Perspectives on the Biological Role of Human Prostasomes

BY
LENA CARLSSON
Prostasomes are extracellularly occurring organelles which are secreted in human semen by the prostate gland. Prostasomes have several known biological activities, but their physiological function is still unclear. In this thesis some new aspects were studied on the biological role of the prostasomes.

The motility-stimulatory effect of prostasomes on cryopreserved spermatozoa was further studied by supplementing the swim-up medium with seminal prostasomes, and with prostasomes purified from a PC-3 prostate cancer cell line (PC-3 prostasomes), on fresh spermatozoa. The recovery of motile spermatozoa after swim-up increased by 50% when the swim-up medium was supplemented with prostasomes. The PC-3 prostasomes bore a functional resemblance to seminal prostasomes as regards various expressions of sperm motility promotion.

Prostasomes proved to have potent antibacterial effects. The effects were not strictly confined to *Bacillus megaterium* since a few other bacteria were also sensitive. The high percentage of patients with anti-prostasome antibodies showed that prostasomes could be one of the major targets for antisperm antibodies (ASA). The results demonstrate that ASA in serum of infertile men and women recognise prostasomes as antigens, and that polyclonal antibodies raised against prostasomes agglutinate human spermatozoa. This suggests that prostasomes contribute at least partly to immunological infertility. Three types of prostasomes (seminal-, native- and metastasis-derived prostasomes) demonstrated similarities regarding a high cholesterol/phospholipid ratio and some marker enzymes. The conclusion is that prostasomes have a common and exclusive prostatic origin in man and that they are internalised in storage vesicles of the secretory cells and released *in toto* by an ordinary exocytotic event.

*Key words*: Prostasomes, cryopreservation, human spermatozoa, sperm motility, anti-prostasome antibodies, PC-3 cells, *Bacillus megaterium*, antibacterial, antisperm antibodies, complement activation, sperm agglutination, infertility.

*Lena Carlsson, Department of Medical Sciences, Clinical Chemistry, University hospital, SE-751 85 Uppsala, Sweden*

© Lena Carlsson 2001

ISSN 0282-7476
ISBN 91-554-5159-4

Printed in Sweden by Uppsala University, Tryck & Medier, Uppsala 2001
"What a piece of work is a man"

Shakespeare, Hamlet, Act ii, scene 2, line 316

To my parents
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I  Carlsson L, Ronquist G, Stridsberg M, Johansson L  
Motility stimulant effects on prostatome inclusion in swim-up medium on cryopreserved human spermatozoa  
*Arch Androl* 1997, 38;215-221

Prostasome-like granules from the PC-3 prostate cancer cell line increase the motility of washed human spermatozoa and adhere to the sperm  

III  Carlsson L, Påhlson C, Bergquist M, Ronquist G, Stridsberg M  
Anti-bacterial activity of human prostatomes  
*Prostate* 2000, 44;279-286

Circulating human antisperm antibodies recognize prostatomes  
*Am J Reprod Immunol* 2001, 46; 211-219

Characteristics of human prostatomes isolated from three different sources  
*In manuscript* 2001

Reprints were made by permission of the publishers
# TABLE OF CONTENTS

### ABBREVIATIONS
- Introduction.......................................................................................................................... 8

### INTRODUCTION
- Seminal Plasma........................................................................................................................ 8
- Seminal Vesicles...................................................................................................................... 8
- Prostate Gland ....................................................................................................................... 8
- Bulbo-Urethral Glands.......................................................................................................... 9
- Secretory Activity of the Accessory Sex Glands................................................................. 9

### THE PROSTASOME
- Prostasomal Membrane Architecture.................................................................................. 10
- Prostasomal Membrane Enzymes....................................................................................... 11
- Neuroendocrine Components of Prostasomes.................................................................. 14

### FUNCTIONAL ROLE OF PROSTASOMES
- Sperm-Prostasome Interaction............................................................................................ 14
- Sperm Motility..................................................................................................................... 16
- Immunosuppressive Activity of Prostasomes...................................................................... 16

### AIMS OF THE PRESENT INVESTIGATION
- Material and Methods.......................................................................................................... 19
- Sample Collection and Sperm Preparation...................................................................... 19
- Prostasome Preparation...................................................................................................... 20
- Antibody Production........................................................................................................... 22
- Sperm Motility Analysis...................................................................................................... 22
- Immunocytochemical Staining............................................................................................ 24
- Sperm Agglutination............................................................................................................ 25
- SDS-PAGE and Immunoblotting......................................................................................... 25
- Flow Cytometry.................................................................................................................. 25
- Assessment of Antibacterial Activity................................................................................. 26
- Scanning Electron Microscopy............................................................................................ 27
- Atomic Force Microscopy (AFM)....................................................................................... 27
- Biochemical Markers........................................................................................................... 28

### RESULTS AND CONCLUSIONS
- Paper I...................................................................................................................................... 30
- Paper II.................................................................................................................................... 30
- Paper III.................................................................................................................................. 32
- Paper IV................................................................................................................................... 34
- Paper V.................................................................................................................................... 35

### DISCUSSION
- Introduction............................................................................................................................ 37

### SUMMARY
- Introduction............................................................................................................................ 48

### ACKNOWLEDGEMENTS
- Introduction............................................................................................................................ 50

### REFERENCES
- Introduction............................................................................................................................ 51

Cover picture: Transmission electron microscopy of seminal prostasomes. Mag x 80 000.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ALH</td>
<td>Amplitude of lateral head displacement</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ASA</td>
<td>Antisperm antibodies</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CASA</td>
<td>Computer assisted sperm analyzer</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CgA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>CgB</td>
<td>Chromogranin B</td>
</tr>
<tr>
<td>CLED</td>
<td>Cystine lactose electrolyte deficient agar</td>
</tr>
<tr>
<td>DPPIV</td>
<td>Dipeptidylpeptidase IV</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s balanced salt solution</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine-tetraacetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FDMEM</td>
<td>Fortified Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GGT</td>
<td>γ-Glutamyltransferase</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphoinositide</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine-aminopterin-thymidine</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NBT/BCIP</td>
<td>Nitroblue tetrazolium/bromochloroindolyl phosphate</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>5’NT</td>
<td>5’Nucleotidase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAD</td>
<td>Pathoanatomical diagnosis</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostate-specific acid phosphatase</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethyleneglycol</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>RIA</td>
<td>Radio immunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute</td>
</tr>
<tr>
<td>STR</td>
<td>Straightness</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodiumdodecanylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>VAP</td>
<td>Average path velocity</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>VSL</td>
<td>Straight line velocity</td>
</tr>
</tbody>
</table>
Fig 1. The urinary bladder, urethra, ductus deferens and the accessory glands. View from behind (from © Liberförlaget AB, Stockholm, Sweden, Anatomisk Atlas, 1993. Reprinted with permission from Liberförlaget AB)
INTRODUCTION

Seminal plasma
The human seminal plasma is a heterogeneous mixture of fluids from the testes, epididymides, ampullae ductus deferentes and the accessory sex glands, viz. vesiculae seminales, prostate, bulbo-urethral and urethral glands (Fig 1). The main contribution is from the prostate (about 30%) and seminal vesicles (about 60%), but even among healthy men there is a large interindividual variation with regard to these proportions.

Seminal vesicles
The seminal vesicles are paired, sac-or tubule-like structures surrounded by a thick coat of smooth muscles. Each seminal vesicle empties separately into the posterior urethra, allowing momentous bulk secretion. The fully developed exocrine glands reside immediately above the prostate gland (Fig 1), and comprise a series of tubular alveoli, lined with a very active secretory epithelium. The secretions are rich in fructose and prostaglandins [1]. The seminal vesicles also produce several androgen-dependent secretory proteins (semenogelins) that are involved in the rapid clotting of the ejaculate [2].

Prostate gland
The prostate gland is located in the space below the bladder and above the urogenital diaphragm (Fig1). The prostate arises from several distinct sets of tubules that evaginate from the primitive posterior urethra. Each set of tubules develops into a separate lobe: the right and left lateral lobes, which are the largest, the medial lobe, and the very small anterior and posterior lobes. The lobes are composed of alveoli, lined with a secretory epithelium that drain through a series of converging tubules into the prostatic urethra. During ejaculation, the prostate acini are compressed by their surrounding fibromuscular coat, emptying their contents into the urethra. The secretory cells of the prostate can be divided into five different functional zones such as the basal, nuclear, supranuclear, Golgi and apical zones, arranged in a polarized manner.
The secretory cells contain a basally located large nucleus. A well-developed Golgi apparatus surrounded by numerous vesicles and vacuoles is located in the supranuclear region.

**Bulbo-urethral glands**
The two bulbo-urethral glands consist of several lobules held together by a fibrous capsule. Its secretory units are mainly tubulo-alveolar in form. The glandular epithelium secretes acid and neutral mucosubstances, and is immunopositive for a number of androgen metabolizing enzymes including 17 β-hydroxysteroid dehydrogenase [3] and blood group antigens [4].

**Secretory activity of the accessory sex glands**
The development and secretory activity of the male accessory glands are dependent on androgens [5]. The secretions are ejaculated in a specific time sequence together with the epididymal spermatozoa during the ejaculation process [6]. The secretion of the prostate gland is emitted first, followed by the sperm rich fluids from the epididymides and the final portion of the human ejaculate, mainly represented by the contributions of the seminal vesicles, flushes the urethra free from spermatozoa. The secretions produced by the prostate gland and the seminal vesicles are the predominant contributors of proteins to human seminal plasma. The protein concentration of this fluid (i.e. the ejaculate devoid of spermatozoa) varies between 35-55 g/L [7]. Albumin is a component (0.5-1.4 g/L) of the seminal plasma proteins and the prostate gland is considered to be the major site of albumin transudation [8,9]. Also the prostate gland has long been recognized for its ability to secrete acid phosphatase in a high concentration [10]. Other major proteins derived from the prostate gland and appearing in the seminal fluid are the prostate specific antigen (PSA) which is a serine protease [11] and a 11 kDa protein called β-inhibin [12,13] or β-micro seminoprotein [14]. The prostate gland is not only secreting soluble proteins but also small organellar structures named prostasomes.
The prostasome

Prostasomes were discovered during the late 1970’s by Ronquist et al [15,16]. They are submicron, membrane-surrounded organelles produced by the epithelial cells of the human prostate gland and are present in appreciable amounts in normal semen. The organelles are encased usually by a lipid bilayered membrane and they have a corpuscular appearance with a mean diameter of 150 nm, range 40-500 nm [17]. The prostasomes have a density of 1.03 when analysed by continuous silica density gradient centrifugation [18] in that respect behaving typically as subcellular organelles. Small spherical particles of approximately 15 nm in diameter may appear in their cytosol [19]. Prostasomes are mostly encased in bigger storage vesicles together with electron dense material in the prostatic secretory cells [18]. The prostasomes may thereby be released as small intact organelles in the prostatic fluid (and semen) by an ordinary exocytotic event involving the membrane surrounding the storage vesicle and the plasma membrane of the prostatic secretory cells [18].

