

Schering Foundation Workshop
4

SPERMATOGENESIS

FERTILIZATION

CONTRACEPTION

Molecular, Cellular and Endocrine Events
in Male Reproduction



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E. Nieschlag
U.-F. Habenicht
Editors

Schering Foundation Workshop 4
Spermatogenesis – Fertilization – Contraception



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Spermatogenesis – Fertilization – Contraception

Editors: E. Nieschlag, U.-F. Habenicht

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Schering Foundation Workshop 4

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E. Nieschlag, U.-F. Habenicht, Editors
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With 77 Figures

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Preface

Investigations into the mechanisms of spermatogenesis, sperm maturation and fertilization provide the basis of our understanding of male reproductive physiology. Since in recent years molecular and cellular endocrinology has provided particularly important contributions to our knowledge, the European Workshops on Molecular and Cellular Endocrinology of the Testis, held regularly during even years since 1980, have become a prominent forum for researchers in the field to discuss recent findings and exchange new ideas. The most recent of these Testis Workshops, the 7th, was held on May 5 – 10, 1992 at Castle Elmau in the Bavarian Alps. The main lectures of this Workshops form the core of this book.

In order to provide a broader view of the current status of male reproduction research, this volume also contains the proceedings of the Schering Foundation Workshop on “Basic Mechanisms of Reproduction and Male Fertility Control” which was held in Berlin on October 31 to November 2, 1991. The topics of the two meetings were planned to complement each other. By combining the proceedings of both workshops, it became possible to produce a book that covers a wide spectrum of current highlights in male reproduction research and provides the reader with an up-to-date review of the field.

The timeliness of the book is emphasized by the fact that it was already available when the 7th European Testis Workshop took place. The contributors deserve our special thanks for adhering strictly to the deadlines and making prompt publication possible. We are also grate-

ful to Susan Nieschlag, M.A. and Kerstin Günther for their enduring editorial and secretarial assistance respectively, which accelerated the editorial process.

We would like to acknowledge the assistance provided by the members of the Standing Committee of the European Workshops on Molecular and Cellular Endocrinology of the Testis, as well as the members of the Organizing Committee as listed below.

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Cell Surface Actions of Steroids: A Complementary Mechanism for Regulation of Spermatogenesis?

Focko F.G. Rommerts

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1 Introduction

The question about the concentration of androgen required for maintenance of spermatogenesis has not yet been solved (Rommerts 1988a). Under normal physiological conditions androgen concentrations in the testis are more than 20-fold higher than in peripheral organs. However, results of various studies show that spermatogenesis can also proceed at approximately 20% of the normal testicular an-

drogen level although the production of spermatozoa is diminished (Sun et al. 1989). In this situation, these reduced local androgen concentrations are still several fold higher than the normal peripheral concentrations. Irrespective of the exact minimal level of testosterone required for maintenance of spermatogenesis (with or without the support of FSH), it is difficult to understand easily why androgen-sensitive cells in the testis are triggered in a manner differing from that of other androgen target cells (Sun et al. 1989). Of special relevance to this question is the current opinion that androgen action in various target cells is mediated by only one receptor (Brinkmann et al. 1992).

It may be possible that the biological effects of this single androgen receptor in testicular cells require a relatively higher saturation level before interaction between transcription factors and DNA sequences can occur which subsequently lead to gene transcription. However, it is not possible to explain a dose-dependent androgen effect with this paradigm when androgen concentrations are higher than the saturation level of the receptor. Under normal conditions all testicular cells are probably exposed to such saturating levels of androgen. Even at 20% of normal testicular testosterone levels the androgen receptor is in a tight nuclear binding form (Blok et al. 1992). Any disturbance in the spermatogenic process which occurs following a specific decrease in the testicular androgen concentration to 20% of normal levels can therefore not be mediated by the conventional nuclear androgen-dependent regulatory systems.

In order to explain androgen-dependent withdrawal symptoms at this high concentration of steroids, one must theoretically postulate an alternative androgen signal transducing system.

In recent years there has been a growing number of observations that a specific class of steroids can rapidly influence functional properties of neurons in the brain. Since these steroids often have a characteristic structure and do not possess genomic stimulating activity, a special name has been coined for these steroids: neurosteroids (Baulieu 1991). During the Fifth Testis Workshop in 1988, it was proposed that the biological concept of the brain-testis axis be restructured. An integrated organ, the neuro testis, was proposed to underline the large number of similarities between the biochemical regulatory systems in the brain and testis. It was also postulated that during evolution many

ancient and foolproof testicular regulatory systems may have been applied in the brain for regulation of neuronal activities (Rommerts 1988b).

The present chapter can be considered as a reversal of this supposed evolutionary process, namely the application of the recently acquired concept and knowledge of neurosteroids action – i.e. cell surface actions of steroids in the brain – to the study of steroid-dependent regulatory systems operating in the testis. These cell surface actions of steroids in the testis could constitute the above-postulated alternative androgen signaltransducing system possibly operating in conjunction with the conventional nuclear androgen actions.

2 Spermatogenesis, a Peculiar Differentiation Process

The development of spermatozoa from spermatogonial stem cells is the result of a series of individual differentiation steps. Compared to other differentiation events, such as the hematopoietic system, spermatogenesis has some unique properties. It is a one-way process without bifurcations, since differentiating spermatogonia are committed to undergo two meiotic divisions and subsequently to follow a spermiogenic differentiation before developing into spermatozoa. In this process hormones can only regulate the efficiency that is the percentage of cells that reaches the final developmental stage. During the spermatogenic process germinal cells seem to have only a binary choice between a developmental programme and a cell death programme (apoptosis).

The kinetics of this long-term differentiation process which requires more than 6 weeks for completion, are remarkably constant (Knobil et al. 1988). Even in the absence of hormones such as following hypophysectomy, the rate of the developmental process is not altered although an increasing number of intermediate cell types degenerates. Our understanding of the regulation of spermatogenesis has not much changed since the investigations of Clermont and Harvey in 1965. With modern techniques we have been able to define better the molecular events in the germinal cells such as gene expression and the synthesis of specific proteins which form the basis for the differentiation

process, but we still do not understand how differentiation or cell death are regulated.

In somatic cells there are many signal transduction pathways for the control of mitosis, development or different states of cellular activity and a variety of hormones and growth factors can activate these cellular activities. So far, such regulatory pathways have not been reported to exist in germinal cells and the development of these cells seems therefore to be regulated mainly by an intrinsically operating programme in a more or less autonomous fashion that has alternatively been described as "cryptocrine regulation". The observation that isolated germinal cells from *Xenopus* testes can develop in a culture medium for a period of 14 days in the absence of Sertoli cells (Risley 1983) supports this view.

Development of germinal cells in mammals can only proceed in close proximity to Sertoli cells. According to current dogma, the Sertoli cells establish a suitable environment for the development of the germinal cells. So far the essential components for establishing this "suitable environment" are unknown, but it is generally accepted that contacts between Sertoli cells and germinal cells, together with a specific composition of the tubular fluid, are of great importance to allow differentiation of the germinal cells. In this model the Sertoli cells receive hormonal and local signals and translate this complex set of regulatory signals into a proper supporting function for the germinal cells. Attempts are still being made to identify the signalling molecules which help in making the binary "d-d" decision of each germinal cell: differentiation or death. Since it is known that androgens play an important role in the regulation of spermatogenesis, there have been many efforts to establish precisely which testicular cells are target cells for androgens. Many of these studies concern the localization of androgen receptors.

In view of the currently recognized autonomous behavior of germinal cells, in retrospect it seems logical that germinal cells are devoid of androgen receptors. However, it took many years of collecting the right experimental data and many discussions before there was agreement that the conventional androgen-dependent regulation of gene expression did not occur in germinal cells (Grootegeed et al. 1977).

3 Olfactory Action of Androstenone

Science progresses with shocks. Depending on new techniques and new concepts, particular areas of biology may receive much attention during a brief period before other compelling subjects or techniques create the next excitement (Tata 1984). In this respect many scientists' interests are greatly influenced by the attractions of trends and fashions. A negative aspect of this attitude is the neglect of other forms and functions in biology. A major and undisputed function of androgens is to regulate gene expression in many different target cells, and studies on this process have contributed enormously to our understanding of androgen action. However, as stated above, exceptions to the general rule should receive more attention. A particularly good example in this connection is the rapid (within seconds) activation of membrane receptors in the olfactory sensory neurons by nanomolar concentrations of androstenone ($\Delta^{16-5\alpha}$ -androsten-3-one) (Snyder et al. 1988; Shepherd and Firestein 1991). This is an example of a neurosteroid effect. At room temperature, small amounts of evaporated steroid in equilibrium with pure androstenone crystals or present in the saliva of boars can be detected by sows. The biological response in sows is very clear: the sows are immobilized, which facilitates fertilization by the boar. Therefore androstenone is a pheromone without androgenic activities for pigs. Man also produces this compound and female social behaviour seems to be altered under the influence of this interesting steroid molecule activating the olfactory system (Cowley and Brooksbank 1991).

This rapid, sensitive and specific effect of the "neuro"steroid androstenone represents not only one of the most simple, rapid and specific *in vivo* bioassays for C₁₉ steroids, but it is above all a good illustration that biologically relevant effects of C₁₉ steroids can operate through cell-membrane receptors independent of nuclear receptors.

4 Different Modes of Steroid Cell Surface Actions

There are two principally different possibilities by which extracellular steroids can modify the functional properties of the plasma membranes, namely a direct and an indirect mode.

In the direct mode, steroids can interact directly with proteins localized in the plasma-membrane and, depending on the functional properties of these proteins, properties of the membrane can be modified. A good example is the gamma-aminobutyric acid (GABA_A) receptor which acts as a chloride channel of which the activity can be modulated by steroids. Alternatively, steroid binding proteins localized in the cell membrane can influence other functional proteins in a similar fashion as receptors for protein hormones, which transduce the hormonal signal via G-proteins to adenylate cyclase, phospholipase C or ion channels, etc.

In the indirect mode, steroids can bind to soluble extracellular binding proteins such as the plasma protein sex hormone binding globulin (SHBG). Due to conformational changes in the binding protein under the influence of the steroid, the extracellular protein may or may not recognize specific proteins in the plasma cell membrane in a manner analogous to that for normal protein hormones. In this model the extracellular steroid binding protein acts as an interface to enable communication between the steroid signal and the membrane components. Supporting evidence for both models will be discussed in the following paragraphs.

5 Indirect Cell Surface Actions of Steroids via Binding Proteins

Proteins that bind androgens and also occur in the testis have received great attention over the past years. Especially in the period when the structure and properties of the androgen receptor were unknown, steroid binding properties of many different testicular protein mixtures were analyzed in order to identify proteins which can play a role in the androgenic regulation of testis function. The properties of the extracellular androgen binding protein (ABP), which differ from those of the

androgen receptor (approximately the same affinity for dihydrotestosterone and testosterone but a very high dissociation rate constant for ABP), initially created confusion, emotions and false claims with respect to the localization of the androgen receptor. After it was realized that ABP was produced and secreted by the Sertoli cell under the influence of hormonal stimulation, many attempts were made to find a biological function for this testicular binding protein which is almost similar to SHBG (Hansson et al. 1975).

It was proposed that the high concentrations of this protein in the tubular fluid were instrumental for the transport to and concentration of androgens around the germinal cells. After it was found that germinal cells could not be considered as classical target cells for androgens because of a lack of conventional androgen receptors, and since efficient transport of androgens in the testis can occur without binding proteins, interest in ABP as an important testicular protein waned. The prevailing view is that ABP, like other steroid-binding proteins in plasma, could act as a buffer to keep the free androgen concentration near target cells constant when fluctuations in androgen production occur. However, recently evidence has been accumulated showing that steroid binding proteins may have other properties in addition to a buffer function (Rosner 1990). There are indications that the binding protein may not only regulate the free steroid hormone concentration, but that the steroids may regulate the conformation and functions of the binding protein. Binding sites for corticosteroid binding globulin (CBG) and SHBG have been identified on plasma membranes of various cell types (reviewed by Rosner 1990) and activation of adenylate cyclase activity by CBG and SHBG has also been reported. These findings are not undisputed, however, and it may be possible that biological effects of steroid binding globulins on membrane properties can only be shown under very strict conditions (Damassa et al. 1991). In any case, these newly recognized possibilities for interactions between steroid binding proteins and cell membranes need proper attention.

So far, biological effects of ABP on testicular cells have not been published; in light of the present findings obtained with CBG and SHBG it seems of interest to reinvestigate the function of ABP. Another reason to undertake such experiments is the earlier observation that ABP can interact with macromolecules in testicular cytosols, lead-

ing to an inhibition of its steroid binding properties (Rommerts et al. 1979, 1980). It could also be shown that this quenching of binding activity is reversible and that a competition exists between ABP, unknown macromolecules (X) and dihydrotestosterone (DHT). The following hypothetical model for these competitive interactions was proposed:



This model for interactions between ABP and macromolecules has only been tested with testicular cytosol preparations and not with intact testicular cells or cell membranes. Thus it seems necessary to reinvestigate, with intact testicular cells, whether steroids can influence properties of cell membranes indirectly via ABP, which may act as a steroid dependent interface.

6 Direct Cell Surface Actions of Steroids

6.1 General Remarks

Apart from the above mentioned effect of androstenone on olfactory cells, it has been known for a long time that steroids can exert rapid effects on properties of cells which cannot easily be explained by the relatively slow genomic effects. The rapid anaesthetic effect of natural and synthetic progestins on brain and the estrogen-induced water inhibition of uterine tissue have been known for many years. However, these and other clear manifestations of unorthodox actions of steroids have not received enough attention. The fact that in many cases relatively high concentrations of steroids were required and that the specificity of these actions was not always characterized may have contributed to our ignorance about the molecular mechanisms of actions of these steroids. This is probably why these steroid effects have been qualified as anecdotal, non-specific and less important.

In the past years there has been a growing number of observations on cell surface actions of steroids, especially in brain tissue (Schumacher 1990; McEwen 1991). Since the structure-function relation-

ships of these cell surface active steroids are different from the steroids that act on gene transcription, the spectrum of steroid dependent cell functions can be enlarged, especially when steroids can modulate different transducing pathways via specific receptors.

The notion that not all actions of steroids are mediated through classical nuclear receptors is also reflected in two discussion articles "at the cutting edge" in *Molecular and Cellular Endocrinology* (Nemere and Norman 1991; Sheridan 1991). From these and other articles it is also clear that cell surface actions are not limited to steroids but that other predominantly nuclear acting compounds like vitamin D₃ and thyroxine may also exert important biological effects at the periphery of the cell (Nemere and Norman 1991; Segal 1990).

6.2 Through GABA_A Receptors

Various steroids derived from pregnane, especially those with a hydroxyl group at C-3 and a keto group at C-20, can rapidly modify activities of neurones in brain tissue. Most of these effects are mediated by interactions of the steroid with GABA_A receptors in the neuronal membrane. This complex receptor molecule forms a chloride-channel and also contains different binding domains for gammaaminobutyric acid (GABA), benzodiazepine and steroids. It has been shown that these pregnane derivatives facilitate the GABA induced chloride influx which renders the cells less prone to excitation. These effects of steroids occur at concentrations of 10-50 nM and require the presence of GABA activated receptors. The GABA_A receptor is composed of 5 subunits and for some subunits there are more than 10 different isoforms (Kirkness 1989; Lüddens and Wisden 1991). The many different subunits and possible combinations thereof may confer a broad spectrum of steroid specificity and sensitivity for regulating the activity of the pentameric GABA_A receptor. Thus via GABA_A activated receptors a highly versatile steroid sensitive chloride transport system can be envisaged which may operate in concert with other hormone dependent ion channels. During evolution the GABA_A receptor system may have adapted to specific modulators since the GABA_A receptors in insects is two to three orders of magnitude less sensitive to the steroids which

are active on mammalian GABA_A receptors. So far no effects of ec-dysone or derived metabolites on GABA_A receptors have been shown (Rauh et al. 1990).

Since GABA_A receptors play an important inhibitory role in the excitability of neurons, most of the investigations on steroid effects on GABA receptors have been carried out in brain tissue (see for examples Table 1). However, GABA not only plays a role as neurotransmitter in the central nervous system but GABA-ergic systems (presence of GABA and GABA_A receptors) have also been identified in peripheral tissues, including endocrine organs (Tanaka 1985; Erdö and Wolff 1990). Although a significant number of investigations has been carried out with peripheral tissues, it is still not known what kind of biological functions are controlled by the GABA-ergic system. Such questions must also be answered for gonadal tissue since there are indications that the GABA-ergic system may also operate in germinal cells. (Erdö et al. 1989; Erdö and Weckerle 1990) (Table 1).

6.3 Through Other Receptors

Certain effects of steroids on functional properties of cells do not require the presence of GABA and GABA_A receptors and many responses are characterized by a rapid influx of extracellular calcium. The structure-function profiles of the steroids which trigger these responses differ from those that are active on the GABA_A receptors. Examples are progesterone and testosterone which can act on spermatozoa and on cultured lymph node carcinoma of the prostate (LNCaP) cells, respectively, and both generate a rapid influx of calcium (Blackmore 1991; Steinsapir 1991). More examples are given in Table 1.

In the brain, membrane effects of dehydroepiandrosterone sulphate, pregnenolone sulphate (Baulieu 1991) and corticosterone (Orchinik et al. 1991) have been shown and a membrane receptor which binds corticosterone has been identified in synaptic membranes. The binding specificity of this membrane receptor does not resemble that of the conventional nuclear receptor and there is a good relationship between the potencies of the steroids in terms of binding and behavioral effects (Orchinik et al. 1991). Specific membrane binding sites have also been

Table 1. Examples of Cell Surface Actions of Steroids

Steroid	Target	Effect	Minimal concentration required (nM)	Reference
progesterone	brain	oxytocin receptors	?	Schumacher, 1990
progesterone	spermatozoa	calcium flux	100	Blackmore, 1991
progesterone	oocytes	cAMP production	100	Finidori, 1981
pregnanolone	brain?	behavior	1	Ramirez, 1987
pregnanolone	pituitary?	gonadotropin secretion	?	Brann, 1990
3 α -hydroxy-progesterone	testis	spermatogenesis	?	Campbell, 1989
3 α -hydroxy-progesterone	pituitary	FSH secretion	0.01	Wiebe, 1987
17 α -,20 β -di-hydroxy-progesterone	olfactory cells	olfaction	1	Dulka, 1987
	sperm duct	sperm mutation	?	Miura, 1991a
aldosterone	leukocytes	calcium flux	1	Wehlin, 1990
corticosterone	brain	binding and behavior	1	Orchimik, 1991
cortisol	pituitary	cAMP production	50	Borski, 1991
5 β -pregnanedione	liver?	heme formation	?	Aragonés, 1991
testosterone	LNCaP cells	calcium flux	0.1	Steinsapir, 1991
testosterone	heart myocytes	calcium flux	1	Koenig, 1989
dehydro-epiandrosterone (sulfate)	brain	neuronal activity	?	Baulieu, 1991

Table 1. (cont.)

androstenone	olfactory cells	olfaction	1	Snyder, 1988
11 keto-testosterone	testis	spermatogenesis	3	Miura, 1991a
17 β -estradiol	pituitary	estradiol binding	0.1	Bression, 1986
17 β -estradiol	endometrial cells	estradiol binding	1	Pietras, 1977
17 β -estradiol	brain	neuronal activity	1	Nabekura, 1986
tamoxifen	breast cells	growth	1	VonderHaar, 1991

detected for tamoxifen. These binding sites may mediate some of the antiestrogenic activities on breast (cancer) cells (VonderHaar and Banerjee 1991). A particularly important observation in the discussion on possible cell surface actions of androgens is the stimulation of calcium fluxes and membrane transport of aminoacids by nM concentrations of testosterone on rat heart myocytes (Koenig et al. 1989).

7 Unorthodox Effects of Steroids on Spermatogenesis

In the prevailing models of the androgenic regulation of spermatogenesis it is postulated that a single androgen receptor is instrumental in conferring all signals from androgens in activation of specific genes in Sertoli cells and peritubular cells. However, since cell surface actions of steroids have been demonstrated in many cell types (Table 1), it cannot be excluded that other testicular cells may also be target cells for cell surface actions of steroids. Not only are there many theoretical arguments for the occurrence of unorthodox steroid effects, there are compelling observations made in fish gonads.

During the breeding cycle in fish dramatic changes occur in endocrine activities. For instance, the conventional nuclear androgen receptor concentration in the brain changes 5-fold (Pasmanik and Callard 1988) and the testis weight increases from less than 0.2% to 6% of the body weight in the period just before spawning (Schulz 1984).

The development of spermatozoa and sperm maturation occur in parallel with high plasma levels of 11-ketotestosterone and later with 17,20 β -dihydroxy-4-pregnen-3-one. This correlation between the appearance of the two steroids which do not bind to the classical receptors for androgens and progestagens and the development of fertile spermatozoa suggests that both phenomena could be functionally connected.

The best evidence that 11-ketotestosterone is the biologically active androgen for stimulation of spermatogenesis in fish comes from *in vitro* studies with testicular fragments from Japanese eels. Development of spermatogonia to spermatids *in vitro* after an incubation period of more than 15 days was only possible in the presence of nanomolar concentrations of 11-ketotestosterone. High concentrations of testosterone and dihydrotestosterone could not support this process (Miura et al. 1991a). Attempts to identify receptor proteins for 11-ketotestosterone have not yet been successful, although androgen receptors in the fish brain show binding properties similar to those of mammalian receptors (Pasmanik and Callard 1988). The molecular basis for the stimulatory effect of 17,20 β -dihydroxy-4-pregnen-3-one on sperm maturation is also unknown, but it may be possible that this steroid and 11-ketotestosterone both act via cell surface actions (Nagahama 1988; Miura 1991b).

These observations do not rule out possible genomic effects of small amounts of testosterone or dihydrotestosterone which are also present in fish. It may be possible that in fish testosterone plays a permissive role and that 11-ketotestosterone controls the development of spermatozoa as a "neurosteroid". In mammals, the permissive and regulatory functions of androgens (nuclear and membraneous effects?) could be combined in the known bioactive steroids.

Although from suppletion studies there are many observations that in mammals testosterone is the major androgen involved in regulation of spermatogenesis, other steroids could act synergistically in a similar fashion as 11-ketotestosterone in fish. In this connection injections of 3-hydroxy-4-pregnen-20-one in rats have shown to be more effective than testosterone in supporting the development of germinal cells after suppression of testicular testosterone (Campbell and Wiebe 1989). This dihydropregesterone could also suppress selectively the FSH se-

cretion by isolated pituitary cells at concentrations of 10^{-11} M (Wiebe and Wood 1987).

When all data from the above mentioned studies are taken together it may be questioned whether the prevailing concept of an exclusive genomic effects of androgens for regulation of spermatogenesis are still valid. The many experimental observations and theoretical arguments warrant more research activities on cell surface activities of steroids. Since membrane effects of progesterone on *Xenopus leavis* oocytes (Finidori-Lepicard et al. 1981) and human spermatozoa (Blackmore et al. 1991) have been observed, it may even be possible that steroids not only have a direct effect on membrane properties of somatic cells, but that developing germinal cells could also be target cells. In normal testicular tissue Leydig cells secrete many intermediates of the biosynthetic pathway in addition to androgens. Thus in testicular tissue there may be enough pregnane derivatives to allow direct non-androgenic regulation of germinal cells via cell surface actions. This would reintroduce the germinal cell as a target cell for steroids, indicating that a broad spectrum of steroid signalling molecules, capable of crossing the blood-testis barrier, could operate in the seminiferous tubules to exert a versatile complementary membrane signalling pathway in conjunction with the genomic pathways (see Fig. 1).

