinical trial was directed against LHRH. It was a semisynthetic vaccine in which glycine at position 6 was replaced by D-lysine which created a functional NH_2 group for linking the carrier either TT or DT [36]. The vaccine was highly immunogenic and induced bioeffective response in rodents [37] and in monkeys [38] with alum alone as adjuvant. Immunization caused cessation of spermatogenesis in rodents along with testosterone declining to castration levels [39]. Spermatogenesis and fertility was regained on decline of antibodies. Immunization against LHRH could also block fertility of female rodents in a reversible manner [40].

In male rats and in monkeys, a drastic atrophy of prostate was observed. The vaccine inhibited the growth of Dunning R3327-PAP tumor implanted in rats by suppression of cell division [41]. Preclinical toxicology studies in India and Austria indicated the safety of semisynthetic vaccine LHRH vaccine. With permission of the Drugs Regulatory Authority in India and Austria and with the approval of the Institutional Ethics Committees, Phase I/II clinical trials were conducted with this vaccine in 28 patients of carcinoma of prostate, 12 patients each at the All India Institute of Medical Sciences (AIIMS) New Delhi, and Postgraduate Institute of Medical Education and Research (PGI) Chandigarh, and four patients at the Urology Department of Salzburg General Hospital. The vaccine was well tolerated in all subjects. No ill effect of immunization was seen. Subjects developing more than 200 pg/ml of antibodies experienced a decline of testosterone to castration levels with marked reduction of PSA. Patients receiving 400 μg LHRH equivalent dose showed better clinical improvement than those receiving 200 μg. Table 19.1 is a summary of observations on 12 patients of

Table 19.1 Observations in clinical trials conducted at AIIMS in patients of carcinoma of prostate after immunization with either 200 or 400 μg of anti-LHRH vaccine

	Dose level of the vaccine	
Effect of immunization	200 μg ($n = 6$)	400 μg ($n = 6$)
Clinically stable/improvement in symptoms	4	5
Reduction in prostatic size/hardness	1	3
Reduction in acid phosphatases	1	4

Vaccine was administered as three primary injections at monthly interval followed by a booster at 8th month

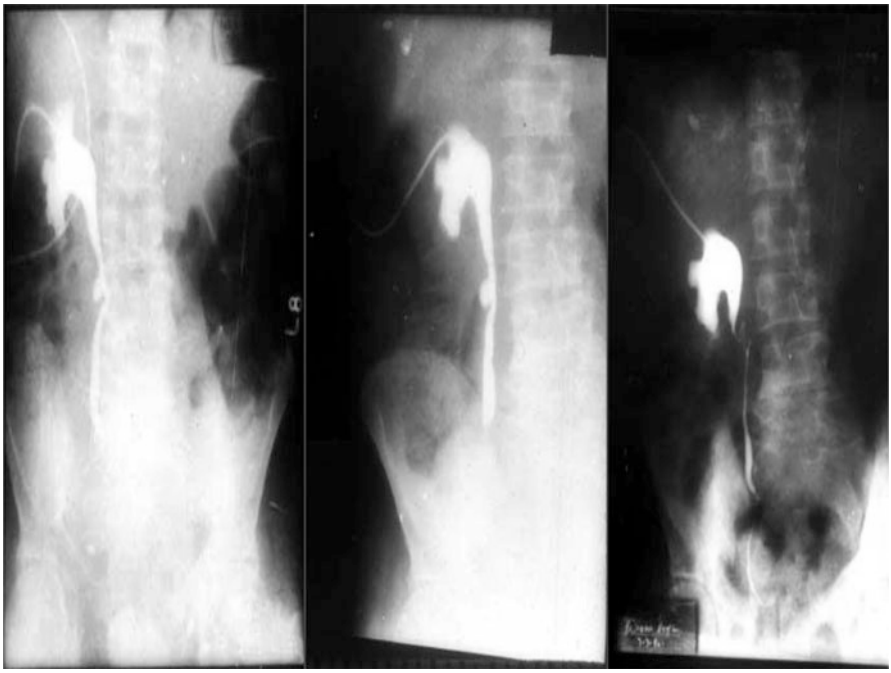

Fig. 19.4 Serial nephrostograms showing the noticeable reduction of prostatic tissue mass at various stages of immunization with anti-LHRH vaccine (Adapted from Talwar et al. [42])

carcinoma of prostate on whom trial was conducted by Prof. S. Wadhwa, at AIIMS. Serial nephrostograms of a patient at PGIMER, Chandigarh, showed clearance of prostatic tissue mass [42] (Fig. 19.4).

Delivery of multiple doses of the vaccine at a single contact point was achieved by encapsulating these in biodegradable microspheres, shortening also the lag period for bioeffective response [43].

19.3.1 Recombinant LHRH Vaccine

A multimer recombinant vaccine has been developed against LHRH in which five units of LHRH are interspersed with four different T non-B peptides from *Plasmodium falciparum* circumsporozoite protein, tetanus toxoid, respiratory syncytial virus, and measles virus. Knowledge-based computer graph of this multimer shows LHRH moieties are exposed to hydrophilic environment, in which the T non-B peptides are embedded. The gene encoding the multimer vaccine was constructed, and the recombinant protein expressed and purified from *E. coli* [44, 45]. This multimer vaccine caused nonsurgical castration lowering of testosterone and drastic atrophy of the rat prostate [46]. Lowell Miller and coworkers at the

National Wildlife Research Center, USA, tested the vaccine in pigs for control of fertility. Given along with Adjuvac, an oily adjuvant approved by USFDA, it sterilized 100 % of the pigs tested [47].

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