A reduced amount of prostasomes in seminal plasma was observed in a patient with Klinefelter’s disease who had the serum testosterone level reduced by 50% [20]. In another patient, with a well differentiated carcinoma of the prostate, the secretion of prostasomes was reduced by 85% after 2 weeks of treatment with an antiandrogenic drug [21]. These observations suggest a role for testosterone in the secretion of prostasomes.

Prostasomal membrane architecture

The prostosome membrane is composite in its architecture and this is valid for both the lipid part and the protein part. Lipid analysis of prostasomes revealed a striking quantitative domination of cholesterol over the phospholipids, the molar ratios of cholesterol/ sphingomyelin/ glycerophospholipids being 4:1:1. Hence, the cholesterol/ phospholipid ratio, being 2.0 [22] was very high in comparison with most other plasma membranes including that of the human spermatozoon being 0.83 [23]. This high ratio was consonant with a high percentage of sphingomyelin in prostasomes rendering the membrane a high molecular ordering as measured by electron spin resonance [22].
Prostasomal membrane enzymes

**ATPase.** Prostasomes contain a rather high ATP-splitting activity which is linked to their membrane [18]. Detergents are potent inhibitors and 0.1% of deoxycholate inhibits 86% of the activity. The Mg$^{2+}$ and Ca$^{2+}$ stimulated ATPase of the prostasomal membrane is calmodulin-dependent [24]. The apparent K$_m$ with regard to ATP of the uninhibited Mg$^{2+}$ and Ca$^{2+}$ stimulated ATPase is 0.82 mmol/L [24]. A Zn$^{2+}$-dependent ATPase of the prostasomal membrane has also been described, this ATPase is inhibited by vanadate and has an apparent K$_m$ of 0.43 mmol/L [25].

**γ-Glutamyltransferase.** Human seminal plasma contains an abundance of γ-glutamyltransferase (GGT) activity which is 200-500 times higher than in normal serum [26]. The prostate is the major source of the enzyme [27] and most of it is bound to the membrane of the prostasomes [28].

**5′Nucleotidase.** 5′Nucleotidase has been shown to be located at the external surface of the prostasome membrane and a 50-60% increase in activity was achieved by the addition of 0.05% Triton X-100 [29]. The enzyme is to some extent linked to the prostasome membrane via a glycoposphoinositide anchor [30].

**Protein kinase.** When prostasomes were incubated in presence of [γ$^{32}$P] ATP the terminal phosphoryl group was transferred from ATP to the hydroxy group of serine and threonine residues of endogenous acceptor proteins in the prostasomal membrane. Serine was most actively phosphorylated in the presence of Mg$^{2+}$ (Ser P/Thr P ratio 3.5) while in the presence of Zn$^{2+}$ the phosphothreonine is the predominant reaction product (Ser P/Thr P ratio 0.19) [31]. This finding indicates the presence of at least two or more isoenzymes. The function of these protein kinases on the membrane of prostasomes is not known [31].

**Arachidonic acid 15-lipoxygenase.** Prostasomes have proven to possess arachidonate 15-lipoxygenase activity [32]. This enzyme catalyses oxygenation of the n-6 carbon of
many polyunsaturated fatty acids and has been implicated in the acrosome reaction of bull spermatozoa [33] and in the lysis of intracellular organelles [34,35].

**Aminopeptidase (CD13).** A hydrolytic activity on the synthetic substrate succinyl(alanine)-paranitroanilide (Suc(Ala)₃ pNA) is also linked to the prostasomes. The enzyme, aminopeptidase or CD13, is a 150 kDa zinc-dependent proteolytic enzyme previously found to be a useful marker of prostasomes [25,36,37]. It is, however, not involved in any ATP-driven protease activity of prostasomes [25].

**Dipeptidyl peptidase IV (CD26).** Dipeptidyl peptidase IV activity was found extremely high in prostasomes [38]. The dipeptidyl peptidase IV antigen (CD26) has a molecular weight of 120 kDa and was identified on prostasomes by a monoclonal antibody [39]. The presence of both the enzyme activity and the antigen was established in prostatic secretion, but absent from that of the seminal vesicles [40]. Dipeptidyl peptidase IV is a membrane-associated proteinase cleaving off dipeptides from peptide chains consisting of three or more amino acid residues with a primary specificity for proline at the penultimate position of the amino terminus [41].

As a serine protease with unique specificity, dipeptidyl peptidase IV participates in the metabolism of peptides during the intestinal digestion and renal transport of polypeptides. In the hematopoietic system, dipeptidyl peptidase IV is involved in the activation of T-lymphocytes and the regulation of DNA synthesis, cell proliferation, and production of cytokines (reviewed by Yaron and Naider [41] and Fleisher [42]). What is more, the enzyme may play a role in HIV infection and apoptosis [43]. The HIV-1 Tat (trans-activating) protein binds and inhibits the activity of dipeptidyl peptidase IV [44], and the HIV-1 envelope glycoprotein gp 120 also interferes with dipeptidyl peptidase IV [45]. Therefore, prostasomes may well bind HIV viruses via prostasome membrane-bound dipeptidyl peptidase IV. It should be mentioned in this context that prostasomes also contain complement inhibitors such as CD46 [46], CD55, and CD59 [47]. Complement inhibitors may be present in semen to protect the spermatozoa, but they may protect the pathogens as well [48]. The interaction between
complement inhibitors and virus has been investigated in *in vitro* experimental systems. The HIV virus, after incubation with CD55 and CD59, acquires these inhibitors in its membrane, and this leads to an increased resistance against attack by complement [49]. Since these complement inhibitors do exist at the prostasome membrane surface, the same working mechanism may be valid in the presence of prostasomes. Accordingly, we may here discern a new principle, by which prostasomes render HIV virus an advantage of survival in human semen.

*Enkephalinase (CD10).* A 100 kDa protein from human prostasomes was recently identified as neutral endopeptidase (NEP, enkephalinase, CD10) using N-terminal amino acid sequencing and mass fingerprint analysis [50]. NEP was initially characterised as a zinc metalloendopeptidase with a specificity similar to a group of microbial enzymes where thermolysin is serving as a characteristic example. NEP was identified as the synaptic peptidase (enkephalinase) involved in terminating the actions of the opioid peptides, the enkephalins [51, 52]. Among its potential substrates are the opioid peptides, tachykinins such as substance P, the natriuretic peptide family, bombesin-like peptides, and chemotactic peptides. A small and inconsistent number of neuroendocrine cells of the epithelial lining of prostatic tissue seemed to contain bombesin-like immunoreactivity [53]. Molecular cloning of NEP revealed it to be a type II integral membrane protein consisting of a short aminoterminal cytoplasmic domain of 27 amino acids, a transmembrane region of 22 hydrophobic residues, and a large extracellular domain of about 700 residues, which contains the active site of the enzyme [54]. This means that NEP is one of the family of membrane-anchored ectoenzymes. It was subsequently shown that NEP was identical to CD 10, a tumour-associated, cluster differentiation antigen expressed on the surface of neutrophils and certain lymphoid progenitors, and also known as the common acute lymphoblastic leukemia antigen (CALLA) [55].

Many other enzymes may be linked to the prostasome membrane, since it has a complex protein pattern showing more than 80 protein entities when analysed by two dimensional gel electrophoresis [56, 57]. For instance phospholipase A2 [56], lactate
dehydrogenase [58] and angiotensin converting enzyme (ACE) [59] are other enzymes associated with the prostasome membrane.

**Neuroendocrine components of prostasomes**

Prostasomes contain the neuroendocrine markers chromogranin B, neuropeptide Y, and vasoactive intestinal polypeptide in about equimolar amounts and chromogranin A in about 2% of that amount [60]. It has also been shown that prostasomes express a newly described common secretory granule protein, *viz.* granulophysin [61]. This molecule has a similar structure as the neuroprotein synaptophysin [62], which has been used as a marker for endocrine, neuroendocrine and neuronal tissue [63]. In neurones, synaptophysin is located in the small synaptic vesicles that contain the classic neurotransmitters, while the chromogranin family of proteins is generally associated with the large dense core vesicles that contain neuropeptides [63, 64]. From that point of view it is possible that prostasomes consist of a mixture of both kinds of vesicles, which also would fit with the very wide range in organellar size. Another possibility could be that prostasomes are a new kind of vesicles sharing properties common to both types of vesicles.

**Functional role of prostasomes**

**Sperm-prostasome interaction**

The prostasomes may play a pivotal role in the normal fertilising process and this implicates a sperm-prostasome interaction. Indeed, prostasomes can adhere to and, at least to some extent, fuse with sperm cells as shown by free zone electrophoresis and electron microscopy [65], octadecyl-rhodamine fluorescence self-quenching [66], and immunofluorescence staining and confocal microscopy [67]. Both spermatozoa and prostasomes display a net-negative surface charge when analysed in free-zone electrophoresis and on comparison prostasomes are slightly less negative than spermatozoa [65]. Accordingly, prostasomes, originally in front of the spermatozoa, were reached by these latter cells, as a result of their faster mobility, already after a 20–25 min run, and the originally two distinct fractions were joined in one common fraction, which was not dissociated [65]. In case of no interaction the spermatozoa
should pass the prostasome fraction and then form its own fraction in front of the prostasomes. The interaction took place regardless of preceding neuraminidase-treatment of prostasomes or of both prostasomes and spermatozoa. This together with the finding that Concanavalin A-pretreatment of prostasomes and spermatozoa did not interfere with the interaction, made it less probable that carbohydrates were involved in the bonding. It was concluded that the bonding most probably was of a hydrophobic nature [65]. This conclusion was also corroborated by the fact that an electron-microscopic demonstration of a prostasome to sperm interaction was only possible when embedding of the fused fraction after free-zone electrophoresis was carried out in a hydrophilic resin [65].

The fusion was shown to be cation-independent, and strictly dependent on pH and required one or more proteins on sperm and prostasome surfaces [68]. This is different from the Ca\(^{2+}\)-stimulated fusion between rat liver liposomes and spermatozoa that does not require any protein and occurs at neutral pH. The fusion observed between prostasomes and spermatozoa is quite a specific phenomenon that is not possible e.g. between leukocytes and prostasomes [68]. Although human seminal plasma has a high buffering capacity, it is possible that pH of the vaginal content is low enough to allow fusion to occur. Membrane fusion is required for many biological events and may represent an important physiological mechanism to help spermatozoa resist the vaginal acidic milieu. Another result of the fusion between spermatozoa and prostasomes could be the transfer of certain enzymes located on the prostasomes to spermatozoa (aminopeptidase, CD13; dipeptidylpeptidase IV, CD26) [69, 70].