8 Conclusions

Of the many steroids that are present in the circulation and in various organs, only a limited fraction is considered to be biologically active. This is supported by our current knowledge on the steroid specificity for regulation of gene expression. The conventional thoughts are that the remaining steroids (quantitatively the majority) are biologically inactive. These steroids circulate as intermediates of the biosynthetic process, such as dehydroepiandrosterone(-sulphate), or as products of catabolism of biologically active steroids, such as many saturated and hydroxylated pregnane or androstane derivatives. In this respect, there appears to be an artificial separation between reactions that lead to the formation of biologically active steroids (biosynthesis) and reactions that convert these compounds to inactive metabolites (catabolism).

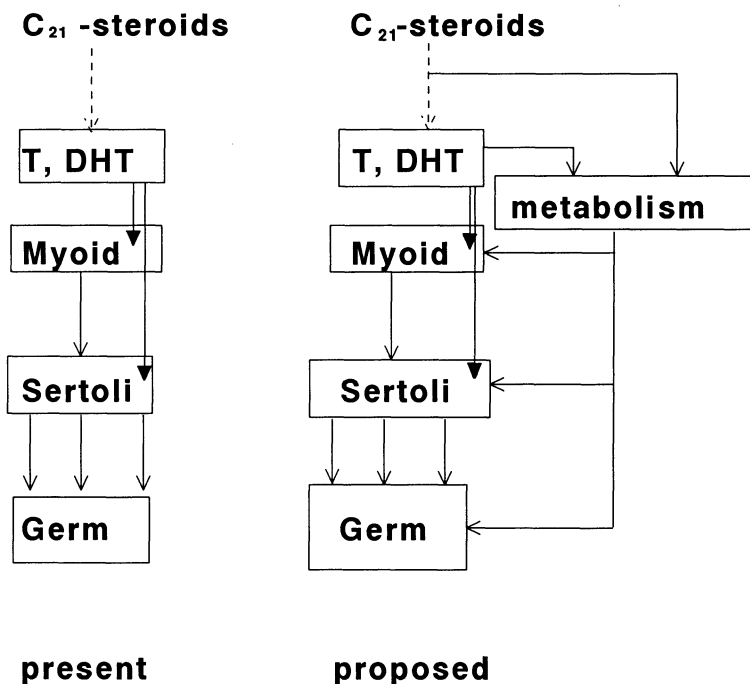


Fig. 1. Two models for regulation of spermatogenesis by steroids. The left model depicts the present notion of an exclusive genomic effect (*black arrows*) of testosterone (T) and dihydrotestosterone (DHT) on somatic cells. In the right model the proposed cell surface actions (*open arrows*) of a spectrum of steroid structures on all testicular cells is incorporated in addition to the genomic effects of androgens

These points of view depend on the biological appreciation of a particular steroid structure; after all any steroidogenic cascade (biosynthetic pathway or catabolism) can be considered as a breakdown of Δ^5 -pregnenolone, the parent compound for all steroids.

Many different enzymes operate in almost any tissue in order to metabolize steroids. From a utilitarian point of view, the many steroids and steroid metabolizing enzymes, as well as the low abundance of biologically active steroids seems to be wasteful and theoretically this re-

dundancy should have disappeared during evolution. On the other hand, the broad spectrum of steroids that occurs in almost all species can make sense when surface actions of steroids with different receptor specificities are also taken into consideration.

The rapid and modulating effects of steroids on cell membranes (see previous paragraphs) could act in concert with the slow and coarse regulating effects of steroids on gene transcription.

To verify whether this complementary regulatory pathway for steroid action is of general biological importance, systems other than the brain such as the testis must be investigated.

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The Structure-Activity Relationships of the Luteinizing Hormone Receptor

Brian A. Cooke and Anthony P. West

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1 Introduction

LH (luteinizing hormone, lutropin) and its homologue chorionic gonadotropin (CG) play a central role in the control of ovulation, pregnancy and testicular function. They belong to a family of polypeptide trophic hormones which also includes follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). An important aspect of the control of their target organs is the dynamic trafficking of their receptors, which, after hormone-induced activation of their transducing systems, may be followed by uncoupling, internalization and recycling and/or downregulation and new synthesis. This trafficking is especially im-

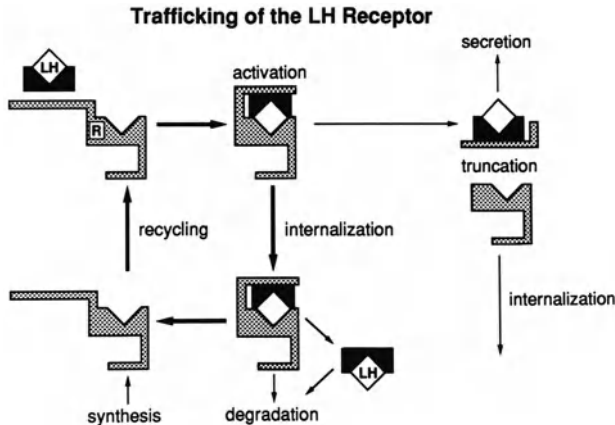


Fig. 1. The trafficking of the LH receptor. LH binds to the extracellular domain of the LHR which presumably causes a conformational change so that activation of the G protein, which in turn activates the adenylate cyclase, can take place. The receptor is thought to be continuously internalized, degraded and/or recycled. Alternatively, the extracellular fragment is cleaved by proteases and released into the extracellular medium

portant for the LH receptor (LHR), which in the Leydig cell of the testis is a continuous process (Fig. 1), whereas in the ovary it is dependent on the stage of the cycle. Very little is known about the mechanisms involved. However, with the recent elucidation of the sequences and structures of these receptors (Loosfelt et al. 1989; McFarland et al. 1989; Minegishi et al.1990; Misrahi et al.1990; Jia et al.1991; Sprengel et al.1990) the mechanisms can now be elucidated. It is the purpose of this short review to highlight the mechanisms and species differences of desensitization and downregulation of the testis Leydig cell LHR. We will describe an adaptation of the antisense oligodeoxynucleotide strategy which we have used to investigate the roles of the C-terminal sequences of the LHR in these processes.

2 Activation and Desensitization of the LH Receptor

LH, via its specific plasma membrane receptor, stimulates testosterone synthesis in the Leydig cell via cyclic AMP and other second messengers (Cooke 1990; Rommerts and Cooke 1988). Continued exposure of the Leydig cells to LH leads to a desensitization of these same responses (Rommerts and Cooke 1988; Cooke and Rommerts, 1988). Previously we demonstrated that desensitization of the rat Leydig cell involves an uncoupling of the LH receptor from its transducing system i.e. a G-protein coupled to adenylate cyclase (Rommerts and Cooke, 1988). This process could be mimicked by stimulation of protein kinase C (PKC) but not cyclic AMP dependent protein kinase (PKA). The LH or phorbol ester induced desensitization led to a 50% decrease in cyclic AMP production, however, in short term studies (2 hours) no decrease in steroidogenesis was found. In contrast, we have now shown that in mouse Leydig cells in the same time period, stimulation of both PKA and PKC leads to complete desensitization with regard to cyclic AMP and steroid production (West et al. 1991a; West and Cooke, 1991a). Thus, there are marked species differences. Although the evidence suggests that the substrate for PKC/PKA mediated phosphorylation is the LHR itself (Rommerts and Cooke, 1988; Minegishi et al. 1989), this has not been directly demonstrated under physiological conditions. The results could equally be explained by phosphorylation of a protein(s) that interacts with the receptor.

2.1 Downregulation of the LH Receptor

It was previously shown that downregulation i.e. loss of the LHR, only occurred after a considerable time period following exposure to LH/hCG i.e. 12-24 hours (Rebois and Fishman 1984; Catt et al. 1979). We have recently shown that there is a rapid proteolytic cleavage of the LHR during culture of rat and mouse Leydig cells which results in the release of the LHR extracellular domain (West and Cooke 1991a) (Fig. 1). This process is continuous in the freshly isolated rat Leydig cells (up to 4 hours), whereas in the mouse Leydig cell it is a cyclic AMP-dependent process. Activation of PKC is ineffective in causing

truncation (West and Cooke 1991a). Paradoxically, inhibition of LHR truncation with protease inhibitors also inhibits cyclic AMP production in the mouse (but not the rat) Leydig cell (West et al. 1991b), indicating that proteolysis of the LHR is required for activation of adenylate cyclase to occur. The depleted receptors in the mouse cells are replenished by a cyclic AMP-dependent process. It is possible, therefore, that loss of receptor in the Leydig cell occurs at two levels: acute, involving truncation, and chronic, involving internalization and degradation and also a decrease of LHR mRNAs (Hu et al. 1990; Wang et al. 1991).

3 The Structure of the LH Receptor

The LHR has been cloned from human and rat ovaries and porcine Leydig cells. Its sequence indicates that it is a member of the 7 transmembrane helix-G protein coupled receptor family (Loosfelt et al. 1989; McFarland et al. 1989) (Fig. 2). The LH (and FSH and TSH) receptors differ from other members of this receptor family in having a very large extracellular domain (50 60kDa), which is the site of the hormone binding (Loosfelt et al. 1989; McFarland et al. 1989; Minegishi et al.1990; Misrahi et al.1990; Jia et al.1991; Sprengel et al.1990) (Fig. 2). There is also very little homology with the other receptors in the transmembrane domains and the C-terminal sequences which are the sites of binding of the other hormones and the G-proteins (Taylor, 1990). A region spanning the 2nd and 3rd transmembrane domains is highly conserved among the LH, FSH and TSH receptors. There is approximately 85% homology between the cloned LHRs. However, antibodies raised against the whole LHR show species and cell specificity (HJ Rajaniemi, personal communication) and the hLHR is unusual in that ^{125}I -hCG binding is not displaced by equine, rat and ovine LH (Jia et al.1991).

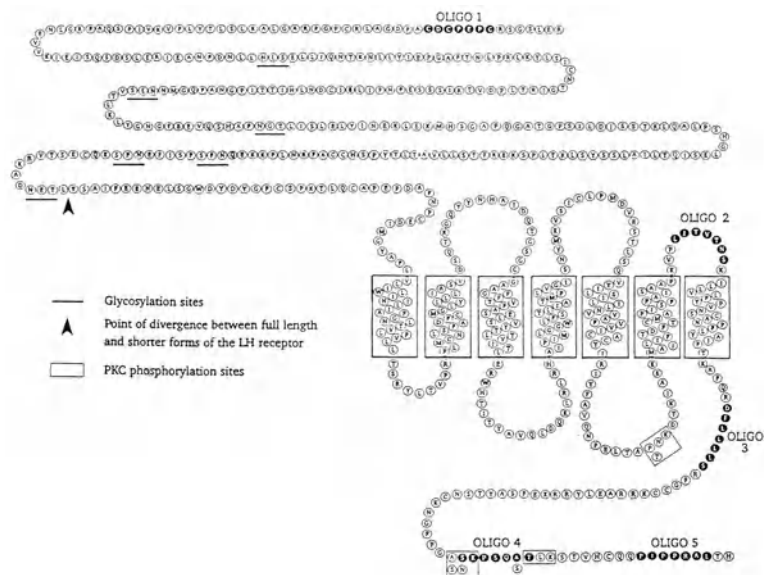


Fig. 2. The amino acid sequence of the LH receptor. The LH receptor has 7-transmembrane domains and a large extracellular domain. The amino acid for the ovarian LH receptor is shown ((McFarland, et al., 1989)). Potential PKC specific phosphorylation sites are indicated by rectangles. AS oligos were synthesised for various sequences of the LHR DNA - the positions of the corresponding peptides are shown by the solid circles

4 The Roles of C-Terminal Sequences of Receptors

There is increasing evidence to suggest that the C-terminal cytoplasmic sequences of regulatory proteins are important in modulating their functional activity e.g. β adrenergic hormone receptor (β AR) (Lohse et al.1989), the glucose transport protein (Lawrence et al.1990) and $G_{\alpha s}$ (Palm et al. 1990). In particular, desensitization and downregulation may be regulated by phosphorylation of the C-terminal sequences of these proteins. In the rat and porcine LHR there are potential PKC phosphorylation site near the carboxyl terminus in the cell cytoplasm (Loosfelt et al. 1989; McFarland et al. 1989) (Fig. 2). Species dif-

ferences in the numbers and sequences of these phosphorylation sites may explain the found species differences in desensitization and down-regulation.

5 LH Receptor mRNAs

Rat ovaries and porcine testes show hybridization of the LHR cDNA to mRNAs of 6.7, 4.3, 2.6 and 1.2 kb (Hu et al. 1990; Wang et al. 1991; Segaloff et al. 1990). The open reading frame of the complete LHR is 2.1 kb. It is not known which of the larger mRNAs is used for the translation of the LHR protein. Alternative splicings resulting in the formation of LHR mRNAs coding for shorter LHR proteins have been found; they all contain the extracellular domain but lack some of the putative transmembrane domains (Loosfelt et al. 1989). When one of these mRNAs (1.2 kb) was expressed in COS1 cells it was found to code for a high affinity soluble LHR. There is disagreement, however, about its secretion from the transfected cells (Xie et al. 1990; Ji and Ji 1991; Tsai-Morris et al. 1990; Lapolt et al. 1991). The 1.2 kb transcript is present in high concentrations in mouse (MA10) tumour cells (Segaloff et al. 1990).

6 Methods of Investigating Receptor Structure-Function Relationships

The generally accepted method of investigating functional activities of cloned receptors is to express receptor genes modified by site-directed mutagenesis in an heterologous expression system. While this gives valuable information, it has disadvantages. For example, the LHR has many factors regulating its function and they are unlikely to be present in these expression systems e.g. when the LHR was expressed in COS-1 cells it could not be downregulated (Segaloff et al. 1990). It is also not known if PKA and PKC mediated desensitization can take place in such expression systems. In addition, since most heterologous expression systems lack the full enzymic machinery for steroid synthesis, regulation by the LHR of steroidogenesis cannot be studied. We have

therefore investigated an antisense strategy in a homologous system to modulate the LHR. We have used the Leydig tumour (MA10) cell line in our studies. These cells retain many of the properties of normal Leydig cells and can also be obtained in large quantities. They can be up and downregulated and undergo PKA and PKC-mediated desensitization with respect to cyclic AMP and steroidogenesis. Similar multiple mRNA LHR species are found in MA10 and mouse Leydig cells (Segaloff et al.1990).

7 The Antisense Inhibitory Strategy

This methodology involves incubating cells with antisense oligodeoxynucleotides (AS oligos) to the gene or mRNA coding for the N-terminal regions of the specific proteins. The AS oligos are taken up by the cells and bind to the DNA/mRNA, preventing transcription/translation and thus *completely* blocking the synthesis of specific proteins. This strategy has been used successfully in many investigations e.g. to study the effects of c-myc (Heikkila et al.1987) and the proliferating cell nuclear antigen (cyclin) on cell proliferation and cell differentiation (Jaskulski et al.1988). It has also been successfully used to determine the role of type IIb regulatory subunit mRNA of PK in the cyclic AMP-induced differentiation of HL-60 leukaemia cells (Tortora et al.1990). We have shown that this strategy will also inhibit LHR synthesis (West and Cooke 1991b). In MA10 cells depleted of their functional LHR by treatment with trypsin, complete resynthesis occurs during subsequent incubation for 48 hours (West et al.1990). When the incubation of the MA10 cells was carried out in the presence of an AS oligo directed against a LHR N-terminal sequence (oligo 1, Fig. 2) no resynthesis occurred and there was a complete loss LH-induced cyclic AMP production and steroidogenesis.

We have *adapted* the strategy in an attempt to produce truncated LHRs with deleted C-terminal phosphorylation sites (West and Cooke 1991b). We used AS oligos, coding for 7 amino acids in the putative 3rd extracellular loop and to different sequences of the intracellular C-terminal loop of the rat ovarian LHR. The presence of the AS oligos 2 or 3 (Fig. 2) was found to have no effect on the replenishment of the

LH receptors, as measured by ^{125}I -hCG binding, or on the LH stimulation of cyclic AMP or steroidogenesis. However, they completely prevented the LH and phorbol ester-induced desensitization of adenylate cyclase by these newly-synthesised (and presumably C- terminal truncated) receptors. Cyclic AMP- and phorbol ester-induced desensitization of steroidogenesis was also prevented. However, no effect was found on LH-induced desensitization of steroidogenesis, thus indicating that LH induces additional desensitization processes which are independent of PKA and PKC. The losses of surface LH receptors caused by LH and cyclic AMP were also investigated. It was found that pretreatment of the MA10 cells with all the C terminal AS oligos, including the sequence coding for the end C terminal peptide (oligo 5), prevented the 50% loss of binding sites detected in the controls.

The mechanisms of the AS oligos against the N-terminus sequence in preventing the synthesis of the LHR can be understood. However, those involved in altering the function of the C terminus are unknown. It is possible that they act as a stop codon on the mRNA so that the LHR is synthesized up to the position where the AS oligo is bound and is then released. Alternatively, mRNAs coding for the LHR lacking the cytoplasmic C-terminus may exist; addition of the AS oligos may completely prevent the synthesis of the full length LHR and thus allow the translation of the mRNAs coding for the truncated receptors. Multiple mRNAs for LHR do exist as noted above.

8 Summary and Conclusions

Because of the marked species differences in the mechanisms of desensitization and downregulation found in the mouse and rat Leydig cells, it is apparent that these mechanisms cannot be studied in only one species. It well established, however, that all Leydig cells contain spare LH receptors (Catt et al.1979); full cyclic AMP and steroid production can be maintained by low occupancy of the receptors. In addition, the antisense experiments in our studies demonstrate that almost complete depletion of the LHR has to occur before cyclic AMP or steroidogenesis is decreased. The acute decreases in the ability of Leydig cells to form cyclic AMP after exposure to LH and other ligands is

therefore not due to a loss in the numbers of LH receptors but to a loss in the ability of the LHR to couple to its transducing systems.

In the rat Leydig cells, acute exposure *in vitro* to LH causes desensitization, but enough cyclic AMP is produced to maintain full steroidogenesis. This desensitization can be induced by PKC activators but not by cyclic AMP. Prolonged exposure is required before downregulation of the LHR and loss of steroidogenesis occurs. However, continuous proteolytic cleavage of the LHR does occur during the first 4 hours of incubation *in vitro*. This results in the excretion of the extracellular binding domain of the LHR into the incubation medium. It is possible that this proteolysis is controlled *in vivo* via regulation with protease inhibitors, perhaps from adjacent cells e.g. seminiferous tubular myoid cells.

In the mouse Leydig cells, desensitization occurs after exposure to LH, cyclic AMP and phorbol esters. This results in very low cyclic AMP and steroid production. Proteolysis of the LHR occurs *in vitro* if internalization is inhibited. This process is stimulated by cyclic AMP but not phorbol esters. Curiously, inhibition of proteolysis inhibits cyclic AMP production; this does not necessarily imply that the proteolysis of the LHR extracellular domain is involved; other changes in the LHR structure or associated proteins may occur.

The anti-sense strategy is obviously a powerful technique for investigating structure-activity relationships of regulator proteins in homologous expression systems. The results of its application to the LHR imply that C-terminal residues are involved in the processes of desensitization and proteolytic cleavage of the extracellular domain. Further work is required to identify the proposed truncated proteins and the mechanisms of inhibition of translation/transcription.

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Development of Antagonists and Agonists of Follicle Stimulating Hormone

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1 Regulating Male Fertility: The Role of Follicle Stimulating Hormone

The initiation and maintenance of spermatogenesis are under the control of follicle stimulating hormone (FSH) and testosterone. For different mammalian species, it is clear that FSH is an important regulator, in particular during the first wave of spermatogenesis, and that testos-

terone is the major stimulator of spermatogenesis in adult males. FSH exerts multiple effects on its target cell, the Sertoli cell, and its action is most evident in testis from immature or hypophysectomized animals (Fritz 1978). However, at all stages of development, the actions of FSH and testosterone seem to be highly integrated. Although the importance of FSH in adult males is not clear, in either the human or in animal species, it appears that FSH is necessary for quantitative maintenance and repair of spermatogenesis. It has been shown that the FSH receptor is carefully regulated in adult rats and, presumably, has an important function in spermatogenesis (Heckert and Griswold 1992). A marked quantitative stimulation of spermatogenesis occurs when FSH is administered to intact adult cynomolgous monkeys (Van Alphen et al. 1988). Administration of anti-FSH antibodies to rats leads to a decrease in spermatogonia (Vaishnav and Moudgal 1992). It cannot be excluded that male fertility will be impaired upon inhibition of FSH action. The possibility of inducing male infertility by blocking FSH action is very attractive for two reasons. First, according to current knowledge, testicular Sertoli cells are the exclusive target cells for FSH in the male, which guarantees specificity. Second, lutropin (LH) action and testicular androgen production would not be affected. Different approaches have been considered to interfere with FSH stimulation of the testis, without acting upon LH stimulation of testicular androgen production. In our laboratory, we are trying to develop FSH antagonistic peptides and peptide vaccines directed against FSH. Synthetic peptides and especially synthetic peptide vaccines instead of "native" protein hormone (vaccines) have been predicted to be especially well suited for mass application in developing countries because peptides are relatively cheap, easy to produce, to store, to transport and to modify. However, a major obstacle to the development of anti-FSH vaccines or FSH antagonistic peptides has been the absence of detailed knowledge about the antigenic determinants and receptor sites on the hormone.

2 Identification of Receptor Sites on FSH

FSH belongs to a group of closely related glycoprotein hormones which contain two noncovalently linked dissimilar subunit chains designated α and β (Pierce et al. 1988; Pierce and Parsons 1981). The pituitary gland synthesizes three members of this family: FSH, LH and thyrotropin (TSH). The fourth member, choriogonadotropin (CG), is a placental product. The α -chain is common to all of these hormones and shows considerable homology from one species to another. The β -chains are unique for LH, FSH and TSH. The β -chain of CG shows 85% homology with the β -chain of LH. Although the primary sequences of FSH, CG, LH and TSH and of their respective receptors are known (for reviews see Ryan et al. 1988; Sprengel and Braun 1992), the structure-function relationships of the hormones remain poorly understood.

The interactions between the glycoprotein hormones and their respective receptors on the plasma membrane of target cells are probably characterized by the same general features as other protein protein interactions that are well understood from studies based upon X-ray crystallography. Electrostatic, van der Waals and hydrophobic interactions are responsible for noncovalent association at protein protein interfaces. Such contact surfaces can comprise structural features that include a sequence of adjacent amino acids in the polypeptide as well as residues that come into close proximity in the folded structure. Such sequences have been extremely useful probes of antigenic structure (Geysen et al. 1984).

2.1 Determination of Antigenic Peptide Determinants on FSH β

Using the Pepscan (Geysen et al. 1984) and polyclonal antibodies raised in rabbits against total bovine(bFSH) and porcine FSH (pFSH) (a kind gift of Dr. Closset, Liege, Belgium), we have characterized two antigenic regions on the β -chain of human FSH (hFSH);

region I: residues 34-45: sequence TRDLVYKDPARP and
region II: residues 87-97: sequence CDSSTDCTVR.