A fusion-like process was also reported by Minelli et al. [67]. In their case they studied the interaction between stallion spermatozoa and prostasome-like vesicles found in horse seminal plasma [67]. They suggested that the interaction CD26/\textit{ecto}-adenosine deaminase (ADA), both found on the vesicles, might be responsible for the fusion. Since adenosine receptors are expressed at the horse spermatozoa surface and said to be second receptors for \textit{ecto}-ADA to form complexes, the interaction CD26/\textit{ecto}-ADA/adenosine receptors during the fusion process could not be ruled out [67].
Sperm motility

The functional capacity of the human spermatozoa has assumed increasing importance. Up to the present, sperm motility has been a critical factor in judging semen quality and the type of this motility influences the fertilizing capability of spermatozoa. In the lower female reproductive tract, motility is needed to penetrate cervical mucus, while in the upper tract, vigorous beating of the sperm tail is necessary for penetration of the zona pellucida [71]. In addition, successful oocyte fertilization in vitro or by artificial insemination requires spermatozoa with adequate motility.

Buffer washings of normozoospermic spermatozoa resulted in a gradual loss of their forward motility [72, 73]. The perturbation that was brought about by the buffer treatment was not of an irreversible nature since the spermatozoa could be functionally restored rather momentaneously by the addition of prostasomes [72-75]. Furthermore, the spermatozoa were metabolically capable of exploiting the energy potential of hexoses as evidenced by the corroborative action by any of fructose, glucose or mannose on prostasome-promotive effect on sperm forward motility [74]. The motility pattern evoked by prostasomes was similar to that of albumin in every respect when compared on an equal protein basis [72, 74]. Prostasomes, however, were more efficient, since they rendered a higher proportion of forwardly motile spermatozoa with a higher amplitude of lateral head displacement at a lower concentration, both parameters being positively correlated to the fertilizing potential of spermatozoa [75]. A higher recovery of motile spermatozoa was obtained after swim-up if albumin in the ordinary swim-up medium was exchanged for prostasomes or if this solution containing albumin was supplemented with prostasomes [75]. The prostasomes also increased the number of hyperactivated spermatozoa, which is thought to be an important parameter for the penetration of the zona pellucida and subsequently for fertilization [76].

Immunosuppressive activity of prostasomes

Human seminal plasma contains a unique range of immunosuppressive compounds, which would prevent cell-mediated and humoral responses to spermatozoa in the female reproductive tract. The prostaglandins of the E series (PGE and 19-hydroxy
Prostaglandins (PGE) present at high levels, are responsible for a wide range of suppressive effects, including suppression of lymphocyte proliferation and natural killer cell activity, and modification of cytokine release from antigen-presenting cells [77]. Prostasomes may play a complementary role to prostaglandins and other compounds in neutralizing the immune defenses of the female genital tract. They were identified as inhibitors of mitogen-induced lymphocyte proliferation and phagocytic cell activity [77, 78]. This activity accounts for a significant proportion of the immunosuppressive activity of human seminal plasma [19]. Since the prostasomes have the ability to adhere to spermatozoa [65], there is the probability that the immunosuppressive effect associated with the prostasomes can be carried up the female genital tract with the spermatozoa [19]. Immunosuppressive activity most likely occurs through phagocytosis of prostasomes or prostasome-to leukocyte pH-dependent adhesion [77]. Confirmation of this hypothesis comes from experiments showing that prostasomes directly inhibit the phagocytosis of latex particles by macrophages, neutrophils and monocytes [78]. Prostasomes also inhibit superoxide anion generation by leukocytes in the basal as well as activated state [78, 79]. Due to their small size, prostasomes may be more readily phagocytosed than the much larger spermatozoa and may be able to reduce the function of the phagocytizing cells. In this way, prostasomes might be able to protect the spermatozoa against phagocytosis, thus facilitating the survival of the sperm [19].

Prostasomes contribute to local modulation of complement activation, providing the C3, C9 and membrane attack complex (MAC) inhibitors. CD55 (decay-accelerating factor) and CD46 (membrane cofactor protein) are C3 convertase inhibitors or inhibitors of complement activation, and CD59 is an inhibitor of MAC. All three proteins are carried on the surface of the prostasomes in a GPI anchor and the spermatozoa may acquire CD59 molecules as a result of the interaction with the prostasomes [47]. Accordingly, prostasomes may represent a pool of CD59 from which protein lost from spermatozoa, possibly as a result of normal membrane turnover or of low level complement attack, may be replenished, thus ensuring that the sperm cells will advance in the female reproductive tract guarded against MAC.
AIMS OF THE PRESENT INVESTIGATION

♦ To investigate the motility-promotive effect of prostasomes on freeze-thawed (cryopreserved) spermatozoa and to elucidate the value of prostasome inclusion in swim-up medium and its benefit in improving results in assisted reproductive technologies using freeze-thawed spermatozoa.

♦ To investigate whether PC-3 prostasomes exhibit similar motility-promotive effects to those of seminal prostasomes on buffer-washed spermatozoa from normospermic semen samples by means of computer-assisted sperm analysis (CASA).

♦ To investigate whether prostasomes exhibit any antibacterial activity in the light of the fact that they contain an unusually high concentration of chromogranin B.

♦ To investigate whether prostasomes could be target antigens for circulating antisperm antibodies (ASA) of immunologically infertile patients.

♦ To compare biochemical and functional characteristics of prostasomes isolated from different sources in order to increase our knowledge about their origin, production and release.
MATERIAL AND METHODS

Sample collection and sperm preparation
Fresh semen samples were obtained from normospermic men, according to WHO laboratory manual, during evaluation for in vitro fertilisation (Papers I-V).

Cryopreserved spermatozoa were obtained from fresh samples, diluted and mixed with an equal volume of a sterile freezing medium. This mixture was drawn up in straws and frozen with a programmable cryopreservation system. After at least 6 months’ storage in liquid nitrogen, cryopreserved spermatozoa were quickly thawed for 10 min at 37°C and washed twice with HEPES buffered Earle’s balanced salt solution containing sodium pyruvate and penicillin. The obtained pellet was carefully and thoroughly resuspended before being counted twice with a Makler counting chamber using a light microscope (Paper I).

Swim-up. Motile spermatozoa were obtained by a swim-up procedure. One ml of liquefied semen was gently layered under 1 ml of Earle’s balanced salt solution (EBSS) medium containing 1% human serum albumin, sodium pyruvate (1 mM) and penicillin G (100 IU/ml) in plastic Falcon test tubes (14 ml). The tubes were placed in air with 5% CO2 at 37°C for 1 h, whereafter the motile spermatozoa in the upper layer of the medium were aspirated, pooled, and washed once with the EBSS medium, and a pellet was obtained after centrifugation at 300 × g for 10 min (Papers I, II, IV and V). To immobilise the spermatozoa they were resuspended in a 50 mM Tris-HCl buffer made isotonic with 138 mM NaCl and 1 mM MgCl2 (pH 7.3) and washed three times at 800 × g for 15 min in that buffer, whereafter the spermatozoa were again resuspended in this buffer solution. After these procedures the spermatozoa showed only weak or no forward motility. Prior to the experiments the sperm concentration was adjusted to 40-60 × 10⁶ /ml. In paper I, the EBSS medium was supplemented with the following effectors: human serum albumin, glucose, adenine and a combination of glucose and adenine to compare the effect with only seminal prostasomes in the EBSS medium. In paper II, the EBSS medium was supplemented with seminal- and PC3-derived prostasomes in different concentrations. In paper V, the EBSS medium was
supplemented with prostasomes derived from seminal plasma, prostate tissue or prostate cancer bone metastases.

Patients’ sera. Sera of patients investigated for infertility were tested for the presence of ASA using the Tray agglutination test (cut-off point of 1:16). Twenty sera of ASA-positive patients (15 men and 5 women) were randomly selected among those who were referred for infertility investigation. Sera of 6 ASA-negative persons (3 women and 3 men) were collected and partly pooled, after individual Tray agglutination tests (Paper IV).

Prostasome preparation

Seminal prostasomes. After liquefaction (30-45 min at room temperature), semen samples were centrifuged for 20 min at 1,000 × g in order to separate spermatozoa and possible other cells from the seminal plasma, which were pooled (12-15 samples) and ultracentrifuged at 10,000 × g for 10 min to pellet possible cells and cell debris. The supernatant was subsequently subjected to another ultracentrifugation for 2 h at 100,000 × g to pellet the prostasomes. The prostasomes were resuspended in a Tris-HCl buffer (30 mM, pH 7.6) made isotonic with 130 mM NaCl. The suspensions were purified by Sephadex G 200 (Pharmacia AB, Uppsala, Sweden) chromatography, to separate them from an amorphous substance [73] at 6 mL/h, and 2 mL fractions were collected. The eluant was the isotonic Tris-HCl buffer, and the eluate was monitored at 260 and 280 nm. Those fractions (5-12) with initial elevated UV absorbances were collected and analysed for aminopeptidase activity, a marker enzyme for prostasomes [36]. Ultraviolet-absorbing fractions with high aminopeptidase activity were pooled and ultracentrifuged at 100,000 × g for 2 h. The pellet representing the prostasomes was resuspended in the isotonic Tris-HCl buffer and adjusted to a protein concentration of 2 mg/mL using a Protein Assay ESL method (Boehringer Mannheim, Germany) (Papers I-V).

PC-3 prostasomes. The human prostatic carcinoma cell line PC-3 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were
maintained in RPMI 1640 cell culture medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine (Sigma Chemicals, MO, USA.). The cells were grown in Falcon Petri dishes (100 mm) at 37°C in a humidified atmosphere of 5% CO₂ in air. When the cells reached 90% confluence, they were recovered by 0.25% trypsin-EDTA and gently scraped. Each plate yielded 2-3 × 10⁶ cells, which were carefully washed in the isotonic Tris-HCl buffer, pH 7.6, and centrifuged. They were again suspended in the Tris-HCl buffer and frozen at -70°C. The frozen PC-3 cells from about 8-10 plates were thawed and pooled and the suspension of disintegrated cells was centrifuged at 1,500 × g for 30 min and then at 6,000 × g for 20 min to remove cell debris. The supernatant was ultracentrifuged at 100,000 × g for 2 h, and pelleted prostasomes were suspended in the isotonic Tris-HCl buffer. The dissociated prostasomes were run through a Sephadex G 200 column (Pharmacia AB, Uppsala, Sweden) at 6 ml/h, and 2 ml fractions were collected. The eluant was the isotonic Tris-HCl buffer, and the eluate was monitored at 260 and 280 nm. Those fractions (5-12) with initial elevated UV absorbance were pooled and ultracentrifuged at 100,000 × g for 2 h and diluted to a protein concentration of 2 mg/mL. These fractions reacting with mAb78 directed against seminal prostasomes were regarded as a suspension of PC-3 prostasomes (Paper II).