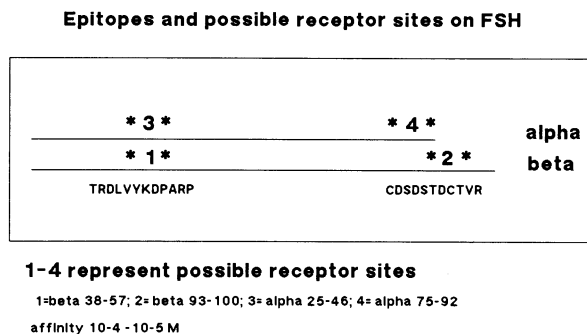


Fig. 1. Follicle stimulating hormone (FSH) consists of two chains, α and β , which are both approximately 100 amino acids long. On both subunits linear domains have been found with low antagonistic activity (sites 1,2,3 and 4). On the β -chain antigenic sites have been detected which overlap with receptor binding sites

The amino acid sequence of region II is conserved among all known species, while there are some small differences in the sequence of region I. In the pFSH β -chain an extra glycine residue is located in the TRDL sequence i.e. TRGDLVYKDPARP. In the bFSH β -chain the lysine residue at position 40 is replaced with an arginine i.e. TRDLVYRDPARP. Strikingly, both regions have also been described as receptor recognition sites (Fig. 1).

2.2 Determination of Receptor Recognition Sites Using Synthetic Peptides

Receptor recognition sites in FSH and other glycoprotein hormones have thus far been identified using synthetic peptides which comprise parts of either the common – or the specific β -chain of the hormones.

At least two receptor sites on the β -chain have been identified in human coriogonadotropin (hCG), which are currently known as the “Keutmann loop” (hCG β -[38-57]; Keutmann et al. 1987; 1988) and the “determinant loop” (hCG β -[93-100]; Gordon and Ward 1985; Keutmann et al. 1989) (Fig. 1). Recently, it has been demonstrated that

a synthetic peptide corresponding to human FSH β -[32-53], *viz.* the “Keutmann loop” in FSH, binds to the FSH receptor and behaves as a partial antagonist of FSH-induced estradiol synthesis at 10^{-5} M (Santa Coloma et al. 1990a). This peptide also was a partial agonist, stimulating basal levels of estradiol synthesis, but not to the maximum levels observed with FSH. Two tetrapeptide sequences from the “Keutman loop” of the β -chain of human (h)FSH, *viz.* TRDL (FSH β -[34-37]) and KTCT (FSH β -[49-52]) compete at very high concentrations (10^{-4} M) with the binding of hFSH to the FSH receptor in a calf testis membrane preparation (Sluss et al. 1986). One of these tetrapeptides, TRDL, also inhibits at 10^{-4} M an *in vitro* biological effect of hFSH, e.g. induction of aromatase activity in rat Sertoli cell cultures (Schneyer et al. 1988). A synthetic peptide corresponding to the human FSH β -chain (β -[81-95]), representing part of the “determinant loop” in FSH (β -[87-94]), did not exhibit an antagonistic but a small agonistic activity at 10^{-5} M (Santa Coloma and Reichert 1990).

On the common α -chain of the glycoprotein hormones, two sites involved in receptor binding have been identified, *viz.* peptides corresponding to residues α -[26-46] and α -[75-92] which inhibit binding of hCG and TSH to their respective receptors at concentrations of 10^{-4} - 10^{-5} M (Charlesworth et al. 1987; Erickson et al. 1990) (Fig. 1).

2.3 Systematic Scanning of FSH β

The reduced potency of the identified peptides relative to the glycoprotein hormones themselves in the utilized assay systems suggests that they represent only a portion of a larger determinant in the intact hormone.

We have scanned the β -chain of FSH for adjacent receptor sites using synthetic peptides. Solid phase synthesis of overlapping nonapeptides was performed according to Fournier et al. (1988) as described (Hage-van Noort 1992). The peptides were acetylated at the amino terminus and amidated at the carboxy terminus, *viz.* Ac-peptide NH₂. The peptides were isolated from the solid phase, lyophilized from 1% acetic acid and stored at -20 C until use.

The peptides have been tested for antagonistic or agonistic activity *in vitro* at concentrations of approximately 10^{-3} , 10^{-4} and 10^{-6} M (final concentrations as calculated from the molecular mass (MW) of the peptide). To study the effects of synthetic peptides we used the FSH-inducible production of cyclic AMP (cAMP) in Sertoli cells isolated from 21 day old rats (Oonk and Grootegoed 1985). Cultures from 21-day-old rats have an important advantage over using cultures from younger rats, requiring only small numbers of readily available animals to provide an adequate number of culture wells (6-9 animals/6x24 well plates). Using this relatively quick (as opposed to FSH induction of aromatase activity) *in vitro* assay system, it is possible to distinguish between agonistic and antagonistic effects of peptides. A highly purified human urinary FSH preparation (hFSH; GT-HMG-52-7) was kindly provided by Organon (Organon bv, Oss, The Netherlands). Ovine FSH (oFSH; NIH-ovine-FSH-S16) was a gift from the Endocrinological Study Section of the National Institute of Health, Bethesda, MD, U.S.A. Non-specific inhibitory effects of peptides were studied by estimation of their effect on cAMP production in the absence of FSH and in the presence of 10^{-4} M forskolin. Forskolin bypasses the FSH receptor and directly activates adenylyl cyclase (Dahl et al. 1988). Furthermore, to rule out toxic effects, the ATP content of the Sertoli cells was estimated after incubation in the absence and presence of peptide. The 28-43 region of the hFSH β -chain was studied more extensively using synthesis of peptides on polyacrylic/ polyethylene pins (Pepscan). Nonapeptides encompassing the amino acids CAGYCY-TRDLVYKDPA with an overlap of eight amino acids, e.g. CAGY-CYTRD, AGYCYTRDL etc., were synthesized simultaneously by the automated Pepscan method on polyethylene rods on which polymers of polyacrylic acid had been formed by radiation grafting (Geysen et al. 1984). The synthesis was started with the tripeptide Asp-Pro-Gly (DPG) (Van der Zee et al. 1988). Using this fast synthetic peptide synthesis method, approximately 10 nmol of peptide could be synthesized on each rod. After deprotection and removal of noncovalently bound impurities, peptides were detached from the polyethylene rods by cleavage of the D-P bonds in 0.2 ml of 70% formic acid for 20 h at 37 °C. Therefore an extra aminoacid (D) is always present on the C-terminus of the peptide, e.g. Ac CAGYCYTRDD-amide etc. The effi-

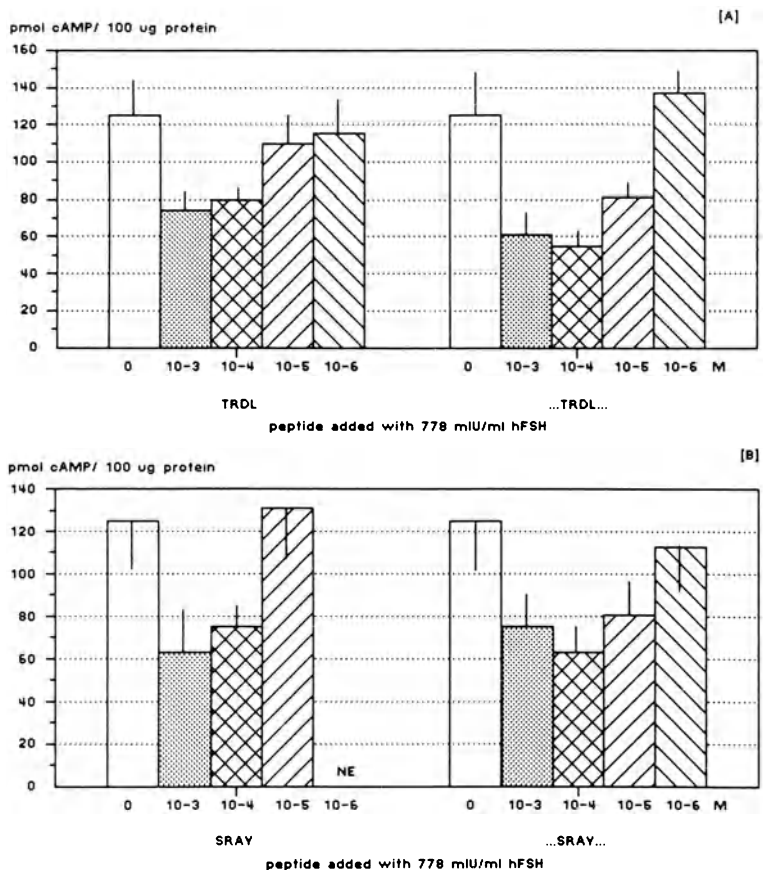


Fig. 2 A,B. Effects on 778 mIU/ml hFSH-stimulated cAMP production by Sertoli cells of the synthetic peptides Ac-TRDL-NH₂ (A) and Ac-SRAY NH₂ (B) and extension of these peptides with amino acids flanking in the primary sequence of the β - and α -chains of FSH respectively e.g. Ac-WCAGYCY-TRDLVYKDPARP-NH₂ (A) and Ac-IYQCMGCCFSRAYPTPLR-NH₂ (B). Sertoli cells were isolated from 21 day old rats. Cells were incubated for 1 hour in the presence of FSH and peptide. Values are mean $\pm$ SD (n=3). The peptides did not effect basal and forskolin induced cAMP production and cellular ATP-content

ciency of peptide detachment was approximately 10%. The formic acid was removed by evaporation/lyophilization. The lyophilized peptides were stored at -20 °C until use.

A systematic scanning of the β -chain of FSH did not reveal other receptor binding determinants on the β -chain than the ones previously published, with the possible exception of FSH β -[13-19]. Certainly no determinants with greater affinity for the receptor were detected. An inhibition comparable to that described by Schneyer et al. (10^{-4} M) was observed using the tetrapeptide TRDL corresponding to the amino acid region β -[(40-43)] from the β -chain of FSH (Fig. 2A). A 10-fold lower dose could be used when in a synthetic peptide the tetrapeptide sequence TRDL was *extended* with amino acids flanking this tetrapeptide in the primary sequence of the hormone, viz. β -[27 45], WCAGY-CYTRDLVYKDPARP, which is in accordance to data previously published by Santa Coloma and Reichert (1990). No significant inhibition of FSH-induced cAMP production was observed using the "determinant loop" sequence CDS DSTDC (β -[87-94]). These data are in accordance to data published by Santa Coloma et al. (1990).

As shown in Table 1, it is possible to scan for receptor binding sites using Pepscan derived peptides (although they are not pure). In future research this may be useful to clarify the importance of individual amino acids in receptor binding sites. Recently, we have developed a method to synthesize a larger amount of peptide (1 mg) on each rod of the Pepscan system. These peptides do not contain an extra amino acid (D) on the C-terminus (Beekman personal communication; Schaaper et al. 1992).

3 Construction of a Three-Dimensional Model for FSH

The receptor binding site of FSH appears not to consist of a stretch of amino acids localized on the hormone specific β -chain, but rather to consist of at least four regions, two on each of the chains of the glycoprotein hormones (Fig. 1). It may be possible to construct synthetic peptides with stronger FSH agonistic or antagonistic action, if we know how these regions can be combined in one construct, namely,

Table 1. Inhibition of the FSH stimulated production of cAMP by nonapeptides encompassing the 28-43 region of the hFSH β -chain. Peptides were synthesized with Pepscan using the tripeptide spacer DPG that can be spliced between the D and P leaving an extra D in the peptides as designated between brackets. Basal cAMP production: 6.2 ± 1.1 pmol/0.5 ml; hFSH induced cAMP production: 110.4 ± 12.8 pmol/0.5 ml; ND = no detectable inhibition.

²⁸ WCAGYCYTRDLVYKDPAR ⁴³ -Derived peptide	%Inhibition
CAGYCYTRD(D)	8
AGYCYTRD(D)	ND
AGYCYTRDL(D)	18
GYCYTRDLV(D)	6
YCYTRDLVY(D)	20
CYTRDLVYK(D)	30
YTRDLVYKD(D)	20
TRDLVYKDP(D)	1
RDLVYKDPA(D)	ND

how these binding sites are structurally located in FSH. Although a paper has been published which attempted X-ray diffraction of dghCG (Harris et al. 1989), both X-ray diffraction and NMR studies have thus far not provided a three-dimensional (3D) structure of glycoprotein hormones. The 3D structure of glycoprotein hormones has thus far mainly been predicted from the assignment of disulfide bonds in their α - and β -chains. This prediction is difficult and many discrepant results have been reported on the assignment of the disulfide bonds; for a review see Ryan et al. (1987). Because disulfide exchange cannot be ruled out, these assignments are uncertain, especially in studies in which reduction and re-oxidation have been used.

A tertiary structure model can also be developed from a primary sequence similarity of glycoprotein hormones with a protein of which the 3D structure has been resolved. Possibly this is related to a similar *in vitro* activity or, more indirectly, to blockade of activity by the same compound. A putative 3D structure model has been developed for hCG, based on the fact that proteinaceous protease inhibitors can block the agonist activity of hCG and are also based on some primary sequence similarity between hCG and the serine protease chymotrypsi-

nogen (Willey and Leidenberger 1989). Thus far no reports have emerged on effective (ant)agonists developed from this model.

We have used the observation that both LH and FSH contain an intrinsic thioredoxin activity to develop a 3D model for the receptor binding site of FSH. Based upon this model both antagonists and an agonist of FSH action have been developed.

3.1 Alignment of FSH β with Thioredoxin

Recently it was shown that both LH and FSH contain intrinsic thioredoxin activity, as measured by their ability to reactivate reduced and denatured ribonuclease (Reichert and Dattatreymurthy 1989; Boniface and Reichert 1990). The LH preparation was even 300 times more active than thioredoxin, while the FSH preparation was 60 times more active.

Thioredoxin is a ubiquitous multifunctional protein, with the conserved active site residues CGPC forming a disulfide bridge in the oxidized form (thioredoxin-S₂). Thioredoxin-S₂ is reduced by NADPH and the flavoprotein thioredoxin reductase to form thioredoxin-(SH)₂, which contains a dithiol and can catalyze dithiol-disulfide exchange reactions (Holmgren 1989). The dithiol-disulfide interchange that is catalyzed by these proteins involves a step at which the two molecules interact in a hetero-dimeric fashion. The thioredoxin-like activity of the hormones seems to depend on the structural integrity of the hormone (LH). Also, the receptor binding potency (of FSH) was unchanged after catalyzing the complete refolding of rRNase. This indicates that the activity of FSH and LH, which is greater than thioredoxin in this assay, probably does not result from involvement of many active disulfides, but rather from only one or a few active sites.

Fig. 3. Alignment of E.coli thioredoxin and E.coli and bovine glutaredoxin with the β - and α -chains of hFSH. Amino acids present in β -sheets (S), α -helices (H) and reverse turns (T) of the "thioredoxin-fold" for thioredoxin-S₂ from E.coli are shown in the first line of the figure. (From Hage-van Noort et al. (1992) Synthetic peptides based upon a three-dimensional model for the receptor recognition site of follicle stimulating hormone exhibit antagonistic or agonistic activity at low concentrations. Proc Natl Acad Sci USA, in press)

	TTTT	TTTT	
	SSSSSS	HHHHHHH	SSSSSSSSSS
1	20	40	60
Thioredoxin E.coli	SDKIIHITDDSFDTLVKADG-AILVDFWAEW--C-GPC----	KMIAPILDEIADEYQGKLTVAKLNI-----	
Glutaredoxin E.coli	MQTVIFGRSG--C-PYC----	VRAKDLAEKLSNE-RDDFQYQYVDIRAEGI-	
Glutaredoxin bovine	AQAFVNSKIOP-GKVVVFIRPT--C-PYC----	RKTOELLSQLPFK-QG--LLEFVDITAAGNI	
1	20	40	60
FSH beta human	1 NSCELTNITIAIEKEEC-RFCISINTTW--CAGYCYTR-DLVYKDPARPKIQ----	KTCYFKELVYETV-	
1	20	40	
alpha human	APDVQ-----DCPECTILOEN--PFFSQPGAPILQC-MGCCF--SRAYPTPLRSKK-----TMLVQKNVT-----		
	HHHHTTTT	SSSS	SSSS
	80	100	
Thioredoxin E.coli	--QNPGTAPKYGIR--GIPTLLLFKNGEVAATKVGLSKGQKKEFLDANLA		
Glutaredoxin E.coli	--TKEDIQQKAGKPVETVPQIFVD-----QQHIGG-----YTDFAAVVKENLDA		
Glutaredoxin bovine	SEIQDYLOQLTGAR--TYPRVFIG-----QECIGG-----CTDLVNMHERGELLTRLKQMGALQ		
1	80	100	
FSH beta human	--RVPGCAHADSL-YTYPVATQCHC-----GKCDSD--STDCTVRGLGPSYCSFGEMKE		
1	60	80	
alpha human	-----SESTCCVAK--SYNRVTVM-G-FKVENHTACHCSTCYHKS		

It has been suggested earlier that FSH interacts with its receptor by dithiol-disulfide interchange. A thioredoxin-like catalysis could occur which then leads to a hormone-induced refolding and thus to conformational changes in the receptor. These changes might be required for postbinding signal transduction.

Based on the homology with part of the human FSH and LH β -chains (residues 90-93 in LH/CG and 84-87 in FSH) it has been suggested earlier that the active S-S refolding region in both glycoprotein hormones is located within the amino acids CGKC for FSH and CGPC for LH (Boniface and Reichert 1990). These sequences are immediately adjacent to the "determinant loop" region which is proposed by Gordon and Ward (1985) as being responsible for conferring biological specificity to these hormones. However, it is difficult to explain that the cysteine residue located at position 93 in LH and FSH is involved both in formation of the "determinant loop" and in formation of the active S-S refolding site. Therefore we have aligned the β -chains of the glycoprotein hormones differently with thioredoxin and other related proteins. Not only are the hydrophobicity index and the length of the thioredoxin molecule (108 amino acids for *E. coli* thioredoxin) comparable with those of the β -chains of the glycoprotein hormones (approx. 115 amino acids) (Boniface and Reichert 1990), all mammalian thioredoxins and glutaredoxins have, in addition to the active center disulfide, two additional structural half cystine residues in the C-terminal half of the molecule (Fig. 3) (Holmgren 1989). We have aligned the active S-S refolding region of thioredoxin with the conserved CAGYC sequence in the β -chains of the glycoprotein hormones (residues β -[28-32] in FSH). The two additional structural half cystine residues in the C terminal half of the mammalian glutaredoxin molecules have been aligned with the cystine residues forming the "determinant loop" region in the glycoprotein hormones (residues β -[87-94] in FSH) (Fig 3.; Hage-van Noort et al. 1992).

3.2 A Three-Dimensional Model for FSH

This alignment has been used to construct a three-dimensional model of the β -chain of FSH based upon the X-ray diffraction data of thiore-

doxin. When the β -chain of FSH is constructed according to this “thioredoxin fold”, the receptor binding sites TRDL and the “determinant loop” described earlier are located within a 10 Å distance from one another (Hage-van Noort et al. 1992). We have also incorporated the α -chain in this model based upon the following assumptions:

1. The 3D-structures of the α - and β -chains of FSH are similar.
2. The α -chain also contains an active S-S refolding region localized in the region α -[32-36] and may also contain a thioredoxin-like 3D structure.
3. The contact sites between the α - and β -chains of glycoprotein hormones are structurally located in the same positions as the contact sites between two thioredoxin (-like) molecules.

For E.coli thioredoxin the contact sites contain residues 33-34, 73, 75-76 and 91-93. Superimposed on the β - and the α -chains of FSH, these contact sites must be located in residues α -[33-35], α -[69-71], [86-88], α -[67] and β -[29-31], β -[76-77], β -[88-90] and in homologous residues in TSH and LH/CG. Several of the contact residues according to the 3D model have been described earlier in literature, such as the region α -[30-50] which is not accessible to trypsin and chymotrypsin in native hCG, in contrast to the isolated α -chain (Mertz 1984); α -[K-67] and α -[Y-69] which can not be titrated or iodinated in intact hormone. The most important contact site on the β -chain is the conserved tetrapeptide CAGY (FSH β -[28-31] (Pierce 1988). β -[D 90] has also been described as an important residue.

To accomplish this contact between the two glycoprotein chains with an assumed thioredoxin-like structure, the β -chain was rotated approximately 180° relative to the α -chain and then moved to the α -chain, so that the contact regions of the α -chain are in contact with those of the β -chain. Strikingly, the contact site between the α - and the β -chain in this model of FSH then consists of many phenylalanine and tyrosine residues, which contain an aromatic side chain. The interaction between both chains in FSH may be stabilized by the noncovalent interactions between these aromatic side chains.

In our model disulfide bridges between cysteines in both the β - and α -chains of FSH can be assigned with ease, without any disruption of

Fig. 4. A ribbon drawing of the hypothetical 3D-structure of two molecules with a "thioredoxin-fold", viz. FSH α -chain and FSH β -chain, in contact. To accomplish contact between these two molecules of which the contact sites are structurally located on the same side, one thioredoxin 3D structure was rotated approximately 180° relative to the other and then moved towards it. The "thioredoxin-fold" consists of a central five-strand β -sheet which is connected by helices and loops. When the amino acid sequences of FSH α and FSH β are superimposed on this structure, the sequences SRAY (α -[34-37]), TRDL (β -[40-43]) and a part of the "determinant loop", represented by the sequence TDSDS rather than by the primary sequence SDSTD β -[87-91], are within 10 Å distance from each other. (From Hage-van Noort et al. (1992) Synthetic peptides based upon a three-dimensional model for the receptor recognition site of follicle stimulating hormone exhibit antagonistic or agonistic activity at low concentrations. Proc Natl Acad Sci USA, in press)

in sheet 1-sheet 4 (α -[11-88]; β -[3-84]), helix 1-helix 3 (α -[14-64]; β -[17-66]), sheet 2-helix 4 (α -[36-91]; β -[20-104]), turn 2 (α -[32-35]; β -[28-32]), sheet 3-sheet 4 (α -[63-86]; β -[51-82]) and sheet 5-helix 4 (β -[87-94]). For the disulfide assignments in the α -chain this totally resembles the assignments made by Combarnous and Hennen (1974). Also for the β -chain, the first three and the last assignments have been generally assumed to be correct (see Ryan 1988). However, since there is no real consensus in the literature about the assignment of disulfide bonds it is difficult to judge the degree of correctness of the model from comparison. Disulfide assignments in glycoprotein hormones based on partial reduction and alkylation experiments might be dangerous because of the observed activity of LH and FSH to catalyze di-thiol-disulfide exchange reactions.

4 Antagonistic Peptides Derived from the Model

In our model of the receptor recognition site of hFSH, the tetrapeptide SRAY of the α -chain (α -[34-37])(which we assume in the α -chain to be located at the same position as TRDL in the β -chain) is now located within 10 Å distance from the tetrapeptide TRDL (FSH β -[40-43]) and the "determinant loop" (FSH β -[87-94]) of the β -chain (Fig 4). The synthetic peptides SRAY and ILQCMGCCFSRAYPTPLR (α -[25-42]), demonstrated antagonistic activity at concentrations of 10^{-4} M and 10^{-5} M respectively (Fig. 2B). The *combined* addition of SRAY with TRDL did not result in a requirement of lower doses (remaining 10^{-4} M) of the respective peptide (Table 2).