**Prostatic tissue and bone metastases.** Tissues from two prostatic glands were obtained from two patients subjected to vesico-prostatectomy due to adenocarcinoma of the urinary bladder with no primary affection of the prostate gland. Tissues from metastases were obtained from 12 patients with bone metastases of prostate cancers (PAD verified, Sahlén et al. Manuscript in preparation 2001). Tissue homogenisations were performed in the isotonic Tris-HCl buffer using an Ultra-turrax homogenisator (Axel Kistner, Stockholm, Sweden) with a rotating pestle (3 x 30 sec). The homogenates were centrifuged 3 times for 15 min at 3000 x g, then twice for 15 min at 10000 x g. Supernatants were ultracentrifuged for 2 h at 100 000 x g. The pellets were resuspended in the isotonic Tris-HCl buffer and applied onto a Sephadex G 200 column and further purification and identification were carried out in accordance with the procedures given above (Paper V).
Antibody production

Two antigens were used: seminal prostasomes (monoclonals) and prostasomes isolated from bone metastatic tissue (polyclonal, chicken antibodies).

Monoclonal antibodies against seminal prostasomes were produced in mice by intraspleenic immunization according to Nilsson et al. [80]. One μg of purified seminal prostasomes was injected intrasplenically 4 times with an interval of 1 month, and a final boost was given 3-4 days before preparing the spleen for fusions. The spleen cells were fused with mouse plasmacytoma cells in FDMEM medium containing polyethyleneglycol (PEG). After 2-3 weeks of HAT selection, the supernatant of the hybridomas was collected and used for screening in an ELISA system using seminal prostasomes as the coating antigen (Papers II and V).

Chicken anti-prostasome antibodies were produced by immunising chickens according to Polson and von Wechmar [81]. Briefly, prostasomes from prostate cancer bone metastatic tissue (2 mg/mL) serving as antigen were emulsified with an equal volume of Freund’s adjuvant. White Leghorn hens were immunized intramuscularly in the breast muscle with 0.5 mL of the emulsified antigen. After the initial immunisation the animal received three booster injections at 2-weeks interval. The first immunisation was performed with Freund’s complete adjuvant and the booster immunisations with Freund’s incomplete adjuvant. Eggs were then collected, labelled and stored at +8 °C until antibody preparation. The antibodies were purified by PEG precipitation (Papers IV and V).

Sperm motility analysis

The development of computer-aided semen analysis (CASA) has made it possible to measure motility characteristics objectively and precisely.

The sperm motility analysis was done in accordance with the guidelines for application of CASA technology. At each time of measurement, at least 200 spermatozoa from each aliquot sample were analysed in order to monitor sperm movement characteristics. This was done with an HTM semi-automated motility analyser, using a micro-cell chamber with a depth of 20 μm. We selected the main analysis parameters.
and conditions of measurement from built-in standard programmes (temp, 37.2°C; frame rate, 20 at 25/s; path velocity (VAP), 10-290 HM/S; straight line velocity (VSL), 0-290 HM/S; track speed (VCL), >90-290 HM/S). The accuracy of the HTM in identifying motile and immotile spermatozoa was checked with use of the play-back function. Data from each individual cell track were recorded and analysed.

The attributes of sperm kinematic parameters evaluated included total cells (x10⁶/ml); motile cells (velocity >10 µm/s, %); rapid cells (velocity>25µm/s, %); average path velocity (VAP, µm/s; the spatially averaged path that eliminates the wobble of the sperm head); straight line velocity (VSL, µm/s; the straight-line distance from beginning to end of track divided by time taken); straightness (STR, %; VSL/VAP × 100%) and amplitude of lateral head displacement (ALH, µm; the maximum width of the sperm head oscillation). These parameters were chosen since it has been suggested that they are of importance for the fertilising capacity of spermatozoa, and they contained significant prognostic information about the time to conception (Papers II and V).

Fig. 2. Parameters of sperm movement (according to HTM manual)
Immunocytochemical staining

The washed spermatozoa mixed with 0.1mg/ml of PC-3 prostasomes or seminal prostasomes were incubated at 37°C for 30 min. Four µl of this sperm suspension were dotted onto slides and air-dried, and the slides were then processed with immune staining. They were first blocked in phosphate buffered saline (PBS), (0.02 M NaH₂PO₄, 0.15 M NaCl, pH 7.2) supplemented with 3% BSA for 15 min and thereafter incubated with the biotinylated monoclonal antibody (mAb78) diluted 1:10 in PBS-BSA for 1 h. A streptavidin complex with alkaline phosphatase diluted 1:500 in PBS-BSA was applied for 1 h. The antibody reaction was visualised by adding a substrate for alkaline phosphatase, diluted 1:100 in 0.1 M Tris-HCl buffer, pH 8.2, for 20-30 min. The mAb78 was raised against seminal prostasomes and detected also seminal and native prostasomes and a secretion in the prostate epithelium. The antibody binds to an antigen, which was distinct from prostate specific antigen (PSA) and prostate-specific acid phosphatase (PAP) [82].

As a control for the specificity of the staining procedure, the washed spermatozoa were mixed with a double volume of non-biotinylated monoclonal anti-prostasome antibody and incubated for 30 min at 37°C, in order to block both the antigen-binding site of any prostasomes that may remain on the washed sperm cells and any cross-reactivity on the sperm cells, then washed three times with the isotonic Tris-HCl buffer and centrifuged at 800 × g for 10 min. Four µl of this sperm suspension were dotted onto slides and air-dried. The slides were then processed for conventional immunostaining as described above. The following three negative controls were run: after incubation in non-biotinylated mAb78, the slides were processed either with streptavidin-conjugated enzyme to check that this complex did not bind unspecifically, or with conventional immunostaining to see that no unmasked prostasomes remained. After addition of 0.1 mg/ml of PC-3 prostasomes to the slides incubated for 30 min, we again checked to see that the streptavidin-conjugated enzyme did not bind unspecifically (Papers II and IV).
Sperm agglutination
Human sperm cells (2 x 10^6) were incubated with increasing concentrations of chicken polyclonal anti-prostasome antibodies (0.08-3 mg/ml) for 30 min at 37 °C. The sperm cells were then observed for agglutination by light microscopy.
Inhibition of anti-prostasome antibody-mediated agglutination was investigated by reacting the chicken polyclonal anti-prostasome antibodies (0.5 mg/mL) with increasing concentrations of seminal prostasomes (0.05-1 mg/mL) for 30 min at 37 °C. The antibodies were then incubated with sperm cells for 30 min at 37 °C and agglutination was observed by light microscopy. An irrelevant polyclonal chicken antibody was used at the same protein concentration as a control for the specificity of the agglutinating effect of the anti-prostasome antibody (Paper IV).

SDS-PAGE and immunoblotting
Sodiumdodecanylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed with Bio-Rad systems (Hercules, CA, USA) according to the instructions of the manufacturer. Purified prostasomes were loaded on a 12-14% polyacrylamide gel. The proteins were visualized by Coomassie staining (20% ethanol, 5% acetic acid, and 0.07% Coomassie Brilliant Blue R250, Bio Rad).
For immunoblotting, the proteins were electro-transferred to nitrocellulose membrane. The membranes were then blocked for 1 h with 5% dry skim milk in PBS. For visualisation of the proteins, the membranes were incubated overnight with biotinylated prostasome antibodies (diluted in PBS 1:5 000 for the monoclonals and 1:10 000 for the polyclonals). The following day, the membranes were washed 3 times with PBS and incubated with streptavidin alkaline phosphatase (Zymed Laboratories Inc., CA, USA) for 1 h. The visualisation was made by NBT/BCIP tablets (Boehringer Mannheim, Germany) (Paper V).

Flow cytometry
The binding of antibodies to different antigens at the surface of the prostasomes or spermatozoa was analyzed using an EPICS XL™ flow cytometer (Coulter, Hialeah, FL, USA). In paper IV, human spermatozoa (5x10^6) were incubated with 50 µL each
of the ASA positive and ASA negative sera for 60 min at 37°C in 5% CO₂ /95% air. After incubation, the cells were washed three times with PBS and resuspended in 50 μL of PBS-1% BSA. The binding of IgG was determined by incubating the sperm cells with chicken antihuman IgG-FITC antibody, and for the binding of complement factor C3, with chicken antiC3-FITC antibody for 1 h at room temperature before analysis on flow cytometer.

In paper V, 7 selected FITC-conjugated, monoclonal mouse anti-prostasome antibodies and one chicken polyclonal anti- prostasome antibody were incubated with 6 μg of prostasomes for 30 min at 20°C before analysis. In controls, the specific antibodies were replaced by an irrelevant chicken anti-insulin antibody or mouse monoclonal antibody at the same concentration (Papers IV and V).

Assessment of antibacterial activity

Bacterial cultures. Bacillus megaterium (strain M A, CCUG 1817), Bacillus laterosporus (CCUG 7421), Bacillus polymyxa (CCGU 7426), Bacillus sphericus (CCGU 7428), Bacillus subtilis (CCUG 1638), Bacillus firmens (CCGU 74118), Bacillus lichenformis (CCUG 7422), Bacillus cerus (CCUG 7414), Klebsiella (ATCC 13883) and capsulated Pneumococcus (clinical isolate) were purchased from the Culture Collection, University of Gothenburg (CCUG), Gothenburg, Sweden, American Type Culture Collection (ATCC), Manassas, VA and stored frozen in aliquots at -70°C. New aliquots were cultured for each experiment. All cultures were performed on CLED agar base (OXOID) at 35°C.

Antibacterial assay. This was a two-step procedure. The first step involved growth inhibition of the bacteria in yeast extract-free Luria-Bertani medium (LB, 1% Bacto-tryptone, 0.5% NaCl, pH 7.5) during incubation with prostasomes under various conditions. Logarithmically growing bacteria in modified LB media at 37°C were diluted to a starting absorbance at 620 nm of 0.017, i.e. an optical density (OD) that corresponded to approximately 1×10⁵ colony forming units (CFU)/mL. The second step implied outgrowth of bacteria from a 40 μL portion of the incubate on CLED
plates over night at 37°C. Microbial growth was assessed by determining CFU on the CLED plates. The isotonic Tris-HCl buffer containing BSA (2mg/mL) was used as a control in each experiment to exclude any possible effect by the buffer on the bacterial growth. With a fixed concentration of bacteria, initial experiments were performed to determine the optimal concentration of prostasomes in the range 0-200 µg/mL. With Bacillus megaterium, a kinetic analysis was performed with a fixed concentration of prostasomes (20 µg/mL) and the logarithmically growing bacteria involving incubations for 0,1,3,5 and 24 h at 37°C. The prostasomes were also subjected to heat-treatment at 80°C in a heat block for up to 60 min prior to incubation for 1 h to test their thermo-stability (Papers III and V).