Using the model, we have synthesized several peptides that encompass the tetrapeptide SRAY, the tetrapeptide TRDL and the pentapeptide TDSDS which represents parts of the "determinant loop" region of the β -chain. The localization and spacing of the amino acid residues were deduced from the constructed model. For spacing we have used synthetic amino acid linkers $\text{-NH-(CH}_2\text{)}_x\text{-CO}$, in which x was deduced from the model. After synthesis the peptides were purified by reverse-phase high-performance liquid chromatography; their homogeneity and amino acid compositions were verified (Hage-van Noort et al. 1992). However the synthetic peptides TDSDS-NH-(CH₂)₅-CO-SRAY

Table 2. Effects of synthetic peptides representing the receptor binding determinants SRAY, TRDL and TDSDS (one-domain peptides) and the effects of linkage of these sequences (two- and three domain peptides), on cAMP production by Sertoli cells. The lowest final concentration at which a peptide added to Sertoli cells was able to inhibit FSH (hFSH 778 mIU/ml)-induced cAMP production (antagonist) or stimulate cAMP production (agonist) is shown. ND= no detectable activity at 10^{-4} M- 10^{-8} M concentrations of peptide

Peptide	Activity on cAMP	
	Antagonist	Agonist
One-domain peptides:		
Ac-TRDL-NH ₂	10^{-4} M	ND
Ac-SRAY-NH ₂ + Ac-TRDL-NH ₂	10^{-4} M	ND
Two-domain peptides:		
Ac-SRAY-NH-(CH ₂) ₄ -CO-TRDL-NH ₂	10^{-7} M	ND
Ac-TDSDS-NH-(CH ₂) ₅ -CO-SRAY-NH ₂	10^{-8} M	ND
Three-domain peptide:		
Ac-TDSDS-NH-(CH ₂) ₅ -CO-SRAY-NH-(CH ₂) ₄ -CO-TRDL-NH ₂	ND	10^{-7} M

and SRAY-NH-(CH₂)₄-CO-TRDL, in which the amino acid sequence SRAY from the α -chain is *linked* to TRDL or to TDSDS from the β -chain using a synthetic amino acid of which the length was derived from the 3D model (two-domain peptides), both exhibit an antagonistic activity at 1000-fold lower concentrations (10^{-7} M). (Fig. 5A, Fig. 5B, Table 2). Hence it can be concluded that the combination of only a few residues from receptor recognition sites described earlier (Fig. 1) according to a model of the receptor recognition site of FSH based on the homology of FSH and thioredoxin (Fig. 4), results in synthetic peptides showing antagonistic activity at much lower concentrations than synthetic peptides described thus far (Table 2).

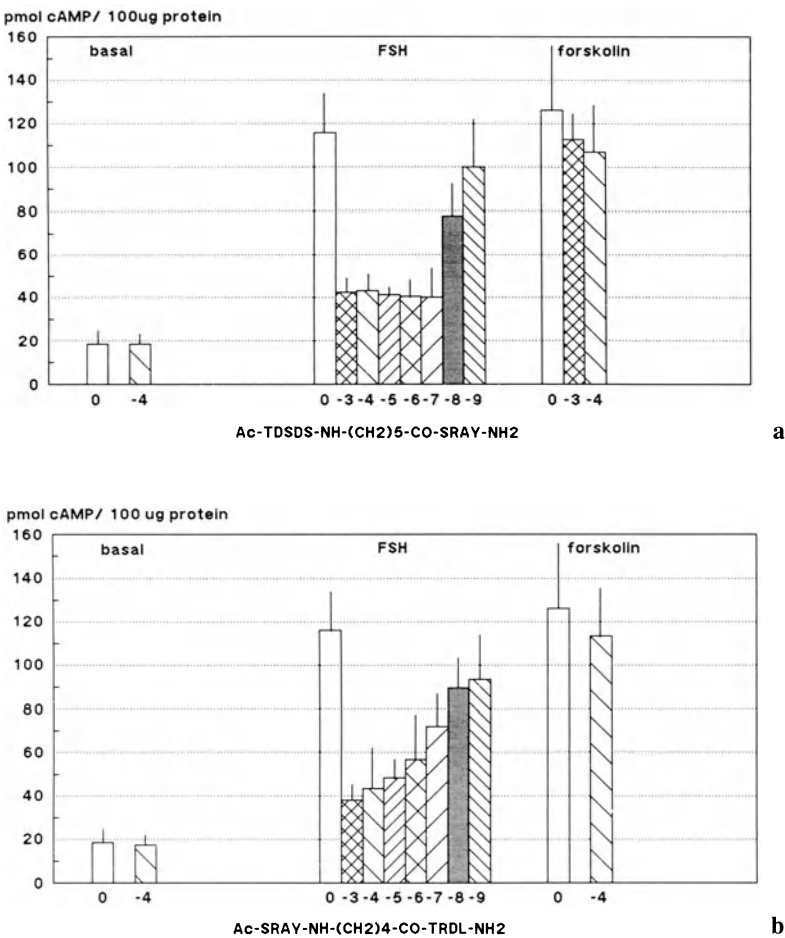


Fig 5 a,b. Effects of different concentrations (10^{-3} - 10^{-8} M) of the *twodomain* peptides Ac-TDSDS-NH-(CH₂)₅-CO-SRAY-NH₂ (a) and Ac-SRAY-NH-(CH₂)₄-CO-TRDL-NH₂ (b) (FSH-antagonists) on basal, 778 mIU/ml hFSH stimulated and 10^{-5} M forskolin-cAMP production by Sertoli cells isolated from 21 day old rats. Cells were incubated for 1 hour in the presence of a phosphodiesterase inhibitor. Values are mean $\pm$ SD (n=3)

5 An Agonistic Peptide Derived from the Model

The peptide TSDS-NH-(CH₂)₅-CO-SRAY-NH-(CH₂)₄-CO-TRDL, in which SRAY, TRDL and TSDS (i.e. amino acids from *three* regions on the α - and β -chains; three-domain peptide) are linked, appeared to be a strong agonist of FSH action as indicated by the ability of this peptide to stimulate cAMP production at concentrations up to 10⁻⁸M (Fig. 6). The addition of either TSDS-NH-(CH₂)₅-CO-SRAY, together with TRDL or SRAY-NH-(CH₂)₄-CO-TRDL, together with TSDS to Sertoli cells did not result in stimulation of basal cAMP production; rather, the antagonistic activity of the two-domain peptides were also maintained in the presence of single peptides representing the third domain (not shown). Synthetic peptides comprising parts of the “Keutmann loop”, the determinant loop and the peptide α -[25-46] did not demonstrate agonistic activity when cAMP production was measured, in contrast to reports demonstrating agonistic activity of the “Keutmann loop” and “determinant loop” when estradiol production

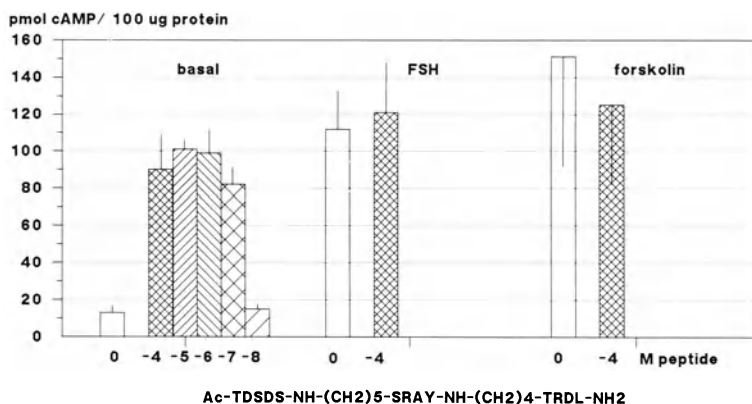


Fig. 6. Effects of different concentrations (10^{-3} - 10^{-8} M) of the *three-domain peptide* Ac-TSDS-NH-(CH₂)₅-CO-SRAY-NH-(CH₂)₄-CO-TRDL-NH₂ (FSH agonist) on basal, 778 mIU/ml hFSH-stimulated and 10^{-5} M forskolin induced cAMP production by Sertoli cells isolated from 21 day old rats. Cells were incubated for 1 hour in the presence of a phosphodiesterase inhibitor. Values are mean $\pm$ SD (n=3)

was measured (Santa Coloma and Reichert 1990; Santa Coloma et al. 1990). This could be due to the relative insensitivity of the cAMP assay relative to induction of aromatase activity. The observation that a synthetic peptide can mimic the action of the glycoprotein hormone FSH at a concentration of 10^{-8} M is surprising since it has been demonstrated that deglycosylated hormones do bind to their receptors but are not able to generate cAMP production. This suggests that the three-dimensional structure of the much larger whole hormone is stabilized by the carbohydrate chains, but that only a few amino acid residues are necessary for transduction of the hormonal signal upon binding to the receptor. It has been reported that correct and complete N glycosylation of the α -subunit, in particular that of the first N glycosylation site (from the NH₂ terminus), is critical for the biological activity of hCG (Matzuk and Boime 1989). The antagonistic effect of deglycosylated (dg) hCG can be reversed by complexing receptor bound dghCG with antibodies (or Fab-fragments of antibodies) specific to the β -subunit of dghCG (Rebois and Liss 1987) and therefore it is thought to be caused by incorrect interactions of dhgCG with the receptor. Recently it has been demonstrated that dghCG and hCG bind to different parts of the receptor (Ji and Ji 1990). This could indicate that carbohydrate chains direct the correct interactions between hormone and receptor, either by binding to the receptor or nearby membrane lectins (Calvo and Ryan 1985) themselves, or by stabilizing the hormone in a certain conformation (Keutmann et al. 1985; Rebois and Liss 1987) which can bind to the correct position on the receptor. That the positioning of certain hormone amino acid residues on the receptor is critical in receptor binding is demonstrated by the present observation that the antagonistic two-domain peptide SRAY-NH-(CH₂)₄-CO-TRDL was converted into an agonistic three domain peptide by addition of the amino acid residues TDSDS. Further experiments indicate that the presence of the arginine residue (R) in SRAY is important for the reported agonistic activity of the three-domain peptide. Finally, linking the "fourth domain" (Fig. 1) to the three-domain peptide yielded an even stronger agonist of FSH action, as indicated by the ability of this peptide to stimulate cAMP production at concentrations up to 10^{-9} M.

6 Summary

The theoretical conformational model of FSH based on the structure of thioredoxin has enabled us to predict a conformational receptor binding site. Based upon this prediction, peptides could be synthesized which demonstrated antagonistic activities (two-domain peptides) or agonistic activities (three-domain peptide) *in vitro* at much lower doses than found earlier for peptides deduced from the linear primary sequence of either subunit chain of FSH. We are therefore confident that this model is a useful tool for the development of peptides with FSH-(ant)agonistic activity. Also, this FSH-model will allow us to define systematically candidate peptides for a synthetic FSH vaccine.

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***The Dialogue Between Late Spermatids
and Sertoli Cells in Vertebrates:
A Century of Research***

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Danielle Hélène Garnier and Françoise Bauché

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1 Introduction

In the nineteenth century, the relationship between spermatids and Sertoli cells had already attracted the attention of scientists (histologists) and much passionate debate. In his review of 1901, Regaud noted that

earlier authors, such as von Ebner, Sertoli, Benda had observed the development of a “substantial union” between elongating spermatids and Sertoli cells. Benda (1887) and von Ebner (1871, 1888) developed the basic concepts of “copulation” and “spermatoblast” (symbiotic relationship) respectively, to characterize the relationship between late spermatids and Sertoli cells. The controversy whether this was true “copulation” (i.e. cytoplasmic continuity between the cells), only ended when Burgos and Fawcett (1955), using the electron microscope, clarified details of the boundary relations between spermatids and Sertoli cells (Roosen-Runge 1962).

A century has elapsed since these early works. The aim of this review is to examine present knowledge of the subject. In particular, this chapter will summarize the anatomical and functional interactions that exist between late spermatids and Sertoli cells in mammals and examine the possible mechanisms underlying the influence exerted by spermatids on Sertoli cell function.

2 The Anatomical Basis of Late Spermatid-Sertoli Cell Dialogue

During spermiogenesis in mammals the position of spermatids within the seminiferous epithelium change markedly. During the early stages of spermiogenesis spermatids occupy a near central position within the epithelium. When nuclear and cytoplasmic elongation begins, the spermatid heads point toward the basement membrane of the tubule and progressively become deeply embedded within the recesses of Sertoli cells. Prior to sperm release, they move rapidly toward the lumen of the tubule and eventually detach as spermatozoa (see reviews by Regaud 1901; Fawcett 1975; Russell 1980, 1984 ; de Kretser and Kerr 1988). In this review the term late spermatids will be used in all species to designate elongating and elongated spermatids. According to von Ebner (1902), from the moment spermatids form bundles, they adhere tightly to Sertoli cells. The “very special surface relationship” between late spermatids and Sertoli cells (Roosen-Runge 1962) first received precise experimental support with the work of Brökelmann (1961, 1963). Using the electron microscope, the authors localized pe-

cular subsurface Sertoli cell structures around the heads of late spermatids in the rat, which they suggested might be involved in the sperm release process. Important progress then followed. The structures that enabled late spermatids and Sertoli cells to communicate were described, their roles became better understood, as was their fate.

Table 1 lists the structural devices identified so far in mammals. They are undoubtedly the most complex set of anatomical structures within the seminiferous tubules and probably within the body. They are thought to be involved either in cell attachment and movement, like the ectoplasmic specializations initially discovered by Brökelmann (1961, 1963), or in both these functions. In addition, they could mediate cell to cell transfer of materials like the tubulobulbar complexes, as well as the Sertoli cell processes and the spermatid-Sertoli cell processes, or they could be solely involved in the transfer of materials, as are the residual bodies. With regard to material transfer, gap junctions, which are known to enable exchanges of small molecules between cells, disappear, as do desmosomes, during the elongation phase of spermiogenesis in mammals (Russell 1980). Their existence is probably not compatible with the profound morphological changes and the large displacements of the spermatids that occur within the seminiferous epithelium during this phase of spermiogenesis.

These different structural devices have been observed in a wide variety of mammals (Sprando and Russell, 1987a; de Kretser and Kerr, 1988). However, they do not follow a strict morphological pattern. For example, ectoplasmic specializations, display a different organization in marsupials (Russell 1984). Several structural devices that help supporting/Sertoli cells and late spermatids to communicate, namely ectoplasmic specializations, "desmosome-gap" junctions and residual bodies (Sprando and Russell, 1987b,c; 1988; Sprando et al. 1988), have also been described in non-mammalian vertebrates. Ectoplasmic specializations have not been found in the bluegill teleost fish (cystic spermatogenesis), but varied types of Sertoli cell-germ cell organizations have been described in an amphibian (frog: spermatogenesis occurs in cysts which open to expose late spermatids to the tubule lumen), reptilia and aves (turtle and chicken: spermatogenesis occurs in seminiferous tubules) (Sprando and Russell 1987b). The ectoplasmic specializations in these three species are highly pleomorphic. In

Table 1. Anatomical basis of late spermatid-Sertoli cell dialogue

Device	Description	Presumed Function	Fate	Selected References
Ectoplasmic specializations (A)	Form within the Sertoli cells; consist of actin filaments sandwiched between the Sertoli cell membrane and cistern of endoplasmic reticulum; these cisterns may be bridged to microtubules by mechanoenzymes	Anchoring devices; May intervene in shaping and positioning the sperm heads; May function as vehicles for microtubule-based transport of late spermatids through the epithelium	Endocytosed by a receptor-mediated mechanism by Sertoli cells; Recycled to earlier generations of germ cells?	Brökelmann, 1963 Russell, 1980, 1984 de Kretser and Kerr, 1988 Resdenbach and Volg, 1991
Tubulobulbar complexes (A, B)	Cytoplasmic evaginations of the spermatid which penetrate into the Sertoli cell cytoplasm; composed of tubular and bulbous portions surrounded by fine filaments and flanked by smooth endoplasmic reticulum. Their cytoplasm is "watery" and organelle-free	Anchor and eliminate excess cytoplasm of spermatids; Indirectly engineer the formation of residual bodies and of the sperm head; may trigger the sperm release mechanism; may permit the concentration of glycolytic enzymes in spermatids to increase	Phagocytosed by Sertoli cells	Russell, 1980, 1984 Grootegeod and Den Boer, 1989

Table 1. (cont.)

Device	Description	Presumed Function	Fate	Selected References
Sertoli cell processes (A, B)	Projections of the Sertoli cell penetrating spermatids. They may contain mitochondria, microtubules, lysosomes, glycogen and vesicles. In the mouse they form a canal complex. The plasma membrane of the spermatid covering the canal complex produces thin tubules which contribute to fragmentation of the spermatid cytoplasm	May allow transport of molecules between Sertoli cells and spermatids; in the mouse serve the removal of the spermatid endoplasmic reticulum; in the mouse and other species, may serve to retain the cytoplasmic lobe of spermatids during movement to the tubule lumen, thus facilitating breakage of the spermatid stalk; may directly be related to the movement of the spermatid head	May become small vesicles which are autolysed and photocytosed by spermatids; may be involved in receptor-mediated endocytosis; the canal complexes are always in continuity with surrounding Sertoli cells; the processes are not pinched off	Clermont and Morales, 1982 Russell, 1984 Sakai and Yamashina, 1990

Table 1. (cont.)

Device	Description	Presumed Function	Fate	Selected References
Spermatid processes (A, B)	Associated finger-like projections of a spermatid and of a Sertoli cell which penetrate into a neighboring spermatid at the same or at different steps of spermatogenesis	Anchoring devices; may transfer spermatid and Sertoli cell materials to other spermatids	Autolysis of the spermatid portion within the Sertoli cell layer followed by degeneration of the latter in the cytoplasm of the spermatid that is penetrated	Segretain and Decrossas, 1991
Residual bodies (B)	Portion of the spermatid cytoplasm left behind the mature sperm as they detach from the apical trunk of the Sertoli cell; their cytoplasm is packed with Golgi remnants, mitochondria, vacuoles, lipid droplets, proteinaceous flower-like structures and high concentrations of ribosomes	Elimination of late spermatid cytoplasm; Transport of spermatid materials to Sertoli cells; may permit an increase in glycolytic enzymes in sperm. Role in the local control of spermatogenesis?	Autophagy and phagocytosis by Sertoli cells	von Ebner, 1888 Regaud, 1901 Roosen-Runge, 1952 Chemes, 1986 Clermont et al., 1987 Pineau et al., 1991b

A: devices involved in cell attachment and/or movement; B: devices involved in the transport of materials from cell(s) to cell

the turtle and chicken they appear at mid spermiogenesis. According to Sprando and Russell (1987b), they may be characterized as an aggregation of filaments (presumably actin) of the Sertoli cell which appose the excentrically positioned spermatid nucleus. As in mammals, they may help position the spermatid head and the spermatid in the epithelium. They may be involved in the spermiation process, since they disappear at late spermiogenesis.

The “desmosome-gap” junctions are particular structures found in the turtle and the chicken (but not in the frog) which may be involved in both anchoring the spermatids to the Sertoli cells and in metabolic exchanges between the two cell types. In the turtle these structures sometimes persist after nuclear elongation begins (Sprando and Russell 1987c). Lastly, Sprando and Russell (1988) and Sprando et al. (1988) have also shown that like mammalian spermatids, spermatids of non-mammalian vertebrates reduce their cytoplasmic volume by eliminating packets of cytoplasm termed residual bodies. In the bluegill, the residual bodies are also phagocytosed by Sertoli cells (Sprando et al. 1988).

The results reviewed here indicate that the structural devices that mediate somatic cell and germ cell interaction are essential to spermatogenesis. The need for a late spermatid-Sertoli cell adhesion and communication system has most probably been preserved throughout evolution; the structure of the system may itself have adapted to different testicular organizations.

3 Evidence for Late Spermatid-Sertoli Cell Dialogue

3.1 Sertoli Cell Influence upon Late Spermatids

It has always been accepted that the influence of the Sertoli cell on late spermatids is essential to spermatogenesis. Russell and Peterson (1984) have shown that the mean elongate spermatid-Sertoli cell ratio is very similar in the vole, the guinea pig, the gerbil, the rat and the hamster (about 10 spermatids per Sertoli cell), higher in the rabbit (12), but lower in the monkey (6), a species in which the Sertoli cell is smaller in both size and volume. For Russell and Peterson (1984), this

ratio is a basic determinant for spermatogenic efficacy, whereas for Orth et al. (1988), the number of spermatids in adult rats depends on number of Sertoli cells produced during perinatal development.

Although it is well known that spermatid maturation is crucially dependent on FSH regulation of Sertoli cell function (Steinberger et al. 1977) and that the most sensitive phase in mammal spermiogenesis is the elongation of spermatid nuclei (Courot et al. 1970; Orth et al. 1988), it is not understood how Sertoli cells exert a favourable influence on the spermatid environment (see below, 4.1).

The position and shape of Sertoli cells within the seminiferous tubules indicate that the Sertoli cells provide germ cells with substances necessary for their division, differentiation and metabolism. According to Regaud (1901) the delivery of nutrients by Sertoli cells to germ cells was clearly postulated by Sertoli in 1871 and for Peter (1899), nurse cells are present in the testis of all vertebrates and in invertebrates. The same author considered that because of the condensed nature of their chromatin, maturing spermatids cannot nourish themselves and have an absolute need for these nurse cells (Sertoli cells); to facilitate this the nuclei of spermatids draw nearer to the Sertoli cell nuclei. If one considers that the nutritive function of the Sertoli cell and more generally, the transport of various substances to germ cells is absolutely essential to spermatogenesis, the number of Sertoli cell products presumably involved in these activities and that have been identified so far seems amazingly limited (Jégou, 1992).

We speculate that the following Sertoli cell products may directly affect late spermatid metabolism and development: tubule fluid (Setchell 1969; Jégou et al. 1982, 1983), glucose metabolites such as lactate, pyruvate and inositol (Robinson and Fritz 1979, 1981; Jutte et al. 1981), lipids (von Ebner, 1902; Marzouki and Coniglio, 1982), the sphingolipid-binding protein named sulfated glycoprotein 1 (SGP₁) and sulfated glycoprotein 2 (SGP₂) (Griswold, 1988; Skinner 1991), vitamin-binding proteins (Skinner 1991), polyamines (Macindoe and Turkington 1973; Tsai and Lin 1985) and glutathione (Den Boer et al. 1989).

Other features of Sertoli cell control of late spermatids concern the spermiation process including the displacement of late spermatids to the tubule lumen and sperm release.

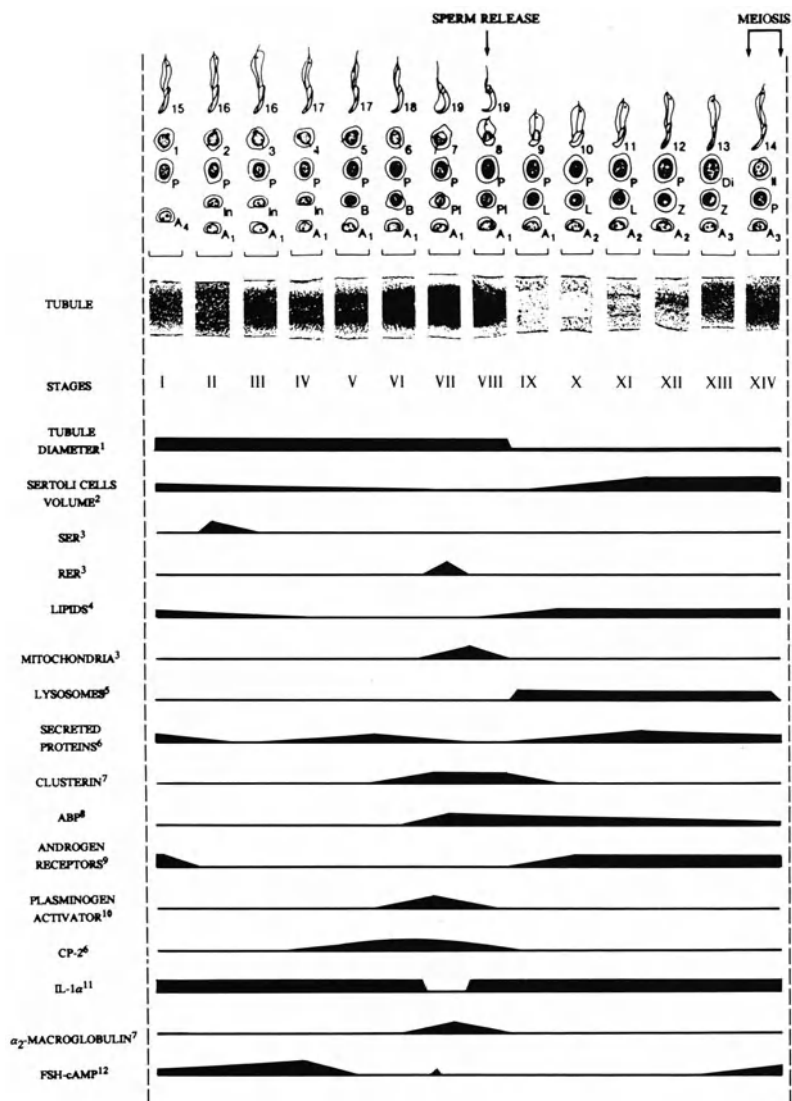
3.2 Late Spermatid Influence upon Sertoli Cell Function

This aspect of the paracrine regulation of testicular function cannot directly be investigated in mammals using cell culture systems since it is impossible to isolate late spermatids without damaging them. Progress has been made by developing other methods of investigation. For example, cyclical changes in Sertoli cell function in vertebrates have been studied and the nature of Sertoli cell dependence on the germ cell complement has been more precisely defined by using various factors which specifically interfere with germ cells.

3.2.1 Cyclic Changes in Sertoli Cells in Vertebrates

It has long been known that in most mammals (but not in humans) the various cells types within the seminiferous tubule form specific and constant cellular associations which succeed one another in time in any given area, in a cyclic fashion (Clermont 1972, Figure 1). Cyclical changes in Sertoli cell cytoplasmic content and morphology in mammals were first described by Sertoli (1875, quoted by Regaud, 1901) and by von Ebner (1888). Since Regaud (1901) formulated the concept of “alternative functioning of Sertoli cell nuclei”, enough evidence has accumulated to confirm the earlier observations of the morphologists.

It is generally thought that the cyclical changes in Sertoli cell morphology and activity directly reflect the influence of germ cells. Figure 1 indicates that Sertoli cells are smallest during stages VI-VIII just prior to and when sperm release occurs, and largest during stages XII-XIV. A significant reduction of the diameter of the seminiferous tubules is also observed after spermiation. The diameter of the tubules begins to increase after stage XIV and decreases after spermiation. The size of the tubule lumen, which probably reflects tubule fluid production (Waites and Gladwell 1982), increases between stages V and VII, corresponding to the periods preceeding sperm release (not shown). It may therefore be concluded that tubule contraction and increased fluid flow may be required for the late spermatids to detach. Both lumen size and fluid production are positively influenced by late spermatids (Table 2). This suggests that late spermatids control their own micro-environment.



An increase in the smooth endoplasmic reticulum (SER), the rough endoplasmic reticulum (RER) and the mitochondria of the Sertoli cell occurs at stage II, stage VII and stages VII-VIII, respectively. Furthermore, shortly after phagocytosis of the residual bodies in the rat, the lipid content of Sertoli cells rises markedly, and reaches a maximum during stages IX-XIV; lipids then decline at stages I-III and remain low after stages IV-VIII.

A stage-dependent variation in the lysosome number has also been observed. The number of secondary lysosomes increases abruptly from stage IX, peaks at stage XIV and then decreases rapidly. Numerous studies have also established that many Sertoli cell products are produced cyclically. Two procedures have been developed to investigate this aspect of tubule physiology. The first one, and the one most utilized so far, was initially set up by Parvinen and Vanha-Perttula (1972). Based on light absorption, a specific transillumination pattern characterizes the tubules at each stages of spermatogenesis (Fig. 1); the corresponding segments of the tubules can be microdissected; Sertoli

Fig. 1. *Top of the figure:* schematic representation of the cycle of the seminiferous epithelium in the adult rat. (Adapted from Dym & Clermont (1970)). A 1-4: A spermatogonia; In: intermediate spermatogonia; B: B spermatogonia; Pl: preleptotene spermatocytes; L, Z, P, Di: leptotene, zygotene, pachytene, diplotene spermatocytes; II: secondary spermatocytes; 1-8: steps 1-8 spermatids, "early" spermatids as defined in this review; 9-19: steps 9-19 spermatids, "late" spermatids here. Transillumination photomicrograph of freshly isolated unstained rat seminiferous tubule (adapted from Parvinen et al. 1986). The successive stages of the cycle as defined by Leblond & Clermont (1952). Below, changes in tubule and Sertoli cell morphology as well as in a number of Sertoli cell parameters, during the seminiferous epithelium cycle. The thickness of each line indicates the onset, duration and size/value of the different Sertoli cell parameters, according to the respective stages. The thickest portions represent maximum values, whereas the thinnest represent basal values or no activity. SER: smooth endoplasmic reticulum; RER: rough endoplasmic reticulum; clusterin = SGP₂; ABP: androgen-binding protein; IL-1: interleukin-1 α ; FSH-cAMP: FSH-stimulated cyclic AMP. These data have been compiled from several articles: Wing and Christensen (1982); Kerr (1988); Ueno and Mori (1990); Kerr et al. (1984); Clermont et al. (1987); Wright et al. (1983); Kangasniemi et al. (1991); Ritzén et al. (1982); Sjögen et al. (1991); Lacroix et al. (1981); Söder et al. (1991); Kangasniemi et al. (1990a); Kaipia et al. (1991)

cell activity at different stages of the cycle can thus be studied. The second procedure originated from the discovery by Morales and Griswold (1987) that, in rats with vitamin A deficiency, vitamin A replacement diet resulted, in a majority of the tubules, in restoration of spermatogenesis which became stage-synchronized (Griswold 1990).

Fig. 1 presents stage specific variations in a selected number of the Sertoli cell products, measured using Parvinen and coworkers' transillumination technique. Total protein secretion varies cyclically, with two peaks at stages VI and XII. At stages VII-VIII, the levels of the following products have been observed to rise: lipid binding protein SGP₁, androgen-binding protein (ABP), cyclic protein 2 (CP2) which is the proenzyme form of the protease cathepsin-L, plasminogen activator (another protease), and the non-specific protease and growth factor inhibitor α_2 -macroglobulin. Rises of CP2 and of the plasminogen activator are interesting since they coincide with the final steps of spermiation. This suggests that these proteases play a role in sperm release into the tubule lumen. The concomitant increase of α_2 -macroglobulin may protect the epithelium against the possible toxic effect of these proteases on the epithelium. Furthermore, the number of androgen receptors rises at stage IX-X and remains elevated until stage XIV and declines thereafter. The production of testicular IL-1 is also stage-dependent. Very low amounts of this cytokine are produced at stage VII which coincides with an absence of DNA-synthesizing cells, unlike stages VIII-XIV and I-V (Söder et al. 1991). Interestingly, the increase in IL-1 levels at stage VIII coincides with the DNA replicating phase of preleptotene spermatocytes. These two events may be directly linked since Parvinen et al. (1991) have shown *in vitro* that IL-1 stimulates the replication of meiotic DNA. The increase of IL-production also occurs when the levels of its potential inactivator, α_2 -macroglobulin, rises.

As suggested below (Table 2), responsiveness of Sertoli cells to FSH is controlled in part by germ cells including late spermatids (see also Le Magueresse and Jégou, 1988a). This is further substantiated by the data in Figure 1 which shows that FSH stimulates cAMP production in the presence of phosphodiesterase inhibitor and varies in a cyclical fashion, being lowest at stages VII-VIII and peaking at stages I-V. This and a report showing that cAMP can inhibit spermiation in the

Table 2. Effects of late spermatid depletion on parameters of Sertoli cell function

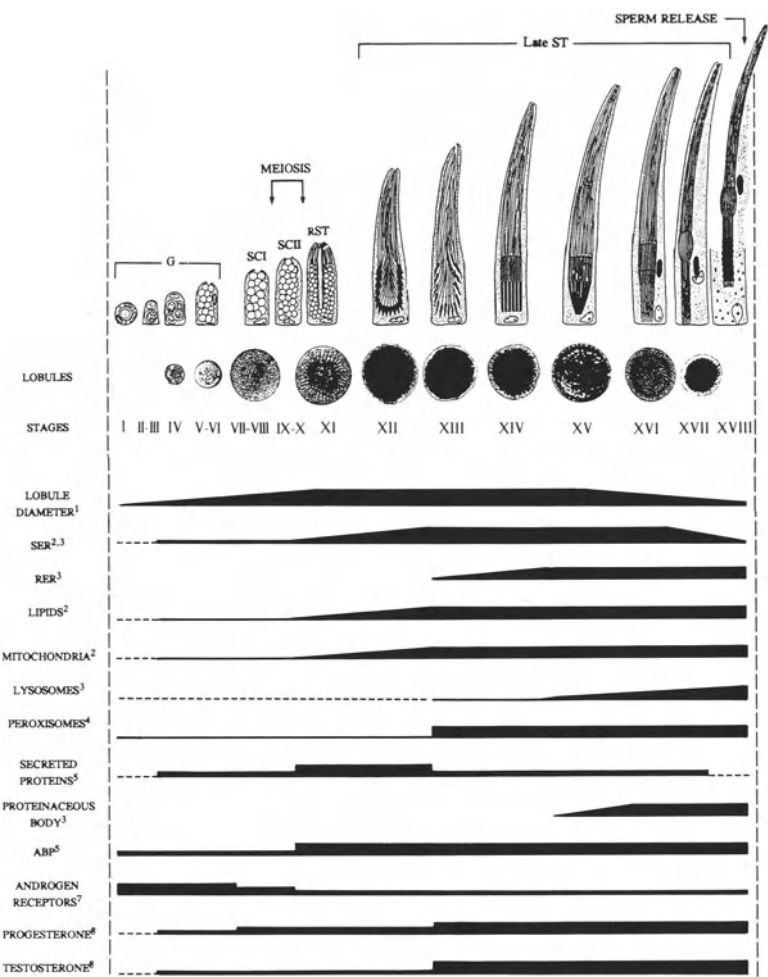
Parameter	Agent	Effect	References	
Number of organelles	γ -rays	Decreased	Pinon-Lataillade et al. 1988	
Vacuoles	γ -rays	Appeared	Pinon-Lataillade et al. 1988	
Tubule lumen size	MAA	Decreased	Sharpe, 1989	
Tubule fluid	Heat X-rays	Decreased	Jégou et al. 1984 Jégou et al. 1992	
Inhibin	X-rays & heat	Decreased	Main et al. 1976	FSH
	X-rays		Hopkinson et al. 1978	
	Heat		Jégou et al. 1984	
	X-rays		Jégou et al. 1992	
	Busulphan		Morris et al. 1987	
	γ -rays		Pinon-Lataillade et al. 1985;1988	
	γ + n & γ -rays		Pineau et al. 1989	
ABP	MAA	Decreased	Bartlett et al. 1988	Inhibin
	Heat		Au et al. 1987	
	MAA		Allenby et al. 1991	
	Heat		Jégou et al. 1984	
	Busulphan		Morris et al. 1987	
FSH receptors	X-rays	Decreased	Jégou et al. 1992	
	γ -rays		Pinon-Lataillade et al. 1988	
	γ + n & γ -rays		Pineau et al. 1989	
	Procarbazine		Velez de la Calle et al. 1988.	
FSH-stimulated cAMP production	Heat	Decreased	Jégou et al. 1984	
	X-rays		Kangasniemi et al. 1990b	
Testins	X-rays	Increased	Kangasniemi et al. 1990b	
		Increased	Jégou et al. 1992	

hamster (Gravis 1980) may indicate that low cAMP levels at stage VIII are necessary for sperm release to occur.

In addition to the cyclic variations in Sertoli cell activity described here, numerous other Sertoli cell products have been found to vary with the seminiferous epithelium cycle (Parvinen 1982; Kangasniemi et al. 1990a). Interestingly, many of the Sertoli cell products investigated to date vary most, whether positively or negatively, during stages VII-VIII. The production of these factors may, therefore, be specifically, at least in part, governed by late spermatids at the latest steps of spermiogenesis. However, since several generations of germ cells are superimposed in the same segment of mammalian tubule, no definitive conclusion can be reached on this point. It is for this reason that Callard's team (Callard et al. 1985; Dubois and Callard 1989) and our own (Sourdaïne and Jégou 1987, 1989; Sourdaïne et al. 1990) independently began to develop a non-mammalian model (the dogfish) in order to study germ cell-Sertoli cell interaction in the testis.

The dogfish model was chosen because the unit structure of elasmobranch fish testis is a lobule rather than a tubule. The lobules, like the tubules, are surrounded by myoid cells; they are of particular interest because they are composed of cysts, and each cyst comprises one

Fig. 2. *Top of the figure:* schematic representation of the cysts at the different stages of spermatogenesis in the dogfish (*Scyliorhinus canicula*) (adapted from Stanley, 1966). G: cyst with spermatogonia; SCI: cyst with primary spermatocytes; SCII: cyst with secondary spermatocytes; RST: cyst with early spermatids; Late ST: cysts with late spermatids. Transillumination photomicrograph of freshly isolated unstained dogfish seminiferous lobules. The successive stages of spermatogenesis as defined by Mellinger (1965). *Below,* changes in lobule and Sertoli cell morphology as well as in a number of Sertoli cell parameters, during spermatogenesis in *Scyliorhinus canicula* and *Squalus acanthia*. The thickness of each line indicates the onset, duration and size/value of the different Sertoli cell parameters, according to the respective stages. The thickest portions represent maximum values, whereas the thinnest portions represent basal values or no activity. Dotted portions: not investigated. SER: smooth endoplasmic reticulum; RER: rough endoplasmic reticulum; ABP: androgen-binding protein. These data have been compiled from several articles: Sourdaïne and Jégou (1989); Pudney and Callard (1984); Collenot and Damas (1980); Mendis-Handagama et al. (1992); Sourdaïne and Jégou (1992); Callard and Mak (1988); Cuevas and Callard (1990); Sourdaïne and Garnier (1992)



Sertoli cell associated with between 1 (spermatogonia) to 64 (spermatids) germ cells (Fig. 2). The cells develop synchronously within the lobules which are arranged in zones corresponding to the different stages of spermatogenesis. The zones radiate from the germinative area of the testis where they are formed, to the opposite margin of the testis where spermiation occurs. As in all known phyla, from nematodes to

mammals, dogfish Sertoli cells form a blood testis barrier which isolates the testicular compartment where meiosis and spermiogenesis occur (de Kretser and Kerr 1988; Jégou 1992). Furthermore, the spermatogenic process of the dogfish is well-documented (Mellinger 1965; Stanley 1966; Holstein 1969). Several studies have shown that a number of parameters vary in a stage-dependent manner as in mammals (Fig. 2). Unlike the rat, the simplified organization of the epithelium in the dogfish allows the association of changes in Sertoli cells to the presence of a specific germ cell type. The lobule diameter progressively increases from the earlier stages of spermatogenesis to stage VII. From this stage to stage XV, the lobule diameter is stable but markedly decreases prior to sperm release. As in mammals, germ cells also influence the morphology of Sertoli cells. Changes in Sertoli cell shape and position occur. Sertoli cell size increases during the progression of spermatogenesis and decreases before sperm release, as reflected by the reduction in lobule size in the final stages. SER begins to increase during stages XI to XII, it peaks between stages XIII and XVI and declines before sperm release. RER progressively rises between stages XIII and XV, when it reaches a plateau. The number of lipid droplets and of mitochondria increases from stages XI-XI, reaches a maximum at stage XIII, and remains elevated until spermiation occurs. A rise in the number of lysosomes and peroxisomes is observed at stages XV and XIII, respectively.

The spatial organization of lobules in the dogfish testis results in zonation on any transverse section of the testis. Thus, three (Callard et al. 1985) or four (Sourdaïne et al. 1990) distinct zones, corresponding to the lobules with spermatogonia, with spermatocytes (primary and secondary), with early spermatids and with late spermatids, can be seen and microdissected. The testicular tissues can then be directly used for measuring Sertoli cell parameters (Sourdaïne et al. 1990) or dissociated to obtain isolated lobules (Sourdaïne and Jégou 1989). Inspired by the approach of Parvinen and coworkers in the rat and based on the marked variation in light absorption of the lobules resulting from the condensation of the nuclei of the late spermatids and to their relation with the Sertoli cells, we have developed a transillumination technique (Sourdaïne and Jégou 1989) (Fig. 2). It has thus been possible to demonstrate that total protein secretion is highest at the be-

ginning of spermiogenesis. During stage XVI a proteinaceous body appears in the cytoplasm of each Sertoli cell. ABP is found to rise when spermiogenesis begins and remains elevated until sperm release, whereas the pattern of variations in androgen receptors was inverse (Callard and Mak 1988; Cuevas and Callard 1990). In the adult dogfish, Sertoli cells are extremely active steroidogenic cells. Interestingly, the lobule content of progesterone progressively rises from the earlier stages of spermatogenesis and reaches its maximum at stage XIII, as does the lobule content of testosterone. In all, in the dogfish it appears that late spermatids most probably induce marked changes in most of the morphological characteristics of Sertoli cells and an increase in their steroidogenic activity.

Although at least 350 million years of evolution separate cartilaginous fish from mammals, similarities do exist in a certain number of parameters at the time of or just before sperm release. These include reduced Sertoli cell size, presence of a developed Sertoli cell RER, numerous mitochondria and maximum levels of ABP. This may indicate that late spermatid control of these parameters has been preserved throughout evolution. The comparative study of cell-cell interaction in the testis in vertebrates is in its infancy. Rat and dogfish models present obvious differences, but, after Peter (1899), Roosen-Runge (1977, review), and others, we hypothesize that some of the important control mechanisms of spermatogenesis may have been conserved throughout evolution. We therefore want to stress the great usefulness of new models for the study of germ cell-Sertoli cell interactions. Many other species are potentially very interesting models, including nematodes (Ward 1987) and amphibians. Several non-mammalian and invertebrate models (e.g. drosophila, xenopus, zebra fish) have played a central role in elucidating the nature of many biologic phenomena.

3.2.2 Effect of Late Spermatid Depletion on Sertoli Cell Function in Mammals

Most of the studies reviewed in this section have been based on the extended use of various physical (irradiation, heat) and chemical (drugs) means which induced the selective depletion of specific germ cell type(s), in order to assess their regulatory influence on Sertoli cells.

Table 2 summarizes the particular effect of late spermatid depletion on a number of Sertoli cell parameters and shows that late spermatids influence both positively or negatively a wide range of Sertoli cell functions.

Although the studies reviewed here have been performed in the rat, Rodriguez-Rigau et al. (1980) have also demonstrated that the current concept of a particular control of Sertoli cell function by late spermatids most probably applies to humans. The authors showed that late spermatid counts, but not other germ cell counts, were negatively correlated with circulating levels of FSH in men undergoing varicocelectomy. This indicates that, in these patients, production of Sertoli cell inhibin was probably controlled by late spermatids. One of the consequences of late spermatid-induced alteration of tubule fluid production is probably the bidirectional secretion of ABP and inhibin (Morris et al. 1987; Allenby et al. 1991). Furthermore, a marked alteration of peritubular tissue morphology was observed when Sertoli cell function was disrupted after depletion of the late spermatid population in irradiated rat testes (Pinon-Lataillade et al. 1988). This illustrates the complete interdependence of the different testicular cell types.

Interestingly, whereas many proteins in cultured media of seminiferous tubules at stages VI-VII were found to decrease or disappear when the number of primary spermatocytes or of early spermatids was depleted, depletion of late spermatids had little effect (Mc Kinnell and Sharpe, 1992, quoted by Sharpe, 1992, review). This has led Sharpe (1992) to hypothesize that late spermatids predominantly control Sertoli cell products that are normally apically secreted, whereas the ones normally secreted via the basal portion of these cells may essentially be under the control of the earlier germ cell types. Further investigations are required to test this hypothesis.

4 Mechanisms Underlying Late Spermatid-Sertoli Cell Dialogue

The precise mechanisms underlying this aspect of paracrine regulation of spermatogenesis are still poorly understood.

4.1 Mechanism of Sertoli Cell Influence upon Late Spermatids

Sertoli cell control mechanisms of late spermatids take the environmental, nutritional and regulatory pathways defined by Skinner (1991). Through the formation of the blood testis barrier the Sertoli cell creates, in all animal species so far studied, the unique microenvironment required for normal spermatogenesis (Fawcett 1975; de Kretser and Kerr 1988; Jégou 1992, reviews). Furthermore, as shown above (2, Table 1), Sertoli cells can interact with late spermatids through an extremely sophisticated set of structural devices.

It is well known that Sertoli cells control the process of spermiation. This has been deduced from the following observations: 1) sperm release is controlled by pituitary and/or testis hormones which act via the Sertoli cells (Russell 1991); 2) it is the Sertoli cells, but not late spermatids, which contain the cytoskeletal agents required for spermatid movement within the epithelium (Russell 1991); 3) Sertoli cells produce several proteinases (Lacroix et al. 1977; Hettle et al. 1986; Sang et al. 1990) which may be needed for sperm to detach from Sertoli cells.

Russell and Peterson (1984), de Kretser and Kerr (1988) and Sakai and Yamashina (1990) have reviewed the different (and often complementary) hypotheses proposed to explain spermiation and therefore will not be discussed here. Two recent studies have brought new insight to bear on this extraordinary process in mammals.

It is generally accepted that ectoplasmic specializations and Sertoli cell cytoskeletal elements are involved in the transport of spermatids through the seminiferous epithelium (Russell 1984; de Kretser and Kerr 1988; Table 1). An elegant model for microtubule-based spermatid transport has been proposed in which Sertoli cell ectoplasmic specializations are considered as vehicles, Sertoli cells microtubules as

tracks and at least one Sertoli cell mechanoenzyme, dynein, as the motor which powers the transport of spermatids along the microtubule tracks (Redenbach and Vogl 1991).

The work of Sakai and Yamashuma (1990) suggests that the Sertoli cell processes which invade the spermatid cytoplasm (Table 1) are dynamic structures which crucially contribute to the adluminal displacement of the spermatid head. In this model, the disappearance of ectoplasmic specializations necessarily precedes sperm release, as previously observed by others in mammalian and in non-mammalian species (Russell 1984; Sprando and Russell 1987b). As stated above (3.1), an essential and general function of germ cell "nutrition" has been assigned to the Sertoli cells. The fact that spermatids are sequestered within the adluminal compartment of the germinative epithelium makes them extremely dependent on Sertoli cells for their survival and development. These cells undergo perhaps the most dramatic morphological changes of all cells in the body; there is no doubt that their structural and metabolic requirements change between the time they are formed and the time they are shed as sperm. Therefore, known Sertoli cell products which influence early spermatids may not affect late spermatids. A good example is transferrin, a well known major Sertoli cell product (Skinner and Griswold 1980). Whereas early spermatids are able to bind and to internalize transferrin by a receptor-mediated endocytic process (Toebosh et al. 1987; Segretain et al. 1992), late spermatids have lost this capacity (Segretain et al. 1992). We have proposed that several Sertoli cell substances may be involved in the nutritional control of late spermatids (above in 3.1). Here we speculate on how these factors may influence late spermatids.