Scanning electron microscopy
Scanning electron microscopy of the bacteria (Bacillus megaterium) was performed in a JEOL SEM 5200 at a voltage of 10 KV. For electron microscopy, prostasomes (200 µg/mL) and bacteria (OD₆₂₀ = 0.170) were concomitantly inoculated on plates and immediately (zero time) and after given times transferred to a fixation solution of 4% glutaraldehyde in PBS (pH 7.5). The specimens were thereafter filtered through a 0.1 µm filter (Millipore) and coated with an atomic layer of gold in accordance with the manufacturer's instructions (paper III).

Atomic force microscopy (AFM)
Bacillus megaterium incubated with prostasomes under various conditions were studied. All AFM scannings were performed on a Nanoscope IIIa multimode AFM (Digital Instruments, Santa Barbara, CA) in tapping mode, using silicon tips with an end radius of 10 nm and a resonance frequency of about 300 KHz. All images were taken with a new tip and with a 4 x 4 µm scan area. The images were analysed with respect to height and roughness (Paper III).
Demonstration of biochemical markers

ELISA plates (F96, Polysorp, Nunc) were coated with 4 µg purified prostasomes, diluted in 100 mM NaHCO₃, pH 9.5 (coating buffer), for 2 h at 37°C. The plates were washed 3 times and blocked for 1 h at 37°C with the coating buffer containing 3% BSA. After blocking, the plates were washed 3 times with 200 µL PBS containing 0.1% Tween (PBS-T).

In paper IV, the plates were incubated with 200 µL of serum samples (diluted 1:5 in PBS) for 2 h at 37°C. After 3 washings with 200 µL of PBS-T, a chicken anti-human IgG-biotinylated antibody (1:500 in PBS) was added and incubated for 1 h at 37°C. The plates were washed 3 times with 200 µL PBS-T, incubated with streptavidin-alkaline phosphatase conjugate (1:500 in PBS) at 37°C for 1 h and then washed 3 times with 200 µL of PBS-T. 200 µL of alkaline phosphatase substrate solution (1mg/mL p-nitrophenyl phosphate in 1M diethanolamine, 0.5 mM MgCl₂, pH 9.8) were added and the plates were incubated for 25 min at room temperature in the dark. The reaction was stopped by 50 µL of 5M NaOH.

In paper V, the plates were incubated with anti-CD10, anti-CD13, anti-CD26, anti-CD46 diluted 1:200 in PBS for 2 h at 37°C. After another 3 washes with 200 µL PBS–T, 200 µL goat-antimouse IgG horse radish peroxidase (HRP) conjugated antibodies, diluted 1:1000 in PBS, were added and incubated for 1 h at 20°C. The plates were washed 3 times with 200 µL PBS-T and incubated with substrate (tetramethyl benzidine, Zymed Laboratories) for 15 min at room temperature and protected from light. The reaction was stopped by adding 50 µL 1.8 M sulphuric acid. The absorbance was measured at 450 nm by an ELISA reader (SPECTRA Max 250, Molecular Devices, Sunnyvale, CA, USA) (Papers IV and V).

Aminopeptidase (CD13)

Aminopeptidase activity was measured using the synthetic substrate Suc(Ala)₃pNA. The absorbance at 410 nm (paranitroanilide) was recorded at 25°C [36] (Papers I-V).
5′nucleotidase (5′NT) and γ-glutamyltransferase (GGT)

5′NT and GGT activities were determined at 25°C with commercially available Sigma kits (Sigma Company, St Louis, MO, USA) (Paper V).

Neuroproteins and neuropeptides

Chromogranin A (CgA), chromogranin B (CgB), synaptophysin (Syn) and neuropeptide Y (NPY) were assessed by radioimmunoassay methods according to Stridsberg et al [83-85] and vasoactive intestinal polypeptide (VIP) was measured by commercially available kit (Euro-Diagnostica AB, Malmö, Sweden) (Paper V).

Lipids

The prostasome lipids were extracted with chloroform/methanol (1:1 v/v). One volume of the prostasome suspension was mixed with 6 volumes of chloroform/methanol. The extracts were enriched with 2 volumes of 0.15 M NaCl acidified to pH 4. Two phases were obtained. The lower, chloroform (non-polar) phase was collected and the upper (polar) phase was washed once with chloroform and the new chloroform phase was combined with the original chloroform phase. The combined chloroform phases were analysed regarding cholesterol and phospholipid contents. Cholesterol was determined enzymatically with the Monotest assay (Sigma) and phospholipids were determined by measuring liberated phosphorous as previously described by Chen et al [86] (Paper V).
RESULTS AND CONCLUSIONS

Paper I
Swim-up medium containing EBSS, albumin (1.25 g/L), and prostasomes was most efficient regarding the yield of motile spermatozoa after thawing of cryopreserved samples. An increased amount of the motile spermatozoa by 50% (p<0.001) was obvious compared to the conventional medium containing EBSS and albumin but lacking prostasomes. Also, replacing 1.25 mg/mL albumin in EBSS with prostasomes (corresponding to 1 mg protein/mL) resulted in a 30% increase (p<0.002) of motile spermatozoa. The prostasome presence in swim-up medium always led to a significantly increased portion of also immotile spermatozoa ending up in a higher absolute amount of spermatozoa. The motile/immotile ratios were 2.5 (albumin) and 2.3 (albumin plus prostasomes), respectively, contrary to only 1.6 in presence of prostasomes alone.

The effect of some other effectors in EBSS-containing swim-up media on the recovery of motile spermatozoa was also tested and compared with the effect of prostasomes. EBSS-glucose was somewhat inferior to EBSS-prostasomes (p<0.002). No further effect was noted by the combination of glucose and prostasomes in EBSS above that achieved by prostasomes alone. Also, EBSS-containing prostasomes was approximately 20% superior to the EBSS-adenine mixture (p<0.001) and, again, no further improvement was observed by the combination of adenine and prostasomes in EBSS. Finally, combining glucose and adenine in EBSS was not enough to surpass prostasomes in EBSS regarding the yield of motile spermatozoa.

These results suggest that prostasome inclusion in swim-up medium might be of benefit in improving results in assisted reproductive technologies using freeze-thawed spermatozoa.

Paper II
A comparison was carried out concerning the effects of different concentrations of PC-3 prostasomes, seminal prostasomes and BSA on sperm motility at 30 min of incubation. The proportion of motile sperm cells increased from about 40% with PC-3
prostasomes at a protein concentration of 0.05 mg/ml to about 55% at a concentration of 0.1 mg/ml (P<0.01), whereafter only minor changes were apparent. Neither was there any significant difference between PC-3 prostasomes and seminal prostasomes at any of the different concentrations tested except at the highest protein concentration (1.0 mg/ml)(p<0.05). Significant increases in motility were found with BSA and seminal prostasomes at all three concentrations as well.

The influence of different concentrations of PC-3 prostasomes (0.05, 0.1, 0.25 and 1.0 mg/ml) on the sperm motility was followed from 30 to 120 min. All concentrations showed a decline in the motility promoting effect over time. A prostasome concentration corresponding to 0.1 mg/ml protein resulted in the best stimulatory effect over time. The lowest concentration of PC-3 prostasomes was apparently counterproductive after 90 and 120 min. Hence, a strict dose/response relationship was not evident with the PC-3 prostasomes.

The effects of PC-3 prostasomes, seminal prostasomes and BSA on sperm cell motility over time were compared at a protein concentration of 0.1 mg/mL. All three effectors significantly stimulated sperm motility (defined as the proportion of motile cells). For instance, at 60 min of incubation, control spermatozoa had a motility of about 15%, while prostasome- or albumin-supplemented spermatozoa had a motility of about 50% (p<0.001). There were no significant differences between the two types of prostasomes in their stimulatory ability, and their efficiency decreased with time in a similar fashion.

The heat treatment did not significantly influence the stimulatory ability of PC-3 and seminal prostasomes at a protein concentration of 0.25 mg/ml, but heat treatment of BSA resulted in a decrease in sperm motility from about 50% to about 15% (i.e. the control value) at 30 min of incubation (P<0.001). However, heat-treated prostasomes showed a decreased activity over time similar to that of non-heated prostasomes. Still a discrepant behaviour was observed between heat-treated albumin (heat-labile) and heat-treated prostasomes (heat-stable) indicating that the sperm motility-promoting effect was not an isolated protein effect.

Immunostaining with anti-prostasome monoclonal antibody (mAb78) revealed that the PC-3 prostasomes and seminal prostasomes adhered to the sperm cells. The staining,
which occurred all over the spermatozoa, was intense on the mid-pieces and weaker on
the sperm heads. The mid-pieces are the locus for sperm mitochondria. Herewith,
some prostasome component may activate the spermatic mitochondrial function, thus
increasing sperm motility.
Both PC-3 prostasomes and seminal prostasomes were able to reactivate washed and
almost immotile sperm cells. Since the prostasomes adhered to the sperm cells, it
seems that the ability to influence sperm cell motility also involves a membrane-to-
membrane contact. Considering that the spermatozoa were suspended in an isotonic
Tris-HCl buffer without any supplementation, the results suggest that PC-3
prostasomes – similar to seminal prostasomes- have a marked ability to restore
different aspects of sperm cell motility. Our results also strengthen the view that PC-3
prostasomes may have some functional properties in common with seminal
prostasomes and that PC-3 prostasomes could substitute for seminal prostasomes in
analyses of the sperm motility promoting effect in such an experimental system. PC-3
prostasomes were in fact superior to human serum albumin in the recovery of motile
freeze-thawed spermatozoa in a swim-up medium (our unpublished data). We
therefore conclude, that PC-3 prostasomes, isolated from in vitro-grown PC-3 cells,
bear a functional resemblance to prostasomes isolated from human seminal plasma.
Therefore, the reactivating effect of prostasomes on sperm motility is a prostasome-
genuine property and not the result of an adhesion of any seminal plasma component
to prostasomes.