Tubule fluid is the vector of Sertoli and even of germ cell secretions in the intercellular spaces and the formation of inter-Sertoli cell tight junctions is essential for the initiation of its production (Jégou et al. 1982, 1983; Jégou, 1992). This Sertoli cell product may therefore transport some of the messages required for spermatid differentiation; it may also intervene in the sperm release process. Sertoli cells are also known to produce lactate and pyruvate from glucose (Jutte et al. 1981). These energy metabolites are able to support the metabolism of early spermatids *in vitro* (Jutte et al. 1981; Mita et al. 1982) and therefore are probably required for late spermatids. However, this requirement

may cease in the later steps of spermiogenesis: very shortly before spermiation, glycolytic enzyme behavior may be changed, enabling spermatozoa to acquire a glycolytic capacity, unlike spermatids (Grootegeed and Den Boer 1989).

Earlier studies have shown that spermiogenesis is highly susceptible to fat deficiency (Hildebrandt-Stark and Fawcett 1978). Furthermore, Lynch and Scott (1951) have suggested that Sertoli cell lipids can be transported to late spermatids. This hypothesis still remains to be proven. However, Sertoli cells produce large quantities of SGP₁ which is a putative lipid binding proteins localized in the sperm and on the sperm plasma membrane (Griswold 1988; Skinner 1991), thereby substantiating this hypothesis. Little is known of the possible transfer of vitamins to late spermatids (and to other germ cells) by the vitamin-binding proteins produced by Sertoli cells (Skinner 1991, review).

Macindoe and Turkington (1973) have proposed that Sertoli cell putresceine may be utilized as a substrate to form spermidine found in spermatids and spermatozoa. However, the presence of ornithine decarboxylase (ODC) mRNA (ODC: the rate limiting enzyme in polyamine biosynthesis) in all tubular cell types, including late spermatids and residual bodies (Alcivar et al. 1989) does not enable easy verification of this hypothesis.

Lastly, Sertoli cells transport of amino acids to germ cells, including spermatids, may be important to glutathione synthesis (Den Boer et al. 1989). Glutathione is essential in many biological processes (e.g. protection of cells against oxydation), consequently this nutritive function of the Sertoli cell may be crucial for optimal sperm fertility.

In addition to these nutritive factors, Sertoli cells produce a number of substances such as growth factors (Bardin et al. 1988; Skinner, 1991) which may act as regulators of germ cell metabolism and function. Whether these substances actually regulate late spermatids is totally unknown. Sertoli cells also secrete polypeptides which stimulate phagocytosis (Russo et al. 1988) and therefore may be involved in the interaction between Sertoli cells and residual bodies.

Interestingly, Gérard et al. (1991a) have shown that ABP and human sex steroid-binding protein (hSBP) could be endocytosed by germ cells including late spermatids and residual bodies, which suggests that steroid-binding proteins may act as transmembrane steroid

carriers during spermatogenesis. This and the findings of Segretain and coworkers on endocytosis in germ cells (Segretain 1989; Segretain et al. 1992) indicates that certain Sertoli cell nutritive and regulatory products may be transferred to late spermatids via endocytosis.

It should be noted that the Sertoli cell factors with putative effects on late spermatids are under humoral control (Bardin et al. 1988; Jégou 1992, reviews). This indicates that Sertoli cells not only control late spermatids directly, but that the control may be exerted and/or modulated via the transduction of systemic hormonal signals such as FSH and testosterone.

The list of Sertoli cell products which act on late spermatids is certainly far from limited to the ones presented here. The exceptionally intimate relationship between Sertoli cells and late spermatids will certainly remain a formidable obstacle to the direct demonstration that a particular Sertoli cell substance is actually transferred to late spermatids. Progress in this aspect of paracrine regulation of spermatogenesis obligatorily requires progress in the elucidation of the specific nutritive and regulatory requirements of late spermatids.

4.2 Mechanism of Late Spermatids' Influence upon Sertoli Cell Function

The earliest direct evidence that spermatids (steps 1-8) may influence Sertoli cell function was provided by Le Gac et al. (1984) and Le Magueresse et al. (1986). The involvement of spermatids (1-8) in the production of a substance(s) which stimulates ABP, transferrin, inhibin, gamma glutamyl-transpeptidase, ceruloplasmin and SGP₁ and SGP₂, production or which inhibits aromatase activity, was subsequently established (Le Magueresse and Jégou 1988b; Le Magueresse et al. 1988; Schteingart et al. 1989; Castellon et al. 1989; Onoda and Djakiew 1990; Pineau et al. 1990). The purification of these early spermatid proteinaceous factors is underway (Pineau et al. 1991a).

Are late spermatids also able to secrete factors which regulate Sertoli cell function? To date we do not know whether throughout spermiogenesis spermatids are able to secrete proteins that regulate Sertoli cell function, or whether the spermatid products are only produced at

certain steps. There is presently no evidence that a late spermatid gene product can be secreted (Hecht 1988). Late spermatid conditioned media cannot be prepared, since isolation of these cells is not possible. Further investigations are required to elucidate this possible aspect of late spermatid control over Sertoli cell function. Of note, however, is that, in contrast to the conditioned media prepared with early spermatids, media conditioned by cytoplasmic fragments of late spermatids and by residual bodies were unable to influence or had only very marginal effects on Sertoli cell function *in vitro* (Le Magueresse and Jégou 1988b; Le Magueresse et al. 1988b; Pineau et al. 1990).

There are several other potential routes whereby late spermatids can influence Sertoli cell gene expression (Jégou 1991):

1. The first pre-supposes that alterations in the type of adhesive contact between late spermatids and Sertoli cells may influence signalling in much the same way as soluble factors do, and also affect Sertoli cell gene expression. For example, the calcium-dependent cell adhesion molecule, N cadherin, dramatically stimulates neurite outgrowth (Doherty et al. 1991). In this instance the effects of N-cadherin are mediated via N and L-type calcium channels. Interestingly, N-cadherin is also present in the testis and is expressed in Sertoli cells and spermatids (Byers et al. 1992). Whether testins which have just been localized to the site of attachment of late spermatids to Sertoli cells (Zong et al. 1992) are involved in such a regulatory signal system remains to be elucidated;
2. A second possible route involves a direct cytoskeletal link between the late spermatid-Sertoli cell interface and the nuclear transcription machinery. This model has been proposed as a potential means whereby externally imposed shape changes, due to cell substratum or cell-cell contact, could directly influence *gene transcription* (Pienta and Coffey 1991). The model requires that a cytoskeletal link be present between the plasma membrane and the *nuclear matrix*. Any changes in cell shape resulting from cell-cell or cell-substratum contact could then be translated via the *cytoskeleton* to the nuclear matrix resulting in an almost physical modulation of transcription (Pienta and Coffey 1991). Although there is no evidence that such a mechanism is used by cells to regulate gene expression,

there are indications that certain components of the model are in place. For example, *vimentin* intermediate filaments interact both with the plasma membrane and with *lamin B* on the nucleoplasmic face of the nuclear envelope (Georgatos and Blobel 1987a,b). Since the nuclear lamina is an attachment site for chromatin, it is possible that alterations in vimentin contact at the plasma membrane could indeed influence chromatin in the nucleus. Sertoli cells do express vimentin filaments and electron microscopic images suggest a close relationship of intermediate filaments with the Sertoli cell plasma membrane and the nucleus (Paranko et al. 1986; Amlani and Vogl 1988). Quite marked changes in Sertoli cell nuclear morphology also take place during spermatogenesis: at certain stages elongated spermatids appear almost to be lodged in clefts in the nuclear envelope (Amlani and Vogl, 1988). In fact, of all germ cells it is the spermatids which exercises the greatest workload on the Sertoli cell (Jégou 1991). In other systems gene expression is influenced by changes in cell shape (Watt et al. 1988; Schmidhauser et al. 1990). For example, the dramatic effects of the extracellular matrix on gene expression have in part been attributed to an indirect effect mediated by ECM-induced changes in cell shape (Watt et al. 1988; Schmidhauser et al. 1990). Whether or not this sort of transducing mechanism mediates the spermatid effects on Sertoli cell gene expression remains to be determined. The striking changes in spermatid and Sertoli cell shape which take place during spermiogenesis indicate that if such a model were correct, it would almost certainly contribute crucially to germ cell modulation of Sertoli cell gene expression;

3. The third possible route involves the structures shed from late spermatids: tubulobulbar complexes and residual bodies. Because they are watery and organelle-free (Table 1), to our knowledge, their bulbous portions have never been supposed to be involved in anything other than autocrine functions: the elimination of excess spermatid cytoplasm prior to sperm release (Russell 1980, 1984) and the concentration in late spermatids of glycolytic enzymes (Groote-god and Den Boer 1989). However, considering the enormous amount of spermatid cytoplasm phagocytosed in this manner by Sertoli cells (as much as 70 % of the total spermatid cytoplasm;

Russell 1980), there is little doubt that they must also influence Sertoli cell function in some ways. Demonstration of a tubulobulbar complex in Sertoli cell control appears, however, extremely difficult to realize technically.

The hypothetical role of residual bodies in local regulation of the spermatogenic cycle was first proposed by Roosen-Runge (1952). This hypothesis was based on the facts that in the rat, the formation and phagocytosis of residual bodies coincide with the occurrence of A spermatogonia mitosis, the entry of spermatocytes into the meiotic prophase, the rapid transformation of early spermatids and marked morphological changes of Sertoli cells. Furthermore, on the grounds that a cyclic change in the lipid content of Sertoli cells exists in the same species, which peaks just after the release of sperm, Lacy (1962) has proposed that the digestion of residual bodies provides Sertoli cells with the precursor required for steroid synthesis. The Sertoli cell steroid(s) thus formed may stimulate the entry of spermatocytes into meiosis thus permitting spermatid renewal for the next cycle.

However, this putative role of residual bodies has never really been accepted. Arguments against it were based on:

1. The cyclical pattern of the seminiferous epithelium observed in the adult mammal emerges before the first generation of spermatozoa is released into the lumen of the tubules (Roosen-Runge 1962);
2. no direct link has ever been established between the dispersion of residual body content and the Sertoli cell lipid inclusions, in any species so far studied (Kerr et al. 1984);
3. it is more likely that lipids derived from residual bodies are degraded by Sertoli cell lysosomal enzymes (Kerr et al. 1984; de Kretser and Kerr 1988).

We believe that the possible involvement of residual bodies in the regulation of Sertoli cell function and therefore of spermatogenesis should be reconsidered (Jégou 1991). As stated earlier, the implication that residual bodies are involved in the synchronization of the spermatogenic process is not necessarily limited to the existence of earlier regulatory mechanisms, which may be sequentially assumed by

younger generations of germ cells as they appear in the tubules during testicular development. Furthermore, lipids are not the only residual body constituents whereby these structures can influence Sertoli cell function.

We have hypothesized that residual bodies may regulate Sertoli cell function via two pathways (Jégou 1991):

1. Residual body mRNA released after residual body lysis within the Sertoli cell may code for factor(s) which control Sertoli cell gene expression; Sertoli cell factors may then regulate different events at the germ cell level. Alternatively, the mRNA residual body translation products could themselves directly influence germ cell division and metabolism.
2. By analogy with the monocyte/macrophage system, where phagocytosis of foreign elements (i.e. bacteria) activates the production of interleukin 1 (IL-1) (Dinarello 1988), we also proposed that phagocytosis of residual bodies by Sertoli cells may trigger the production of factor(s) able to regulate Sertoli cell and/or germ cell functions.

Verification of these hypotheses required the development of an *in vitro* model. An obstacle to this was the obtainment of a pure population of residual bodies (RB). Meistrich et al. (1981) have shown that the "residual bodies" prepared *in vitro* are in fact essentially cytoplasts from elongated spermatids (CES) that are formed by the rupture of the cytoplasmic lobes of spermatids at steps 10 to 18 of spermiogenesis (rat). Rupture occurs during the mechanical and enzymatic treatments of the tubules. We recently confirmed this observation (Pineau et al. 1991b). However, our studies have indicated that *in vitro* the different steps of the digestion of CES/RB very closely resemble the different steps of residual body digestion *in vivo* (Pineau et al. 1991b). We therefore adopted this *in vitro* system to study late spermatid effects on Sertoli cells; we found that CES/RB adhere much better to Sertoli cells than early spermatids (steps 1-8) (Le Magueresse et al. 1986; Pineau et al. 1991b). On this basis and the finding that plasma membrane antigens specific to residual bodies do exist (Byers et al. 1992), we recently proposed that the binding of late spermatid cytoplasmic lobes

and of residual bodies does not involve a single adhesive molecule, but is the result of a strictly regulated cooperation between molecules. Some of the molecules may be present at earlier stages of germ cell development, their partitioning at the cell surface being modified as previously suggested by Millette (1979); other(s) may be specifically expressed just prior to sperm release (Byers et al. 1992).

Our first attempts to influence Sertoli cell secretory activity *in vitro* by coculturing them with CES/RB were disappointing. CES/RB had no significant effect on transferrin, inhibin and estradiol production and only marginally influenced ABP secretion, whereas spermatocytes and early spermatids were very active on Sertoli cell function; the CES/RB effect could be attributed to the early spermatids contaminating the CES/RB fractions used (Le Magueresse et al. 1986; 1988; Le Magueresse and Jégou 1988b; Pineau et al. 1990).

Interleukin 1 (IL-1) was recently found to be produced *in vitro* by seminiferous tubules (Syed et al. 1988) and Sertoli cells (Gérard et al. 1991b). We decided to investigate the effect of phagocytosis of CES/RB on this factor because its production is triggered by the phagocytosis of bacteria by macrophages (Dinarello, 1988). Our results show that whereas IL-1 was not detectable in Sertoli cell culture media from 20 day-old rats, the production of IL-1 was activated by CES/RB. This was specific since pachytene spermatocytes or early spermatids did not induce any comparable effect (Fig. 3). CES/RB also stimulated prepubertal (35 days) and pubertal (45 days) Sertoli cells (Gérard et al. 1992). Furthermore, activators of monocytes/macrophages like lipopolysaccharides and latex beads were also found to be extremely active on Sertoli cells (Gérard et al. 1992). Of note, however, is the fact that, contrary to the other phagocytic cells where IL-1 β is predominant, Sertoli cells only produce IL-1 (Gérard et al. 1991, 1992). These experiments were the first to demonstrate that Sertoli cell phagocytic function cannot be reduced only to a function of disposal of degenerating germ cells or of excess germ cell materials; it is, in fact, a very potent regulatory system of Sertoli cells. The induction of IL-1 production by residual bodies, *in vitro*, is compatible with the rise of IL-1 which occurs in rat seminiferous tubules, between stages VII at VIII of the cycle (Söder et al. 1991; Fig. 1).

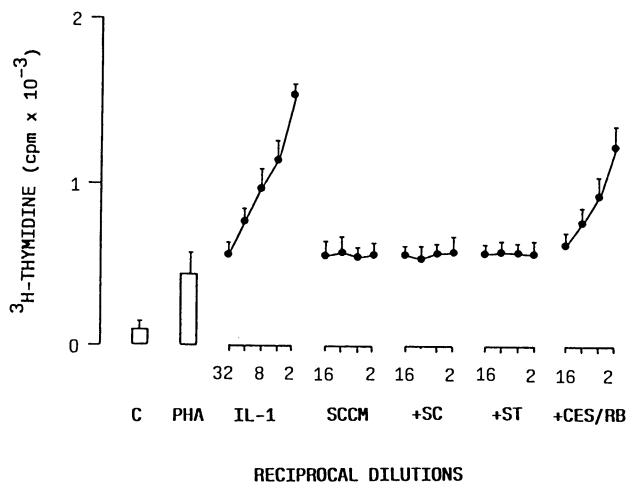


Fig. 3. Effects of enriched fractions containing pachytene spermatocytes (2×10^6 ; + SC), early spermatids (8×10^6 ; + ST) and cytoplasm from elongated spermatids/residual bodies (8×10^6 ; CES/RB) on interleukin-1 (IL-1) secretion by rat Sertoli cells over a period of 20 h. Sertoli cells were cultured as previously described (Gérard et al. 1991). IL-1 activity was determined in the culture media using the murine thymocyte assay. Mouse thymocytes were cultured in the presence or absence of test material for 46 h. ^3H -thymidine was added to cells and incubated for 2 h. The cells were then harvested and the incorporated radioactivity determined (Scale and Oppenheim, 1983). C: control thymocytes; PHA: thymocytes plus phytohemagglutinin; SCCM: media of Sertoli cells cultured alone

IL-1 is a cytokine with a multitude of biological effects including mitogenic and cell differentiation; IL-1 is also able to stimulate mitotic and meiotic DNA syntheses in the tubules, *in vitro* (Parvinen et al. 1991). We can therefore conclude that phagocytosis of residual bodies, via IL-1 production, is an essential regulatory system of the spermatogenic process. However, further investigations are required to demonstrate an actual involvement of residual bodies in the synchronization of the cycle of the seminiferous epithelium.

5 Concluding Remarks

The dialogue between late spermatids and Sertoli cells has always attracted the attention of morphologists and knowledge of the anatomical support of this aspect of the paracrine regulation of Sertoli cells by germ cells has progressed tremendously. However, the mechanisms underlying this dialogue are still very poorly understood and biologists of all disciplines should now join their efforts in unravelling them. After a century of research, it is remarkable that we still have no precise answers to the fundamental questions: What are the mechanisms controlling spermatid movements in the epithelium and sperm release? What is the nature of the Sertoli cell factors required for late spermatids? How do late spermatids influence Sertoli cell function? How is the synchronization of the seminiferous epithelium cycle controlled?

Data have, however, begun to accumulate on some aspects of the Sertoli cell control by late spermatids and a broad range of techniques are now available. Furthermore, important breakthroughs have been made in other domains of cell biology which are fundamentally relevant to some aspects of late spermatid-Sertoli cell dialogue. Rapid progress should, therefore, be made in deciphering the language used by these two cell types.

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Structure and Function of the Human Androgen Receptor

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1 Introduction

Androgens play an essential role in several stages of male development [e.g. sexual differentiation, initiation and maintenance of spermatogenesis, development and maintenance of secondary male characteristics, feedback regulation of gonadotrophin secretion](Griffin and Wilson 1989). The action of androgenic hormones (e.g. testosterone and 5 α -dihydrotestosterone) is mediated by the intracellular androgen receptor, which belongs to a superfamily of ligand-dependent transcription

factors. This family also includes the other steroid hormone receptors and the thyroid hormone and retinoic acid receptor subfamilies (Evans 1988; O'Malley 1990).

The androgen-receptor complex transduces the extracellular androgenic signal via binding to specific enhancer sequences referred to as androgen responsive elements present in the flanking regions of androgen target genes and is supposed to control transcription through protein-DNA interactions and protein-protein interactions with other transcription factors (Beato 1989; Wahli and Martinez 1991). This could result in the formation of a stable pre initiation complex near the transcription start site of the target gene, which allows efficient transcription initiation by RNA-polymerase II. The androgen receptor might achieve this by stimulating the assembly of the preinitiation complex or by stabilizing the complex. This interaction can be direct or indirect, involving coactivators that mediate synergism between different transcription factors.

The cloning and characterization of cDNA encoding the human androgen receptor have greatly increased our knowledge of the functional

Gene:

Single Copy on X-Chromosome

Eight Exons:

Exon 1 : Regulatory Domain

Exons 2 & 3: DNA-Binding Domain

Exons 4-8 : Androgen Binding Domain

mRNA:

Two Major Species:

11 and 8.5 kb

High expression in prostate cell lines androgen regulated

Protein:

910 Amino Acid Residues

N-Terminal-Domain: DNA-Binding Domain: Androgen-Binding Domain:

Gln and Gly stretches Cys/Arg/Lys rich Hydrophobic

Pro, Glu, Gly

and Ala rich

Hydrophilic

Fig. 1. Summary of the structural characteristics of the human androgen receptor gene, mRNA and protein

structure and expression of the human androgen receptor (Chang et al. 1988; Lubahn et al. 1988; Trapman et al. 1988; Faber et al. 1989; Tilley et al. 1989). In Fig. 1 the present knowledge on the structural aspects of the human androgen receptor gene, mRNA and protein has been summarized. In human prostate tissue two androgen receptor mRNAs have been demonstrated with different sizes (11 and 8.5 kilobase (kb) respectively). The two mRNAs are generated by alternative splicing in the 3'-untranslated region of the mRNA (Faber et al. 1991). The cDNA sequence of the human androgen receptor reveals an open reading frame of 2730 nucleotides encoding a protein of 910 amino acid residues with a calculated molecular mass of 98.5 kilodaltons (kDa). The single copy gene is located on the X-chromosome at the Xq11-12 locus and has a size of >90 kb (Kuiper et al. 1989; Brinkmann et al. 1989; Brown et al. 1989). The information for the protein coding region is separated over eight exons. The sequence encoding the N-terminal domain is present in one large exon (exon one) (Faber et al. 1989; Faber et al. 1991), whereas the DNA-binding domain is encoded by exons two and three. The information for the steroid binding domain is distributed over five exons (exons four to eight) (Kuiper et al. 1989; Lubahn et al. 1989; Marcelli et al. 1990a).

The recent availability of specific monoclonal antibodies against the human androgen receptor has greatly enhanced the characterization of the androgen receptor protein with biochemical and immunohistochemical techniques (Van Laar et al. 1989; Ruizeveld de Winter et al. 1990; Ruizeveld de Winter et al. 1991; Van Laar et al. 1991; Zegers et al. 1991). It is possible now to immunoprecipitate selectively the human androgen receptor protein from total cell lysates even if present in low concentrations.

In this overview we will focus on the functional domain structure of the wild-type human androgen receptor, on the synthesis and phosphorylation of the androgen receptor protein and finally on the molecular structure of the androgen receptor in individuals with the androgen insensitivity syndrome. Throughout the text, the numbering of the different codons is based on a total number of 910 amino acid residues in the human androgen receptor. This number differs from the amino acid content published by others: 917 residues (Tilley et al. 1989); 918 residues (Chang et al. 1988) and 919 residues (Lubahn et al. 1988). These differences are caused by the variation in length of the polyglu-

tamine and polyglycine stretches in the N-terminal domain of the receptor reported by the different research groups.

2 Functional Domain Structure

Human androgen receptor mutants were transiently expressed in COS and HeLa cells to establish the domains involved in hormone binding and transactivation. The hormone binding domain is encoded by approximately 250 amino acid residues in the C-terminal region. Deletion of amino acid residues 651-712 or 712-731 in this domain as well as truncation of the last 257, 197 or 114 C-terminal amino acid residues, abolishes hormone binding completely (Fig. 2). In contrast to

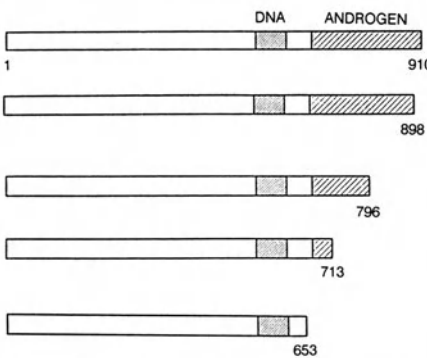
		FUNCTIONAL ACTIVITIES OF HUMAN ANDROGEN RECEPTOR MUTANTS		
		STERIOD BINDING	CAT ACTIVITY % -R1881	+R1881
	1	+	< 1	100
	898	-	< 1	< 1
	796	-	< 1	< 1
	713	-	40	40
	653	-	90	110

Fig. 2. Steroid binding and transcription activation activity of human androgen receptor C-terminal deletion mutants. Androgen receptor proteins were expressed in COS cells and hormone binding was determined by whole cell labeling with [³H]-R1881. Transcriptional activity was examined by cotransfection of androgen receptor expression plasmids and an androgen responsive element containing reporter plasmid (GREtkCAT). CAT activity was determined from cell lysates of transfected HeLa cells cultured in the absence (-R1881) or presence (+R1881) of 10 nM R1881. CAT activity induced by the wild type androgen receptor in the presence of 10 nM R1881 was arbitrarily set at 100%

the estrogen receptor, where truncation of even a C-terminal fragment of 61 amino acid residues does not affect ligand binding (Lees et al. 1989), the truncation of only 12 amino acid residues in the human androgen receptor results in complete loss of binding of methyltrienolone (R1881). Deletions in the N-terminal and DNA-binding domain do not affect hormone binding (Fig. 3 and Jenster et al. 1991). These results are in agreement with mutational analysis studies performed on the other members of the steroid hormone receptor family (Kumar et al. 1987; Giguere et al. 1986; Dobson et al. 1989).