**Paper III**

A dose-response relationship was apparent for incubation of prostasomes (0-200 µ
g/mL) with *Bacillus megaterium* for 1 h. Prostasome concentrations less than 20 µ
g/mL had no visible growth inhibitory effect on the bacteria, mean value ± SD, 505 ±
5 CFU. A clear cut effect was however seen at a prostasome concentration of 20 µ
g/mL with a mean of 9 ± 2 CFU. A complete inhibition of growth was obtained with
prostasome concentrations of 30 µg/mL and above that (CFU = 0).
The effect of incubation of prostasomes (20 µg/mL) for various times on the growth of
*Bacillus megaterium*, resulted in an effective inhibition already after 1 h of incubation
with a CFU mean of only $7 \pm 2$. After 3 h of incubation with prostasomes (20 $\mu$g/mL) a complete growth inhibition was attained with no formed colonies at all. A zero time value of $501 \pm 15$ CFU was obtained by briefly mixing prostasomes with the bacteria in LB medium followed by immediate application of the mixture for outgrowth on CLED plates over night at 37°C. Heat treatment of prostasomes at 80°C for up to 2 min prior to incubation did not reduce their bacterial growth inhibitory effect. With increasing heat-treatment times from 5 min and more, a successive weakening of the prostasome effect was however apparent. After 20 min of heat treatment a complete abrogation of the anti-bacterial activity had taken place, resulting in a bacterial growth that was equivalent to the control, $500 \pm 14$ CFU. Millipore-filtered or dialyzed prostasomes had an undiminished bacterial growth-inhibitory effect. Also, the prostasomes subjected to detergent treatment and ultrasonication giving rise to (after ultracentrifugation) a supernatant (dialyzed) and a pellet (washed) demonstrated a preserved antibacterial activity. When adjusted to an equal protein content the supernatant and pellet phases had about the same antibacterial effects. This was indicatory of prostasome intactness not being a necessary prerequisite for the antibacterial effect. The effect was not due to the detergent since the buffer control (originally containing the same concentration of detergent and then dialyzed in the same fashion) did not affect the bacterial growth.

Ultrastructurally, increasingly irregular contours and a loosening of the smooth surface were observed combined with a fragmentation of the bacteria. After prolonged contact with the prostasomes the bacterial surface became irregularly deformed and disrupted. Small caveola were identified with a diameter of about 100-150 nm, i.e. corresponding to the mean diameter of prostasomes. Among 9 other bacterial strains tested, a complete growth inhibition by prostasomes was attained in 3 strains while the other 6 were unaffected at the prostasome concentration used (30 $\mu$g/mL). Incubation with small amounts of human prostasomes results in a marked decrease or a total abrogation of outgrowth of *Bacillus megaterium*. This antibacterial activity is associated with bacterial membrane deformation in which process the creation of membrane cavities appeared to be essential. When pore formation is complete, any action potential across the cell membrane is untenable since electrochemical and
chemical gradients are dissipated, resulting in bacterial cell death. The antibacterial effect of prostasomes was not strictly confined to *Bacillus megaterium* since a few others were also sensitive. Therefore, it is reasonable to conclude that the prostasomal effect, primarily studied on *Bacillus megaterium*, has some general significance. The present finding shows that prostasomes, or proteins or peptides derived from prostasomes, can have antibacterial effects. Since peptides with anti-microbial functions have been identified in lower animals and insects, this may be considered as a genuine though unspecific, but still important, defense mechanism.

**Paper IV**

The addition of polyclonal chicken anti-prostasome antibodies caused approximately 80% of the spermatozoa to agglutinate. The agglutination displayed several types of sperm formation; mostly tail to tail contacts, but the type of interaction was dependent upon the concentration of the anti-prostasome antibody. When anti-prostasome antibodies were pre-incubated with high concentrations of prostasomes no agglutination was observed during the subsequent contact with spermatozoa. Nor was any agglutination observed when using an irrelevant chicken antibody.

IgG antibodies against sperm cells were detected in all of the patients’ sera. In the majority of cases (90%), the sera of the patients caused complement activation, measured by the deposition of C3 on the sperm cells. The twenty sera used in these experiments had also been analysed for ASA titre by the Tray agglutination test. No significant correlation was found between sperm-bound IgG and ASA titre, $r = 0.40$, whereas significant positive correlations were found between sperm-bound C3 and ASA titre, $r = 0.43$ and especially between the deposition of C3 and IgG, $r = 0.79$.

The twenty Tray agglutination test-positive patients’ sera were analysed by ELISA for the presence of antibodies against prostasomes. Binding of IgG antibodies to prostasomes was clearly visible although there were variations in binding capacities. The absorbance values were significantly higher than those with normal serum as a negative control. No significant correlation ($r = 0.30$) was found between sperm cell agglutination capacity and prostasome binding affinity of patients’ sera.
The high percentage of patients with anti-prostasome antibodies in this study shows that prostasomes could be one of the major targets for ASA. The results of the present study demonstrate that ASA of the IgG type in serum of infertile men and women recognise prostasomes as antigens, and that polyclonal antibodies raised against prostasomes agglutinate human spermatozoa. This suggests that the prostasomes are strongly immunogenic and that they can at least partly contribute to immunological infertility.

**Paper V**

Flow cytometry showed that prostasomes derived from seminal plasma, prostate tissue and prostate cancer bone metastases had a similar size distribution, although that of native prostasomes was somewhat wider. Separation of prostasomal proteins by SDS-PAGE revealed similarities between the types mainly found in the high molecular weight range with consistent occurrence of bands at about 150 kDa, 120 kDa, 90 kDa and 60 kDa. In the low molecular weight range, three distinct bands of approximately 18 kDa, 7 kDa and 5 kDa were typical for metastasis-derived prostasomes. Prostate tissue (native) prostasomes displayed a broad, distinct band of 6 kDa and both seminal and native prostasomes contained a protein band of approximately 10 kDa. In Western blot, all 9 monoclonals recognized a protein with a molecular weight of approximately 60 kDa in all 3 types of prostasomes. In 5 of the monoclonals, we found an extra band of 7 kDa in the metastasis-derived prostasomes. The polyclonal antibody recognised a wide range of proteins in all 3 types of prostasomes with the 10 kDa band being predominant in the seminal and native prostasomes while metastasis-derived prostasomes lacked it. Incubating the prostasomes with 9 monoclonal antibodies and 1 polyclonal antibody resulted in a binding of all 10 antibodies to the three prostasome types suggesting the presence of common surface antigens. The antibodies showed no differences in binding capacity between the types.

The content of known biochemical markers for seminal prostasomes was analysed with ELISA and radioimmunoassays (RIA). Four known CD markers were tested by ELISA coated with the 3 different prostasome types. The same inter-relation of the 4 CD markers was seen in each prostasome type. Metastasis-derived prostasomes had a
much lower overall content of all 4 CD markers. Seminal and native prostasomes showed a similar content of CD 10 (enkephalinase). The absorbance value for CD26 (dipeptidylpeptidase IV) was about the double in the native prostasomes compared to the value of seminal prostasomes, whereas CD13 (aminopeptidase) showed a reversed distribution. The CD46 (membrane co-factor protein) showed about equal amounts in all 3 prostasome types. All three prostasome types displayed an unusual CgB/CgA ratio with CgB in abundance over CgA, shown earlier [60]. Prostasomes derived from the bone metastasis contained a 10-fold higher amount of CgA compared to the other 2 prostasome types.

Aminopeptidase activity was highest in seminal prostasomes while the corresponding activity was less than 10% in metastasis-derived prostasomes. The native prostasomes were somewhere in between in this regard. On the other hand, this latter prostasome type showed the highest 5´NT activity. The GGT activities were rather similar among the 3 types. All 3 prostasome types had a very high cholesterol/phospholipid ratio, which previously was found to be characteristic for the prostasome membrane [22, 87].

Seminal and metastasis derived prostasomes showed the same promotive effect on the spermatozoa, whereas native prostasomes had no additional effect compared to control. All three prostasome types showed an antibacterial activity against Bacillus megaterium at a given concentration (30 μg/mL). A complete inhibition of growth (CFU=0) was obtained after 1 h of incubation with the bacteria.

We conclude that there are strong similarities between prostasomes isolated from seminal plasma, prostate tissue and prostate cancer bone metastasis and that this was valid for both size distribution, biochemical and functional properties. We also conclude that prostasomes have a pure prostatic origin and that an apocrine secretory mechanism can be ruled out. Instead it is assumed that the prostasomes are internalised in storage vesicles of the secretory cells and released in toto by an ordinary exocytotic event.
DISCUSSION

Motility stimulant effects

In spite of the important uses of cryopreserved spermatozoa, little is known about the physical and biochemical events which occur during sperm freezing, storage and thawing. Spermatozoa from most species survive current cryopreservation protocols very poorly and best efforts usually result in recovery of only around 40% of the initial semen motility. Sperm function is also impaired, as manifested after thawing by shorter longevity and reduced membrane stability. The process is complicated by the biochemically diverse compartments of the spermatozoa (acrosome, nucleus, mitochondrial-flagellar network (Fig. 3 A and B), all of which may respond quite differently to freezing and thawing. The respiratory metabolism of sperm cells has been found to be altered after freeze-thawing [88, 89]. A release of hyaluronidase, aspartate aminotransferase and lactic acid dehydrogenase from the cells has also been detected [90]. Of about 60 enzymes examined, only two showed diminished activity, viz. the first two enzymes of the hexose monophosphate pathway [91].

The tail also appears susceptible during freezing, especially the axial filaments [92-94]. Impairment of tail function would account for the increased incidence of erratic and circular movements [95]. Freezing and thawing does not appear to damage the DNA nor is there evidence that genetic changes occur after freezing [96]. The sperm are also exposed to reactive oxygen species (ROS) during their transit through wide temperature changes [97].

It is generally recognized that the fertility potential of frozen-thawed human semen is reduced, compared to fresh semen. This incontestable drop in fertility is indeed more marked in human semen than for the majority of the domesticated animals, especially cattle. Comparisons are not easily made and the variables are many but the overall pregnancy rate with frozen human semen is approximately 10-15 % lower than with fresh semen [98]. It has also been concluded from studies of several properties of semen that the zona pellucida penetration was the most significant and gave the highest correlation with the conception index [99].
Prostasomes have proven to promote forward motility in human spermatozoa rendered immotile by buffer washings [72-74]. This favourable effect was preserved even after heat treatment of the prostasomes at 80°C for up to 20 min [72]. Swim-up media containing prostasomes significantly increased the recovery of motile spermatozoa and also the percentage of spermatozoa displaying progressive motility after 1 h of incubation [75]. Further, a beneficial effect of prostasomes was noted on lateral head displacement and percentage of hyperactive spermatozoa during the first 6 h of incubation [75]. This beneficial effect of prostasomes was also confirmed in our study involving cryopreserved spermatozoa (paper I). We showed that prostasomes not only dramatically increased the number of post-thawed motile spermatozoa when included in the swim-up medium, but also the total number of cells, i.e. the sum of dead and
alive spermatozoa was increased. The reason for that phenomenon is not evident at present. One explanation could be the above mentioned prostasomal induction of hyperactive spermatozoa [75] with a concomitant hypermetabolic state, followed by an energy crisis and necrosis of the already damaged post-thawed spermatozoa. Whatever the explanation is, the dramatic increase in the number of post-thawed motile spermatozoa by prostasome inclusion in swim-up medium is evident, and this adds credence to the view that prostasomes could be of benefit in increasing the fertilization rate achieved in assisted reproductive technologies.

Also, the prostasome-like vesicles from the PC-3 prostate cancer cell line (PC-3 prostasomes) showed a beneficial effect on washed spermatozoa (paper II). We found that the addition of PC-3 prostasomes increased the proportion of motile spermatozoa from about 15% to 50-70%, which was satisfactory. The positive effect of the PC-3 prostasomes on the spermatozoa lasted only for a limited period of time, most probably because of a lack of fuel, and generally there was little motility after 2 h [cf.:74]. Thus, the PC-3 prostasomes possess a motility stimulating ability similar to that of seminal prostasomes.

**The mechanism behind motility-stimulation**

The mechanism by which non-heated prostasomes initiate forward motility in buffer-washed spermatozoa is not known, nor is it known whether the mechanism requires a membrane contact or not [100]. However, since prostasomes were found to adhere to sperm cells when subjected to free zone electrophoresis in a previous study [65] and when observed with immunocytochemistry in the present study (paper II), it seems likely that the working principle may include a close prostasome-sperm cell contact. For instance, sperm membrane properties, such as membrane permeability to Ca²⁺ and H⁺, may be influenced. Other effectors in this context could be thermostable peptides, *viz.* neuropeptide Y or vasoactive intestinal peptide (VIP), which are indeed components of the prostasomes [60]. The peptide VIP has been shown to be a potent stimulator of adenylate cyclase activity in many target tissues [101], an enzyme that synthetizes the second messenger cyclic AMP (cAMP) from ATP. An important role for cAMP in the initiation and maintenance of sperm motility has been known for a
long time [102-104]. Protein kinase activation and enhanced phosphorylation of endogenous proteins are concomitant events associated with elevated cAMP. It has been shown that elevated cAMP results in increased phosphorylation of multiple proteins [105]. A dominant soluble phosphoprotein, axokinin, is associated with this activation in mammalian sperm, later proved to be the type II regulatory subunit (RII) of cAMP-dependent protein kinase. Other soluble factors involved in activation of sperm motility have been identified in bovine and trout sperm (reviewed by Tash [106]).

Comparing the literature to date, a number of observations is beginning to emerge that suggests some common aspects concerning the regulation of axonemal movement. Although there might be slight species differences, many axonemal systems share an ability to respond to elevated cAMP:

1. In most cases, elevated cAMP is associated with stimulation of sperm movement [106]. Human sperm, containing certain parameters of movement associated with hyperactivation in response to Ca\(^{2+}\) are also attenuated by cAMP [107].

2. In order to respond to cAMP, these cells must contain cAMP-dependent protein kinase.

3. cAMP-dependent protein kinase stimulates phosphorylation of axonemal components resulting in changed axoneme movement.

4. The population of proteins that can be phosphorylated by cAMP-dependent protein kinases in the axoneme is a subset of all phosphoproteins, which are in turn a subset of all axoneme proteins. There are approximately 200 proteins that comprise the basic axoneme, of which about 80 are known to be phosphorylated [108].

It should be kept in mind, however, that prostasomal VIP may have other functions as discussed in a recent paper by Carlsson et al. [109].

Further, we found previously by molecular biological methods that dipeptidyl peptidase IV (also known as CD26) was a component of the prostasomes [39]. Since it has become apparent that dipeptidyl peptidase IV is able to mediate binding of cells to extracellular matrix proteins [110-112], we assumed that this peptidase may play a role...
in the prostasome-sperm interaction. Our immunostaining study (paper II) revealed that the whole sperm cell was stained and that the stain was most intense on the mid-piece. This indicated that PC-3 prostasomes (and seminal prostasomes) could adhere to the sperm cell. Since the mid-piece is the region where the mitochondria are located, it is possible that some prostasome component activates the mitochondrial function, with increased sperm motility as a result. Since substance P, which regulates the activation of acetylcholine, can be cleaved by dipeptidyl peptidase IV, the prostasome-bound activity of dipeptidyl peptidase IV may influence the regulatory effect of acetylcholine on sperm motility [113]. The prostasome-associated activity of dipeptidyl peptidase IV might therefore represent the molecular link through which the prostasomes exert their promotive effect on sperm forward motility. It is known that prostasomes fuse with spermatozoa at slightly acidic pH values [68] and that this fusion means a transfer of lipids and proteins to spermatozoa membranes [66]. However, since our experiments were carried out at pH 7.6 a fusion process was less probable. Also, a recent work [114] showed that the sperm motility-promotive effect of prostasomes was not pH dependent, ruling out a fusion being an obligatory step preceding the sperm motility stimulation.

The antibacterial activity of prostasomes

Our findings demonstrate that prostasomes, proteins or peptides derived from prostasomes, can have antibacterial effects (paper III). This antibacterial activity is associated with bacterial membrane deformation in which process the creation of membrane cavities appeared to be essential. When pore formation is complete, any action potential across the cell membrane is untenable since electrochemical and chemical gradients are dissipated, resulting in bacterial cell death. The antibacterial effect of prostasomes was not strictly confined to Bacillus megaterium since a few others were also sensitive. Therefore, it is reasonable to conclude that the prostasomal effect, primarily studied on Bacillus megaterium, has some general significance. This bactericidal effect of prostasomes differs mechanistically from that of neutrophil granulocytes. The principle of action of these latter cells is based on the generation of reactive oxygen species (ROS). Due to their paucity in cytoplasm and antioxidant
mechanisms, spermatozoa have little defence against these ROS, which damage their membrane and DNA [115]. Accordingly, it appears functional that prostasomes rather than neutrophil granulocytes serve as antibacterial agents in semen. What is more, prostasomes themselves act as antioxidants and may counteract the formation of ROS in leukocytes [79].

Interestingly, a C-terminal fragment of chromogranin B has been shown to have potent antibacterial activity [116]. This peptide, called secretolytin, forms a 3-D structure similar to the insect-derived proteins, cecropins, which possess antibacterial activity [117]. The biological activity of these peptides results from their ability to form channels through the bacterial wall and membranes. Recently, also other parts of both chromogranin B and chromogranin A proved to possess antibacterial activity [118].

We have previously demonstrated that prostasomes contain large amounts of chromogranin B [60]. Also in this study (paper V) we demonstrate this unusually large amount of chromogranin B in the prostasomes isolated from different sources.

All three prostasome types (seminal, native and metastasis-derived) proved to possess an equally strong antibacterial activity against *Bacillus megaterium*. It is possible that the observed lytic activity of prostasomes may involve chromogranin B or peptides derived from proteolytic cleavage of chromogranin B. However, the chromogranins are heat-stable proteins [63] but the observed lytic effects of the prostasomes were indeed heat-labile. A conceivable explanation may be that chromogranin B or chromogranin B-derived peptides form heat-labile complexes with other compounds in order to achieve the antibacterial effect or that the generation of biologically active chromogranin B-derived peptides is inhibited by the heat treatment. Hence, a heat-labile proteolytic event might be an obligatory step preceding the lytic effect by prostasomes. In this context it is worthy of note that prostasomes are rich in proteolytic enzymes [70]. Additionally, intact prostasomes were not a prerequisite for the studied antibacterial effect, since fractions obtained after detergent- and ultrasonication treatment of prostasomes were also fully active. An enigma not yet resolved is the selectivity of action of prostasomes in this regard. While *Bacillus megaterium* and some other bacteria are rapidly and readily destroyed, others are not (paper III).
Further, prostasomes may interact harmlessly with spermatozoa (paper II), erythrocytes [119] and leukocytes [19, 120].

The integrity of the human reproductive system against potentially invasive pathogenic microorganisms is crucial. Readily available, preformed antimicrobial proteins of the nonadaptive immune system serve as the body’s first line of defense, while the adaptive immune system becomes involved if the pathogens start to invade. In recent years, several components of the human nonadaptive immune system have been isolated and characterized, among them the only member of the cathelicidins known to exist in humans, the human cationic antimicrobial protein (hCAP-18). The protein was present at high concentration in seminal plasma and also, a strong association of the protein with the surface of spermatozoa was demonstrated [121]. However, no hCAP-18 could be detected in the prostate gland with immunohistochemical methods. There may be other antimicrobial proteins in seminal plasma. The beta defensin hBD-1 occurs in the human testis [122] and the antibacterial peptide seminal plasmin has been isolated in seminal plasma from cattle [123].

In our study we have demonstrated the antibacterial activity of human prostasomes and since the activity was also present at the same high degree in both native and prostate cancer metastasis derived prostasomes (paper V), the biological activity most likely originates from the prostasome itself and is not the result of associated seminal plasma compounds.

**The immunogenic capacity of prostasomes**

The contact of antibody-forming cells with their respective antigens during fetal life induces a state of immune tolerance to autoantigens. Because spermatozoa are not produced in women and are not present in men until puberty, spermatozoal antigens are foreign to the immune systems of both adult men and women. Autoimmunization to sperm antigens is normally blocked because of the sequestration in the blood-testis barrier and in the epididymis by tight junctions. However, antisperm antibodies (ASA) may develop in women after spermatozoal deposition into the genital tract with a compromised epithelial barrier, the peritoneal cavity or the gastrointestinal tract. The susceptibility of some women and not others for mounting an immune response to
sperm antigens is incompletely understood but may relate to a defect in the presence of or response to immunosuppressive factors in semen [124]. In men, obstruction or injury to the male reproductive ducts is associated with ASA formation [125]. Also antibodies against spermatozoa appear in the serum of at least 50% of vasectomized males [126].

The complexity of the prostasomal membrane with over 80 protein entities visible when running 2D electrophoresis [56], render the prostasome highly immunogenic. The strong immunity-inducing capacity of prostasomes has also been confirmed in this study by the production of monoclonal antibodies against prostasomes resulting in clones with high titers (paper V and [127]). Their immunogenicity and the fact that they interact with spermatozoa, and perhaps even fuse [66, 67] make their contribution to the antigenicity of spermatozoa possible. A prostasome coat on swim-up sperm cells was found when we immunostained our specimens with 7 different monoclonal antibodies, raised against prostasomes according to a reported method [128]. Considering that all the monoclonal antibodies tested demonstrated a similar staining pattern, that prostasomes from the cancer cell line PC3 also coated sperm cells, and that a prostasome-sperm binding was observed using other methods [65-67], we believe that prostasomes do coat the swim-up sperm cells.