Transcriptional activation by androgen receptor wild type and mutants was investigated after co-transfection in HeLa cells of the androgen receptor expression plasmids and a plasmid containing an androgen-regulated reporter gene construct. The reporter construct

FUNCTIONAL ACTIVITIES OF HUMAN ANDROGEN RECEPTOR MUTANTS

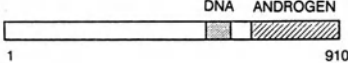
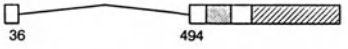
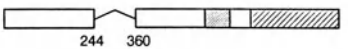
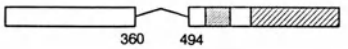
	STERIOD BINDING	CAT ACTIVITY % -R1881 +R1881
 1 910	+	< 1 100
 36 494	+	< 1 < 1
 51 211	+	< 1 < 5
 244 360	+	< 1 100
 360 494	+	< 1 100

Fig. 3. Steroid binding and transcription activation activity of human androgen receptor N-terminal deletion mutants. Androgen receptor proteins were expressed in COS cells and hormone binding was determined by whole cell labeling with [^{3} H]-R1881. Transcriptional activity was examined by cotransfection of androgen receptor expression plasmids and an androgen responsive element containing reporter plasmid (GREtkCAT). CAT activity was determined from cell lysates of transfected HeLa cells cultured in the absence (-R1881) or presence (+R1881) of 10 nM R1881. CAT activity induced by the wild-type androgen receptor in the presence of 10 nM R1881 was arbitrarily set at 100%

contains two androgen responsive elements in front of a thymidine kinase promoter linked to chloramphenicol acetyltransferase (CAT) reporter gene. Wild type androgen receptor induces CAT expression 50-fold in the presence of 10 nM R1881. This activity was arbitrarily set at 100%. C-terminal deletions of either the last 12 or 114 amino acid residues inactivate the receptor (Fig. 2). Interestingly, a human androgen receptor without the hormone binding domain is functionally active (Fig. 2). Deletion of this domain leads to a constitutively active androgen receptor protein, which has wild type trans-activation capacity, even in the absence of hormone. Thus, it appears that in the absence of hormone the hormone binding domain acts as a repressor of the trans-activation function.

The N-terminal domain of the human androgen receptor is characterized by the presence of several homopolymeric amino acid stretches e.g. three polyglutamine stretches with different lengths, a long polyglycine stretch, a polyproline stretch of eight residues and a polyalanine stretch of five residues (Faber et al. 1989). The exact function of these amino acid repeats is still unknown, although glutamine stretches, designated as opa-repeats have been found in proteins which are involved in developmental control and/or regulation of gene expression (Wharton et al. 1985; Duboule et al. 1987). A mutant androgen receptor lacking a large part of the N-terminal domain (amino acid residues 37-494) is still able to bind androgen, but does not induce CAT activity anymore (Fig. 3). Narrowing down the region in the N-terminal domain which is indispensable for transcription activation results in the identification of a transcription activation unit encoded by amino acid residues 51-211. This region contains the three polyglutamine stretches. The length of the longest polyglutamine repeat varies in different normal individuals from 17 to 29 residues (La Spada et al. 1991; McPhaul et al. 1991; Sleddens et al. unpublished data). This type of polymorphism offers the possibility of linkage studies in families with supposed defective androgen receptors. Deletion of the longest glutamine stretch, however, did not affect the transactivating function of the androgen receptor in test systems. Deletion of a region in the N-terminal domain (residues 360-494) containing the polyglycine stretch is also not deleterious for transcription activation by the androgen receptor (Fig. 3).

3 Androgen Receptor Synthesis and Phosphorylation

In previous studies we have demonstrated that the androgen receptor from human LNCaP cells has different isoforms, which migrate as a closely spaced doublet of 110-112 kDa on SDS PAGE (Van Laar et al. 1990). Both isoforms were able to bind ligand specifically as was assessed by photoaffinity labeling with radioactive methyltrienolone (R1881). To investigate the origin of the 110-112 kDa androgen receptor proteins experiments were designed to study the synthesis of both isoforms in more detail with the aid of ^{35}S -methionine incorporation and specific immunoprecipitation with a monoclonal antibody against the human androgen receptor. Incubations of hormone-depleted LNCaP cells with radioactive labeled methionine for 5, 10, 15 and 20 minutes, respectively, results in a rapidly labeled androgen receptor protein of 110 kDa within 5 minutes and virtually no labeling of the 112 kDa form (Fig. 4). After longer incubation periods a gradual increase in the amount of the slower migrating androgen receptor protein (112 kDa) was observed. The different kinetics of the *de novo* syn-

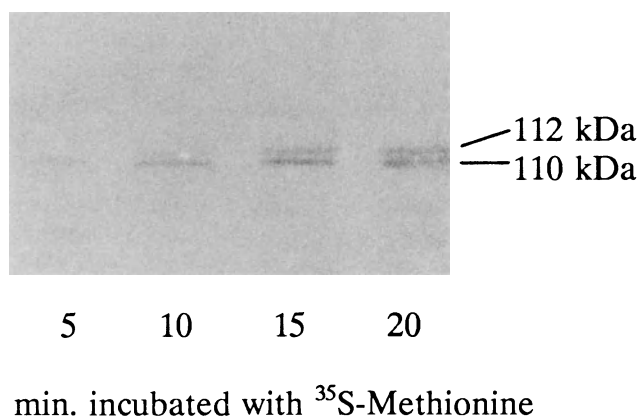


Fig. 4. Androgen receptor synthesis in LNCaP-cells. LNCaP-cells, pre-incubated for 60 minutes in methionine-free medium, were incubated for different time periods with [^{35}S]-methionine. Androgen receptors were immunoprecipitated from total cell lysates and analyzed by SDS-PAGE and blotting. Incubations with radioactive methionine were for 5, 10, 15, and 20 minutes

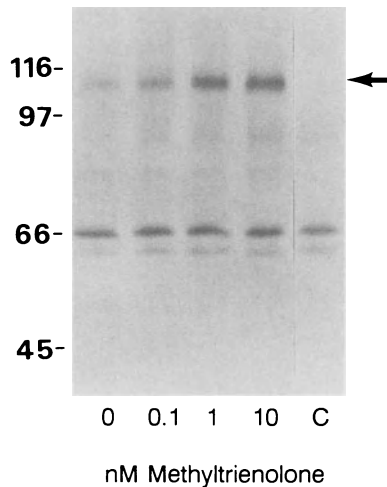


Fig. 5. Hormone-dependent androgen receptor phosphorylation. LNCaP-cells, cultured for 4 h with [^{32} P]orthophosphate (0.15 mCi/ml), were incubated in the absence or presence of 0.1, 1, or 10 nM R1881. R1881 was added 30 minutes before the end of the incubation with radiolabeled phosphate. Receptors were immunoprecipitated from total cell lysates with a specific monoclonal antibody or with a non-specific mouse IgG (lane C). Isolated proteins were separated on a 7% SDS-polyacrylamide gel, transferred to nitrocellulose. The protein blot was used for autoradiography

thesis of the 110 kDa and 112kDa androgen receptor proteins strongly suggests a time dependent conversion of the 110 kDa androgen receptor protein into the 112 kDa form. In separate experiments it was established that both isoforms can undergo a hormone-dependent transformation to a tight nuclear binding form, indicating that the 110 kDa to 112 kDa conversion is not an obligatory step for hormone binding nor for receptor transformation (Kuiper et al. 1991).

Evidence that the androgen receptor is a phosphoprotein has been obtained by metabolic labeling studies with radioactive orthophosphate in LNCaP-cells (Van Laar et al. 1990; Van Laar et al. 1991). The phosphorylation of the human androgen receptor could be enhanced considerably by addition of increasing concentrations of R1881 (0.1, 1, and

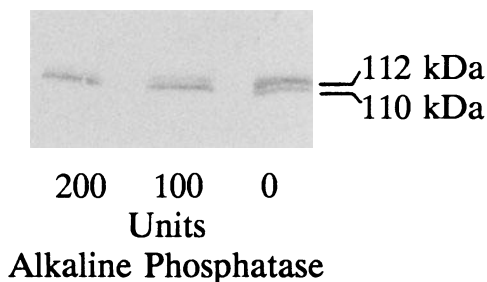


Fig. 6. Androgen receptor dephosphorylation. LNCaP-cells were incubated for 60 minutes with 10 nM R1881. After homogenization of the cells cytosol was prepared and incubated with either 0, 100 or 200 units alkaline phosphatase before immunoprecipitation. The isolated proteins were separated on a 7% SDS-polyacrylamide gel and transferred to nitrocellulose

10 nM) 30 minutes before the end of a 4 h metabolic labeling period with ^{32}P -orthophosphate (Fig. 5).

The observed micro-heterogeneity of the androgen receptor protein might be linked to a differential phosphorylation status of the receptor protein as has been observed for other steroid hormone receptors and other phosphoproteins. Alkaline phosphatase treatment of cytosolic fractions from LNCaP cells containing both androgen receptor isoforms resulted in the selective elimination of the 112 kDa form with a concomitant increase of the 110 kDa form (Fig. 6). The effect of alkaline phosphatase could be blocked completely by the simultaneous addition of phosphatase inhibitors, indicating that the apparent upshift in molecular mass from 110 kDa to 112 kDa is caused by a phosphorylation of the 110 kDa androgen receptor protein.

The androgen receptor doublet is not a specific feature of the LNCaP-cell, because a similar doublet is observed in transfected COS cells expressing wild type androgen receptor cDNA (Fig. 7). This finding indicates that a similar post-translational process is operating in both cell types. Interestingly, a 84 kDa androgen receptor mutant lacking amino acid residues 51-211 does not display doublet heterogeneity on SDS-PAGE after expression of the mutant protein in COS cells (Fig. 7). This result indicates that possible phosphorylation sites caus-

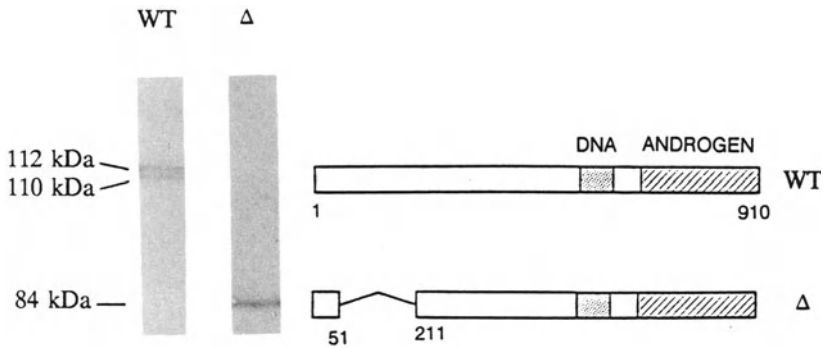


Fig. 7. Immunoblot of wild-type androgen receptor (WT) and a mutant androgen receptor lacking amino acid residues 51-211 (Δ). The receptor proteins were expressed in COS-1 cells and immunoprecipitated with a specific monoclonal antibody, separated by SDS-PAGE, transferred to a nitrocellulose filter and immunostained with a polyclonal antibody against the human androgen receptor.

ing the doublet are located in the deleted region (e.g. amino acid residues 51-211).

Partial proteolytic digestion of the androgen receptor with chymotrypsin results in the generation of a 45 kDa fragment which contains the Androgen- and DNA-binding domains (De Boer et al 1987). From double labeling experiments with the human androgen receptor from LNCaP-cells using ^{32}P -orthophosphate for metabolic labeling and [^3H]-R1881 as photoaffinitylabel, it could be calculated that 10 times more radioactive phosphate residues were incorporated in the intact receptor than in the 45 kDa chymotrypsin-generated fragment. Furthermore, a relatively high ^{32}P -phosphate content could be identified in receptor fragments originating from the N-terminal domain. This indicates that the androgen receptor in LNCaP cells is preferentially phosphorylated in the N-terminal domain (Kuiper et al. unpublished data).

4 Naturally Occurring Androgen Receptor Mutants

4.1 Androgen Insensitivity

It is generally accepted that in the X-chromosome linked androgen insensitivity syndrome, defects in the androgen receptor gene can prevent the normal development of both internal and external male structures in 46,XY individuals (Pinsky and Kaufman 1987; Griffin and Wilson 1989). Information on the molecular structure of the human androgen receptor has facilitated the study of molecular defects associated with androgen insensitivity. These naturally occurring mutations in the androgen receptor gene are an interesting source for the investigation of receptor structure-function relationships. In addition, the variation in clinical syndromes provides the opportunity to correlate a mutation in the androgen receptor structure with the impairment of a specific physiological function of the androgen receptor.

4.2 Deletions of the Androgen Receptor Gene

Only three cases have been reported with partial or complete deletion of the androgen receptor gene, indicating the relatively low frequency of this type androgen receptor defect (Brown et al. 1988; French et al. 1990; Trifiro et al. 1991a). All individuals in the reported cases are completely androgen resistant. In one person a complete deletion of the gene was found and in two other individuals a deletion of exons five to eight and exons three to eight respectively have been described (French et al. 1990). So far mutations in the androgen receptor promoter region or in the 5'- and 3'-untranslated regions of the gene have not been reported.

4.3 Mutations in the N-terminal Domain

The N-terminal domain of the human androgen receptor is characterized by the presence of several homopolymeric amino acid stretches. The exact function of these amino acid repeats is still unknown. In a

Table 1. Locations of different amino acid substitutions in the Androgen Binding Domain due to point mutations in exons four to eight in the androgen receptor gene of individuals with the complete (CAIS) or partial (PAIS) androgen insensitivity syndrome

Position	Amino acid wild-type mutant		Nucleotide change	Pheno- type	References	
Exon 4	668	Leu	Pro	(T -> C)	CAIS	Belsham et al. 1991
	686	Asp	Asn	(G -> A)	CAIS	Ris-Stalpers et al. 1991
	686	Asp	His	(G -> C)	CAIS	Ris-Stalpers et al. 1991
	709	Trp	Stop	(G ->A)	CAIS	Sai et al. 1990
Exon 5	733	Met	Val	(A -> G)	PAIS	Trapman et al. unpubl.
	750	Ser	Phe	(C -> T)	CAIS	De Bellis et al. 1991
	754	Tyr	Cys	(A -> G)	PAIS	McPhaul et al. 1991
Exon 6	765	Arg	Cys	(C -> T)	CAIS	Brown et al. 1990
	765	Arg	His	(G -> A)	CAIS	Trifiro et al. 1991a
	787	Trp	Stop	(G -> A)	CAIS	Marcelli et al. 1990b
	805	Ser	Asn	(G -> A)	PAIS	Trifiro et al. 1990
Exon 7	822	Arg	Stop	(C -> T)	CAIS	De Bellis et al. 1991
	822	Arg	Leu	(G -> T)	CAIS	Trifiro et al. 1991a
	822	Arg	Gln	(G -> A)	CAIS	Brown et al. 1990
	831	Arg	His	(G -> A)	PAIS	De Bellis et al. 1991
	831	Arg	Cys	(C -> T)	PAIS	Trifiro et al. 1991a
	846	Arg	Cys	(C -> T)	CAIS	De Bellis et al. 1991
	846	Arg	His	(G -> A)	PAIS	Chang et al. 1991
	855	Asp	Gly	(A -> G)	CAIS	De Bellis et al. 1991
	857	Val	Met	(G -> A)	CAIS	Brown et al. 1990
857	Val	Leu	(G -> T)	PAIS	Trifiro et al. 1991a	
Exon 8	874	Lys	Stop	(A -> T)	CAIS	Trifiro et al. 1991b

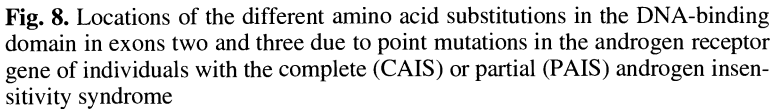
family with androgen resistance associated with profound hypospadias the length of one of the polyglutamine repeats has been reported to be shortened (only 12 glutamine residues; McPhaul et al. 1991). The shortened glutamine stretch as such is not the cause for the androgen resistance, but seems to increase the thermolability of the androgen receptor in combination with a point mutation present in exon five (Tyr -> Cys; codon

754; ligand binding domain; see also Table 1). This point mutation in exon five causes rapid ligand dissociation but no thermolability. These data indicate that two mutations in different functional domains affect each other and suggest an interaction of the two separated regions in the androgen receptor. An interesting aspect is that the defect becomes critical in only part of the androgen target tissues because of the partial character of the androgen resistance found in this family (McPhaul et al. 1991).

Another example of a tissue-specific effect of a defective androgen receptor is the Kennedy syndrome. This rare, X-linked motoneuron disease becomes manifest in men between the age of 30 and 40 and is characterized by a progressive spinal and bulbar muscular atrophy associated with signs of androgen insensitivity and infertility (Harding et al. 1982; Arbizu et al. 1983; Warner et al. 1991). The length of the polyglutamine stretch in the androgen receptor in all investigated Kennedy syndrome patients ranges between 40 and 52 (La Spada et al. 1991a). Whether a doubling of the length of the polyglutamine stretch directly affects motor neuron function is still unclear. Possibly, a change in the structural constraint of the N-terminal domain could result in a modified interaction with motoneuron-specific transcription factors and consequently in an altered assembly of the transcription initiation complex. Interestingly, the length of the glutamine stretch seems to correlate with the severity of the disease (La Spada and Fishbeck 1991 b).

4.4 Mutations in the DNA-Binding Domain

The DNA-binding domain of the human androgen receptor reveals a strong homology (~80%) with those of the human glucocorticoid and progesterone receptors (Trapman et al. 1988). It is characterized by a high content of basic amino acid residues and by nine conserved cysteine residues. It has a compact, globular structure in which two substructures can be distinguished. Both substructures contain centrally one zinc atom, which interacts via coordination bonds with four cysteine residues. The two zinc clusters are structurally and functionally different and are encoded by two different exons (exon two and exon



Recently, two different point mutations have been reported in the same cysteine codon(567) in exon two of the androgen receptor gene

of two unrelated individuals with the complete androgen insensitivity syndrome (Chang et al. 1991; Zoppi et al. 1991 and Fig. 8). This residue is one of the four conserved cysteines which are involved in coordination bonding with a zinc atom. Both receptor proteins displayed normal ligand binding characteristics, but have impaired biological activity. Another point mutation in this exon in codon 550 (Fig. 8), which changes a cysteine residue into a tyrosine residue also results in complete androgen resistance (Zoppi et al. 1991). A fourth mutation in exon two in a partial androgen resistance individual is reported at codon 559 which substitutes a glycine residue by a valine residue (Chang et al. 1991 and Fig. 8). Recently, the deletion of either codon 573 or 574 (encoding a phenylalanine residue) has been reported in a family with complete androgen resistance (Trifiro et al. 1991b and Fig. 8). The phenylalanine residues are highly conserved among the members of the steroid receptor family and are supposed to take part in the interaction between the zinc cluster substructures of the DNA-binding domain via the aromatic side chain (Luisi et al. 1991).

Deletion of exon three results in complete androgen insensitivity as has been recently demonstrated in two 46, XY children with a female phenotype (French et al. 1990). The deletion of exon three still allows the synthesis of an androgen receptor protein with an intact ligand binding domain. In genital skin fibroblasts of both affected siblings androgen receptor levels were found to be two-fold increased.

A premature termination due to a point mutation at codon 581 (Lys) in exon three of the androgen receptor protein has been reported in a 46, XY individual with complete androgen resistance (Marcelli et al. 1990a, and Fig. 8). The receptor protein is C-terminal truncated and lacks the ligand binding domain and most of the second zinc cluster structure. The predicted protein is non-functional as was established after co-transfection studies with a reporter gene construct (Marcelli et al. 1990a). In a family with a high incidence of perineoscrotal hypospadias a point mutation in codon 587 in the androgen receptor gene was found in three affected children. This mutation resulted in the substitution of an alanine residue by a threonine residue in the second zinc cluster structure of the DNA-binding domain (Kaspar et al. 1991 and Fig. 8). Interestingly, this alanine residue is also present at the same position in the glucocorticoid, progesterone and mineralocorticoid re-

ceptors and is supposed to take part in protein-protein interactions between the DNA-binding domains of two receptor molecules (Luisi et al. 1991).

In two children with partial androgen resistance and with ambiguous genitalia a substitution of a leucine residue (codon 607) with an arginine residue has been reported (De Bellis et al. 1991). The consequence of this mutation is three consecutive arginine residues near the C terminal of the second zinc cluster (Fig. 8). The clustering of three basic amino acid residues might be deleterious for the structure of the predicted α -helix in this region (Luisi et al. 1991) and might induce instability in higher order structures as well.

In a receptor positive (normal ligand binding) complete androgen insensitive person a point mutation has been reported in codon 608 converting an arginine residue into a proline residue (Marcelli et al. 1991a and Fig. 8). The position of this codon is at the 3'-terminus of exon three. The arginine residue can be considered as part of the bipartite nuclear targeting signal (amino acid residues 608-624), which is conserved in all steroid hormone receptors (Robbins et al. 1991). Whether this arginine residue is indispensable for nuclear targeting in the human androgen receptor is still not known. Extensive mutational analysis performed for a similar signal in the nuclear protein nucleoplasmin, revealed that a single mutation of the basic amino acid residue at the arginine position partly inhibits nuclear import (Robbins et al. 1991). Another possibility is that the proline substitution disrupts the structure of the α -helix which is located at the C-terminal side of the second zinc-cluster (Luisi et al. 1991) and negatively influences intramolecular interactions and receptor dimerization. In all cases, the net result is a functional defective androgen receptor.

The arginine residue at position 606 is also indispensable for proper androgen receptor functioning. Deletion or substitution by a histidine residue of arginine 606 results in complete androgen resistance (Trifiro et al. 1991b and Fig. 8). The arginine residue at position 606 is highly conserved among the members of the steroid receptor family and is supposed to be involved in the interaction with phosphate groups at specific sites on the DNA (Luisi et al. 1991).

4.5 Mutations in the Androgen-Binding Domain

The androgen binding domain is encoded by part of exon four, the complete exons five, six and seven and part of exon eight. The published mutations in the androgen binding domain have been summarized in Table 1 and will be discussed in this paragraph.