The results of our study (paper IV) give evidence to the fact that prostasomes are highly immunogenic, interact with spermatozoa and could be one of the major targets for ASA.

The origin of prostasomes

The origin of prostasomes has been under debate. In a preceding paper [57], a polyclonal rabbit antiserum was used against a highly purified preparation of prostasomes from human semen. The antiserum reacted in the supranuclear and apical region of human prostate epithelial secretory cells [57] in a fashion similar to the one that has been described using a monoclonal antibody against prostasomes [127]. The monoclonal antibody used recognized another prostasome - derived antigen [127, 128] than did the polyclonal antibody [57]. Furthermore, a strong reaction was reported with the polyclonal antibody in human epididymis (contrary to testis), and a less
intense one in seminal vesicles. The antiserum also reacted with bile canaliculi in human liver and with cells from collecting ducts in the kidney [57]. A labeling was also prominent in acinar secretory cells of the parotid and submandibular glands as well as in parietal cells of the stomach [57]. The conclusion was that the immunoreactivity with the prostasome antiserum of epididymal and vesicular epithelial cells clearly indicated the heterogeneous origin of components forming the prostasomes during ejaculation. Therefore, due to this varied background of prostasomes, these organelles should rather be designated aposomes or seminosomes indicating that their origin from not only the prostate gland but also other sources could be valid, as the vesiculae seminales and the spermatozoa [57]. A cross-reactivity was also observed with the monoclonal antibody to i.a. syncytiotrophoblast cells of first trimester placenta, to exocrine cells of the pancreas, and to some cells of the pancreatic islets [127]. Hence, polyclonal and monoclonal antibodies against prostasomes react dispersedly with antigens on other cell structures than the prostatic gland in the male urogenital tract as well as outside it. A reaction with macrophages was also evident [127]. The organelles have a pure prostatic gland origin [16, 17, 20, 129, 130], and in that respect the prostatic gland is exceptional in the sense that membrane-surrounded organelles occur extracellularly under physiological conditions. There are no indications of a production and secretion of any organelle in the other accessory genital glands in man [20, 129]. A strong support of human prostasomes deriving solely from the prostate gland and not from the seminal vesicles is the finding of Wilson et al [40]. They measured the activities of dipeptidylpeptidase IV (DPPIV) and its molecular forms using immunoblotting of seminal plasma from men who were vasectomized and from those with different sperm concentrations, and in prostatic and seminal vesicle secretions of men undergoing prostatic surgery. The DPPIV antigen (CD26) and enzymatic activity were present in prostatic secretion, but absent in the secretion of the seminal vesicles. Since DPPIV and CD26 are intimately linked to prostasomes [38, 39], prostasomes do derive from the prostate gland but not from vesiculae seminales. These findings are consonant with the present study showing presence of CD26 on prostasomes regardless of type. What is more, no decrease at all
of a Mg$^{2+}$ and Ca$^{2+}$ dependent ATPase activity (a marker enzyme of prostasomes) was observed when comparing prostasomes isolated from ejaculates of 13 men before and after vasectomy [131]. This finding rules out any significant contribution by the testes and epididymes in the formation of prostasomes. A bovine organelle production and secretion in seminal vesicles have been described with no corresponding formation in the prostate gland and these organelles were denoted vesiculosomes [132].

There is no strong support to the idea of an apocrine secretion of prostasomes. The membrane of these organelles, regardless of their isolation from seminal fluid, prostate tissue or prostate cancer bone metastasis, exhibited a very high cholesterol/phospholipid ratio (about 2:1) (paper V) contrary to plasma membranes in general and the corresponding ratio for human benign prostatic hyperplastic epithelial cells was 0.5:1 [133]. Similarly, this latter ratio agrees with that of human spermatozoa [23, 134]. Hence, there is little basis for the belief that membrane particles derived from the apical plasma membrane of prostatic secretory cells, fragments or portions from epididymal stereocilia and membrane elements detached from spermatozoa were contributory to the formation of prostasomes as has been claimed [57]. Since prostasomes have a net negative surface charge [65], we do not exclude the possibility of the attachment by electrostatic forces of cationic proteins, secreted especially from the seminal vesicles, to the prostasomes [30]. The typical strong quantitative predominance of cholesterol over phospholipids was established in all 3 prostasome types (paper V) and such a lipid composition accounts for a high molecular ordering of the prostasome membrane. The high cholesterol/phospholipid ratio also supports the assumption that prostasome vesicle formation actually takes place in the Golgi apparatus followed by an internalization in larger storage vesicles whereafter they can be displaced in toto from the interior to the acinar lumen by an ordinary exocytotic event [17]. Since the cholesterol/phospholipid ratio of the prostatic epithelial plasma membrane is below 1 [133], a suggested apocrine secretion, i.e. the release of prostasomes through apical protrusions or blebs [57, 135, 136] is ruled out as the secretory mechanism of these organelles from the prostate gland.
Fig 4. Supranuclear parts of epithelial cells in benign hyperplastic human prostate. The secretory cells, which are filled with storage vesicles, have apical surfaces bulging into the duct lumen. The storage vesicles are filled with prostasomes of varying size. Mag. x 66 000. (Published with courtesy by Ove Nilsson).
SUMMARY

The results in this thesis have shown that:

♦ Swim-up media supplemented with prostasomes were superior in comparison to the other effectors investigated in the recovery of motile spermatozoa for insemination. These results suggest that prostasome inclusion in swim-up medium can be of benefit in improving results in assisted reproductive technologies using freeze-thawed spermatozoa.

♦ Immunostaining with anti-prostasome monoclonal antibody revealed that the PC-3 prostasomes and seminal prostasomes adhered to the sperm cells and that the stain was most intense on the mid-piece. This indicates that the PC-3 prostasomes can adhere to the sperm cell. Since the mid-piece is the region where the mitochondria are located, it is possible that some prostasome component activates the mitochondrial function, with increased sperm motility as a result.

♦ CASA did not reveal any difference in ability to activate sperm cells between PC-3 and seminal prostasomes when used at certain concentrations. Further, the motility pattern evoked by PC-3 prostasomes was similar to that of BSA in every respect concerning the effects on various sperm movement characteristics. However, after the moderate heat treatment, the motility promoting effect of PC-3 prostasomes remained, while that of albumin greatly diminished, indicating different mechanisms of motility promoting action. Accordingly, the prostasome effect does not seem to be due to a pure protein action.
Prostasomes, proteins or peptides derived from prostasomes, have potent antibacterial effects. This antibacterial activity was associated with bacterial membrane deformation in which process the creation of membrane cavities appeared to be essential. The antibacterial effect of prostasomes was not strictly confined to *Bacillus megaterium* since a few others were also sensitive. Therefore, it is reasonable to conclude that the prostasomal effect, primarily studied on *Bacillus megaterium*, has some general significance.

The high percentage of patients with anti-prostasome antibodies showed that prostasomes could be one of the major targets for ASA. The results demonstrated that ASA of the IgG type in serum of infertile men and women recognised prostasomes as antigens, and that polyclonal antibodies raised against prostasomes agglutinated human spermatozoa. This suggests that the prostasomes are strongly immunogenic and that they can at least partly contribute to immunological infertility.

Three types of prostasomes (seminal prostasomes, native prostasomes and metastasis-derived prostasomes) demonstrated similarities regarding a high cholesterol/phospholipid ratio, marker enzymes and neuropeptides. This finding was the basis for our belief that they had a common and exclusive prostatic origin. Further, an apocrine secretory mechanism of prostasomes was ruled out. Instead it was assumed that the prostasomes are internalised in storage vesicles of the secretory cells and released *in toto* by an ordinary exocytotic event.
ACKNOWLEDGEMENTS

This thesis were financially supported by grants from the Swedish Medical Research Council and Swedish Cancer Foundation.

I wish to express my sincere gratitude to all those who have guided, supported and assisted me during the course of this work. First of all, I would like to thank:

Gunnar Ronquist, my supervisor and mentor, for introducing me into “the world of prostasomes”, for sharing your profound knowledge in clinical chemistry, for your encouragement and thoroughness, for teaching me the difficult art of scientific writing, and also for reading my manuscripts always arriving in the last minute.

Professor Ove Nilsson, my assistant supervisor, for scientific guidance and support, for teaching me immunohistochemistry and, last but not least, for all the French chocolate boxes!

Mats Stridsberg, my assistant supervisor and co-author, for valuable discussions and constructive criticism.

Anders Larsson, co-author, for your impressive knowledge, close collaboration and never failing optimism.

MonaLill Lundquist, my co-author, and all the staff at the Reproduction center, for good collaboration and for providing me with the most important - all the seminal plasma and sperm samples.

Calle Pählson, for introducing me into the world of “baggar”, sharing your knowledge in microbiology and for valuable discussions.

My co-authors, Lars Johansson, Jian Wang, Cinzia Allegrucci, and Magnus Bergquist, for good and stimulating collaboration.

Göran Sahlén, for providing me with all the tissue material from the Central operation.

Pia Lög Dahl and Lena Lennartsson, for teaching me cellculturing and all the tricks with monoclonal antibody production.

U-B and Inger for sharing lab and running chromogranins.

Gunnilla Strömstedt and Barbro Bjurhäll, for kindness and excellent secretarial help.

Kristina Seton, Eva Lindmark, Jonas Byström, Anders Målarstig, Matilda Johnell, and David Carlander my PhD fellows and friends, for making daily life at work more fun.

Taavo, Lena M, Lena H, Kerstin, IngBritt, Birgitta, Agneta, Ingrid, Ulla-Britta, Karin, Christina, Adil, Xiao, Sheng, Linshu, Lixin, Gosia, Mia, Charlotte and all the rest at the department, for friendship and for creating such a good atmosphere.

Åke Ericson, my room mate, close friend, and group member!!, for all the help, laughs and discussions, and for providing me with maps and train timetables whenever I was going somewhere.

All my friends and family, for reminding me of the outside world.

Henrik, for encouragement and love, and for just being a part of my life.

My parents, Sonja and Bo Carlsson, for making it all possible.
REFERENCES


29. Fabiani R and Ronquist G: Characteristics of membrane-bound 5’nucleotidase on human


43. Morimoto C, Lord CI, Zhang C, Duke-Cohan JS, Letvin NL, Schlossman SF: Role of


69. Arienti G, Carlini E, Verdacchi R, Cosmi EV, Palmerini CA: Prostasome to sperm


83. Stridsberg M, Öberg K, Li Q, Engström U, Lundquist G: Measurements of chromogranin A, chromogranin B (secretogranin I), chromogranin C (secretogranin II) and pancreastatin


126. Law HY, Bodmer WF, Mathews JD, Skegg DCG: The immune response to vasectomy
and its relation to the HLA system. Tissue Antigens 1979; 14:115-139.