Several functional and structural aspects have been ascribed to exon four, which harbors the major part of a highly conserved nucleoplasmic like bipartite nuclear localization signal (residues 608-624: -Arg-Lys-Cys-Tyr-Glu-Ala-Gly-Met-Thr-Leu-Gly-Ala-Arg-Lys-Leu-Lys Lys-), the hinge region and part of the androgen-binding domain respectively. Mutations in exon four therefore could affect different functions of the androgen receptor. All reported mutations to date in exon four concern individuals with the complete androgen insensitivity syndrome. In three siblings a mutation in codon 709 (Trp) has been described, which changes this codon in a translational stop codon (Sai et al. 1990). Theoretically, the predicted receptor protein should have full constitutive transcription-regulating activity (see also Fig. 2). However, the phenotypic expression of this C-terminal truncated androgen receptor *in vivo* does not support the presence of a constitutive-active molecule. Whether a very short-living protein is synthesized in this particular case has not been investigated.

In a kindred with the receptor-negative complete form of androgen insensitivity, a point mutation was found at codon 668, which replaces a leucine residue by a proline residue (Belsham et al. 1991 and Table 1). This particular leucine residue is highly conserved among the members in the steroid receptor family. The mutation creates a MspI-site which allows mutation detection in this family by RFLP analysis.

In the sequence of the androgen receptor gene from a person with the complete form of androgen insensitivity, a G to T mutation on position 1 in the splice donor site of intron four was found (Ris-Stalpers et al. 1990). The consequence of this point mutation on RNA-splicing was investigated after amplification of cDNA from the affected person using specific primers for exons four and five. This resulted in the identification of a mutant fragment, which was shorter than the corresponding fragment from the wild-type androgen receptor. This finding indicated abnormal androgen receptor mRNA splicing. Sequence ana-

lysis of the mutant fragment revealed the use of a cryptic splice donor site in exon four of the human androgen receptor gene which is normally inactive. The use of this cryptic splice site results in the deletion of 123 nucleotides from the mRNA and translation of this mRNA will consequently result in an in-frame deletion of 41 amino acid residues in the androgen receptor protein from this person. Transient expression of the mutant androgen receptor resulted in the synthesis of a protein which was approximately 5 kDa smaller than the wild-type receptor. The mutant receptor was unable to bind androgens and did not activate transcription of an androgen regulated reporter gene construct (mouse mammary tumor virus promoter linked to the chloramphenicol acetyl transferase gene) [MMTV-CAT] (Ris-Stalpers et al. 1990).

In two unrelated individuals with the complete form of androgen insensitivity, two different point mutations were found at the same nucleotide in codon 686 (aspartic acid) of exon four (Ris-Stalpers et al. 1991 and Table 1). One mutation (G → C) results in an aspartic acid → histidine substitution. In this person the androgen receptor displays low to negligible androgen binding. The other mutation (G → A) leads to an aspartic acid → asparagine substitution. The androgen receptor in the person with the latter mutation shows normal androgen binding capacity but the half life of the hormone-receptor complex is five times shorter as compared with the wild-type hormone-receptor complex. Both mutant androgen receptors displayed the same altered binding characteristics after transient expression as those measured in genital skin fibroblasts from the affected subjects. In co-transfection studies, using two glucocorticoid responsive elements linked to the thymidine kinase promoter and to the chloramphenicol acetyl transferase gene (GRE-tk-CAT) as a reporter gene, both mutant receptors were devoid of any transactivation activity in the presence of physiological hormone concentrations (Ris-Stalpers et al. 1991).

Exon five forms an essential part of the steroid-binding domain of the androgen receptor. Three different point mutations in this exon have been reported. One mutation at codon 754 (Tyr) has been discussed already in combination with a shortened glutamine stretch in exon one. The consequence of the mutation at codon 754 alone is a rapidly dissociating androgen receptor-ligand complex (McPhaul et al, 1991). A second, more severe mutation in exon five at codon 750

(Ser → Phe) has been reported recently (De Bellis et al. 1991). Ligand-binding by this receptor protein in the affected individual is lost completely and has resulted in a complete androgen resistance (Table 1).

A third mutation in exon five has been detected at codon 733 of the androgen receptor gene of a person with partial androgen resistance (Trapman et al. unpublished data). The mutation creates a substitution of a methionine residue by a valine residue. The consequence of this substitution is a diminished transactivating activity of the receptor.

Three reported mutations in exon six have been found in androgen receptors from individuals with the complete form of androgen insensitivity (Table 1). One single nucleotide substitution was identified in codon 787 (Trp) and changes its sense into a stop codon (Marcelli et al. 1990b). The truncated protein was unable to activate transcription. The second mutation described in exon six refers to codon 765 (Arg) which is converted to a cysteine in the androgen receptor of a person with the receptor-negative form (no detectable androgen binding) of complete androgen resistance (Marcelli et al. 1991b). Interestingly, the mRNA levels of this mutant receptor were found to be seven-fold reduced in genital skin fibroblasts of the affected persons as compared with those of normal persons. Another mutation in the same codon 765 has been detected by others (Trifiro et al. 1991b and Table 1). This mutation results in the substitution of the arginine residue by a histidine residue. The clinical phenotype of the affected person is complete androgen insensitivity. A fourth mutation in exon 6 has been reported in two unrelated families with incomplete androgen resistance (Trifiro et al. 1990 and Table 1). The mutation was discovered in the highly conserved codon 805 and changed the sense of this codon from serine to asparagine. Interestingly, the mutation impairs receptor affinity for 5 α -dihydrotestosterone but not for testosterone, suggesting a selective conformational restriction for the binding of one of the two natural androgenic ligands.

Sequence analysis of exon seven in several persons with the complete androgen insensitivity syndrome has resulted in the detection of six different point mutations in four different codons (codon 822: Arg → Stop; codon 822: Arg → Gln; codon 822: Arg → Leu; codon 846: Arg → Cys; codon 855: Asp → Gly and codon 857: Val → Met) (Brown et al. 1990; De Bellis et al. 1991; Ris-Stalpers et al. un-

published data; Trifiro et al. 1991b and Table 1). For all reported mutant receptors no specific androgen binding could be detected. In four other persons with partial androgen resistance two arginine residues (codon 831 in two individuals and codon 846 in another person) and one valine residue (codon 857) were substituted. The reported mutations resulted in a considerable reduction in binding capacity of the mutant receptor proteins as compared with the wild-type receptor (Chang et al. 1991; De Bellis et al. 1991; Trifiro et al. 1991b and Table 1). Obviously, a relatively large number of CGX codons (arginine) is susceptible to mutagenesis in exon seven. In the whole steroid binding domain 82% of the reported point mutations affects a G or C (Table 1).

Recently, one mutation in exon eight has been published in a family with complete androgen insensitivity (Trifiro et al 1991c). It is a non-sense mutation in codon 874. The mutation predicts the synthesis of a truncated receptor protein that lacks 36 amino acid residues at the C-terminal end of the androgen binding domain. It is known that this kind of receptor truncation abolishes hormone binding and transcriptional activation completely (Rundlett et al. 1990; Jenster et al. 1991).

5 Conclusions

Mutational domain analysis of the human androgen receptor has revealed that a large C terminal region constitutes the hormone-binding domain and that a central basic domain is responsible for DNA-binding. In addition, separate domains responsible for transactivation and nuclear translocation have been identified.

The human androgen receptor in the prostate tumor cell line LNCaP is a heterogenous protein, which is synthesized as a single 110 kDa protein, but becomes rapidly phosphorylated to a 112 kDa protein.

The information on the molecular structure of the human androgen receptor has facilitated the study of molecular defects associated with androgen insensitivity. Reports to date on androgen receptor gene structure in individuals with the complete and incomplete form of androgen insensitivity indicate that gross deletions within the androgen receptor gene are infrequent. The locations of the different mutations reported cannot be assigned to a single site but are spread throughout

the ligand-binding and the DNA-binding domains. Further investigations on mutations in different androgen receptor-linked forms of androgen resistance will certainly yield important new insights into the nature of the androgen receptor and will help to unravel the molecular basis of androgen action in normal and abnormal growth and development.

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Androgens and the Brain: Role of Testosterone Metabolism

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1 Introduction

There is no doubt that the central nervous system (CNS) is an important target for practically all hormonal steroids; specific receptors for androgens, estrogens, progesterone, and corticoids have been shown to be present in several brain regions (Martini 1978). The CNS also represents an area of intense metabolism of steroid hormones. The study of the metabolism of androgens, and especially of testosterone, has been the subject of particularly active research; however, some studies have also been devoted to analyzing the metabolism of estrogen, progesterone and corticoids (Martini 1982). Moreover, it has recently been shown that the brain is also able to synthesize dehydroepiandrosterone, delta-5-pregnenolone and their respective sulfates (Baulieu and Robel

1990). All these observations underline that, at present, the brain must be viewed not only as an important target for steroid effects, but also as a structure which possesses the machinery for synthesizing hormone precursors and for metabolizing both these precursors and hormones originating in the peripheral steroidogenetic glands, in order to fulfill specific functional requirements.

2 Metabolism of Androgens

In the brain, androgens may be metabolized according to two different pathways. The first one, known as the aromatase pathway, transforms testosterone into estradiol and androstenedione into estrone (Martini 1982). The second one, which is similar to that present in the majority of the peripheral androgen-dependent structures (prostate, seminal vesicles, etc.) (George and Peterson 1988; Shima et al. 1990), transforms testosterone into 5 α -androstane-17 β -ol-3-one (dihydrotestosterone, DHT) through the action of one or more 5 α -reductases (Martini 1982) (Fig. 1). DHT may be further metabolized to yield 5 α -androstane-3 α -,17 β -diol (3 α -diol) as well as minute amounts of 5 α -androstane-3 β -,17 β -diol (3 β -diol); the enzymes involved in these processes are two (3 α - and 3 β -)-hydroxysteroid dehydrogenases (Martini 1982). In the brain, testosterone may also be converted into delta-4-androstene-3,17-dione (androstenedione), through a reversible process catalized by a 17 β -hydroxysteroid dehydrogenase. Androstenedione may be subsequently 5 α -reduced to yield 5 α -androstane-3,17-dione and androsterone, the two 17-keto derivatives corresponding respectively to DHT and 3 α -diol (Fig. 1) (Martini 1982). Previous work of this laboratory has indicated that there might be two different enzymes for the 5 α -reduction of 17-OH and 17-keto androgens (Motta et al. 1986); this hypothesis has recently found support by the cloning of two cDNAs, encoding for two enzymes named respectively 5 α -reductase I and 5 α -reductase II. Previous work performed has shown that the 5 α -reductase of the brain (and of the pituitary) is also able to convert progesterone (Stupnicka et al. 1977; Zanisi and Martini 1979, Campbell and Karavolas 1989) and corticosterone (Kraulis et al. 1975) into the corresponding 5 α -reduced metabolites, independently of the sex of the

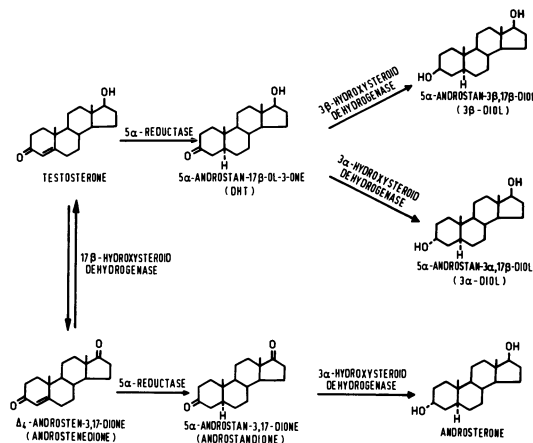


Fig. 1. The 5α-reductase pathways (from Martini 1982 with permission)

animals. These observations suggest that, physiologically, the enzyme (or enzymes) might utilize different substrates in the two sexes (testosterone in males? progesterone in females? corticosteroids in both sexes?). Only further studies may be able to clarify this issue.

In the brain, the two androgen metabolizing pathways (aromatization and 5α-reduction) probably serve different functions. It has been known for some time that the aromatization of androgens to estrogens, which occurs in the CNS during the fetal or neonatal life, is a crucial process for the “organization” towards male patterns of the brain centers which control sexual behavior and gonadotropin secretion (Martini 1982). In adult males, the process of aromatization appears to be important for androgens to exert their typical “activational” effects on male behavior (Martini 1982). This is true for the majority of the species studied; there are, however, relevant exceptions since, in some species, DHT (a non-aromatizable androgen) is also effective (Connolly and Resko 1989; Davidson et al. 1990). The effects of estrogens derived from androgens in the “organizational” or in the “activational” processes involve the binding of the resulting molecules to the estrogenic receptor (Bonsall et al. 1990); these effects are probably linked to the capability of estrogens to influence neuronal sprouting,

the density of dendritic spines (Frankfurt et al. 1990) and the development and remodelling of synapses in particular brain areas (Naftolin et al. 1990; Perez et al. 1990; Leedy et al. 1987; Olmos et al. 1987).

The physiological significance of the 5α -reduction of androgens in the brain is less clear. On the basis of the observation that the 5α -reduced androgens are more potent than testosterone in suppressing LH secretion in a variety of animal models, it had been suggested that the 5α -reduction of testosterone might play an important role in the feedback mechanisms controlling LH secretion (Martini 1982; Zanisi et al. 1973). In support of such a theory, it has been shown that, in castrated male rats, the concomitant administration of testosterone and of a 5α -reductase blocker will decrease the ability of testosterone to inhibit LH secretion (Zoppi et al. 1988). Whether this concept may be applied also to humans is still an open question. One may recall that serum levels of LH are usually elevated in subjects with the syndrome of 5α -reductase deficiency (Imperato-McGinley syndrome) (Martini et al. 1979); however, recent data have shown that in men treated with an inhibitor of the 5α -reductase used for the management of benign prostatic hyperplasia (finasteride-MK 906) serum LH remains unaltered, in spite of a very significant decrease of serum DHT levels (Imperato-McGinley et al. 1990).

On the basis of the information available, it is difficult to assert whether the two processes (aromatization and 5α -reduction) are co-localized in the same nervous nuclei, or whether they are confined to different brain regions. Probably both possibilities exist. However, the aromatase system seems to be particularly concentrated in the hypothalamus and in the limbic system (Martini 1982). Moreover, Selmanoff et al. (1977) have found a significantly higher aromatase activity in the medial rather than in the lateral hypothalamus. On the contrary, the 5α -reductase appears to be more elevated in the lateral than in the medial hypothalamus (Martini 1982; Melcangi et al. 1985). A functional link between the two systems seems to exist; the aromatase activity seems to be, at least in the brain of some species, under androgenic control (Hutchison et al. 1990; Roselli et al. 1987a; Steimer and Hutchison 1989; Connolly et al. 1990). Recent data seem to indicate that the aromatase and the 5α -reductase coexist in neurons, while glial cells apparently possess the 5α -reductase (Melcangi et al. 1990c; Celotti et al. 1991).

The 5α -reductase and the 3α - 3β -hydroxysteroid dehydrogenase systems are present also in the anterior pituitary; this appears to be the only system metabolizing androgens in the gland, since the aromatase is not present (Martini 1982). In the pituitary, the activity of the enzyme is clearly influenced by the endocrine status; castration significantly increases this enzymatic activity in the gland, while the administration of either testosterone or estrogens reestablishes the original situation (Martini 1982). From these and other observations, two general conclusions seem to emerge; namely that: 1) the anterior pituitary might be the most important site for the feedback effect exerted by the 5α -reduced androgens on LH secretion; and 2) the 5α -reductase is probably present almost, if not exclusively, in the gonadotrophs (Martini 1982). Contrary to what happens in the pituitary, the 5α -reductase system of the brain, and in particular of the hypothalamus, does not seem to be sensitive to humoral signals. Castration and steroid administration do not influence the activity of this enzyme, either in the whole hypothalamus (Melcangi et al. 1987), or in those hypothalamic nuclei where the enzyme is particularly concentrated (Melcangi et al. 1985). Also neural inputs seem to be totally ineffective in modifying the 5α -reductase activity of the hypothalamus, since the pharmacological interruption of cholinergic, adrenergic, serotonergic and opiate inputs to the hypothalamus are without any effect (Celotti et al. 1983). Moreover, the total surgical deafferentiation of the hypothalamus, performed according to the technique of Halasz and Pupp (1965), does not change this enzymatic activity (Celotti et al. 1983). These results, while suggesting that the hypothalamus might not be the major site for the feedback effect of the 5α -reduced androgens on gonadotropin secretion, open the possibility that the process of 5α -reduction in the brain might be important for non-endocrine processes.

3 Presence of the 5α -Reductase in the White Matter

Some early studies from this laboratory had shown that, in the rat brain, the subcortical white matter dissected macroscopically proved able to 5α -reduce testosterone with much higher yields than the cerebral cortex (Celotti et al. 1986; Celotti et al. 1987); also the amounts of

DHT formed by tissue punches microdissected from frozen sections of the corpus callosum and the optic chiasm (two white matter structures) have been found to be respectively 3 and 6 times higher than those formed by punches of the superficial layer of the cerebral cortex (Cecchetti et al. 1984). It was consequently decided to analyze this phenomenon in more detail. For this purpose, the 5α -reductase activity has been evaluated in different white matter structures microdissected using the punch technique of Palkovits (1973). In these experiments (and in the subsequent ones) the formation of the 5α -reduced metabolites was measured *in vitro* using labelled testosterone as the substrate. Punches were obtained from the following brain structures: the anterior commissure, fornix, habenulo-interpeduncular tract, corpus callosum, stria medullaris, optic chiasm, fimbria of the hippocampus, cerebral peduncle, pontine fibers, cerebellar medulla and corticospinal tract. The data have shown that the formation of DHT is significantly greater in all the white matter structures studied than in the cerebral cortex. Also the formation of 3α -diol is generally higher in white matter than in cerebral cortex (Melcangi et al. 1988b). The ability of the corpus callosum to form 5α -reduced metabolites was also noted by Hanukoglu et al. (1977) in the female rat, using progesterone as the substrate; and by Sholl et al. (1989) in male and female fetal monkeys. On the basis of these findings, it is likely that the enzymatic activity present in different brain areas might be strongly influenced by the respective content of white matter. The high levels of 5α -reductase activity observed in white matter-rich structures like the midbrain tegmentum (Snipes and Shore 1982), the midbrain, the pons (Krieger 1983) and the spinal cord (MacLusky et al. 1987) support this conclusion. It must be pointed out, however, that autoradiographic studies have never indicated the presence, in the white matter, of significant numbers of receptors for androgen (Sheridan 1984), corticosterone (Stumpf and Sar 1975), or progesterone (Sheridan 1984), three possible substrates for the 5α -reductase (Massa and Martini 1971/1972). This suggests that DHT formed in the white matter might exert its possible functions through mechanisms not mediated by the classical intracellular steroid receptors. Non-genomic effects, involving the interaction of 5α -reduced androgens, progestagens or corticoids with the surface of the cell, or with membrane-bound receptors may be postulated. Of significance, in this

respect, are the observations which indicate that some of the 5α -reduced derivatives of progesterone may bind to the GABA_A receptor (see for references Celotti et al. 1991).

It is interesting to underline that a peripheral nervous structures rich in myelin (the sciatic nerve) also possesses a significant 5α -reductase activity (Melcangi et al. 1990b).

4 Presence of the 5α -Reductase in Myelin

Since white matter structures are mainly composed of myelinated axons, it was thought of interest to investigate whether the myelin might possess a 5α -reductase activity. The formation of the 5α -reduced metabolites of testosterone in brain homogenates (the starting material for the isolation of myelin) and in purified myelin was consequently evaluated. Myelin was purified from the whole brain of adult male rats by sucrose density gradient ultracentrifugation (Melcangi et al. 1988b). The results are provided in Fig. 2. It is apparent that the purified myelin preparation possesses an extremely active 5α -reductase, which is 8.3 times higher than that of the brain homogenate. It is interesting to note that 3α -diol does not appear to be formed by myelin incubates, while minute amounts of this steroid are formed by the brain homogenate. The purity of the myelin preparation used was investigated by electron microscopy (see for details Melcangi et al. 1988). The purity of the myelin was subsequently checked by the evaluation of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP, which is generally considered a marker for myelin membranes) and of cytochrome-C-reductase, an enzyme present in microsomal membranes. The enrichment of the purified myelin membrane preparations for CNP activity was 5-7 fold vs. the brain homogenates, the starting material for the isolation of myelin. On the contrary, the cytochrome-C-reductase was undetectable in the myelin preparations, indicating the absence of a significant contamination by microsomal membranes.

The study of the kinetic parameters of the enzymes in the white matter and in the myelin has indicated that they possess similar K_m values, but different V_{max} values (white matter: 19.572.36; myelin: 34.405.54) (Poletti et al. 1990). These data indicate that the enzyme in

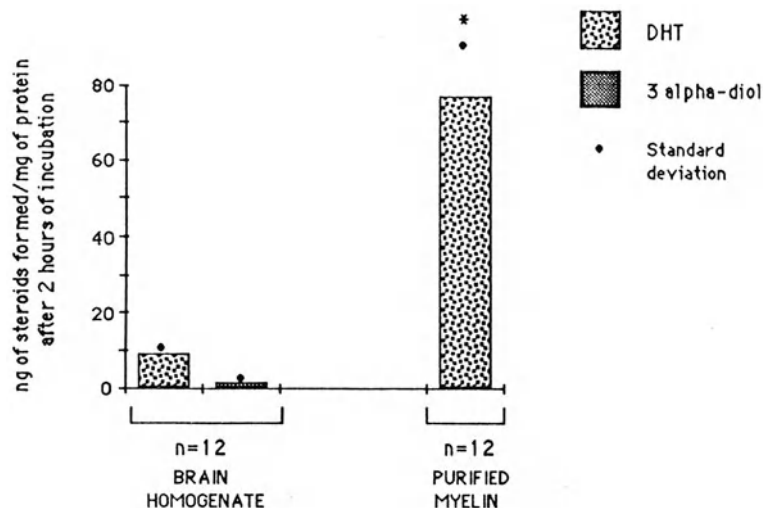


Fig. 2. DHT and 3 α -diol formation in whole brain homogenate and in the purified myelin, measured *in vitro* after incubation with [4-¹⁴C] testosterone (3.16×10^{-6} M). Data are means $\pm$ SEM * $p < .01$ versus brain homogenate (from Melcangi et al. 1988b with permission)

the different nervous structures has the same affinity for testosterone (Poletti et al. 1990), may be present in the same form, but that the myelin possesses a concentration of 5 α -reductase which is much higher than that present in the white matter (Poletti et al. 1990). The fact that, at least in the rat brain, much of the 5 α -reductase activity is associated with myelin membranes reinforces the argument that the amounts of the enzyme present in the different CNS structures may be highly influenced by the presence of myelinated fibers.

The physiological meaning (if any) of the presence of the 5 α -reductase in the myelin is obscure at the moment. It is certainly premature to predict a role of myelination for 5 α -reductase and for the steroids originating from this process. However, it may be pointed out that a few studies have underlined the presence of a peak of 5 α -reductase activity in the rat brain (cerebral cortex, hypothalamus, etc.) (Massa et al. 1975) and in the purified myelin (Melcangi et al. 1988a) in the early postnatal period, i.e. at the time at which the myelination process is be-

ginning. The hypothesis that the 5α -reduced steroids locally produced play a role in the process of myelination is certainly supported by data discussed below showing that the oligodendrocytes (the glial cell which form the myelin) possess considerable 5α -reductase activity.

5 Presence of 5α -Reductase and of Aromatase in Neurons and in the Glia

In subsequent studies, it was decided to verify in which of the two major cellular components of the brain (neurons and glia) the 5α -reductase is present (Melcangi et al. 1990a). The ability to metabolize testosterone was studied in freshly isolated cell preparations, and in well characterized cell cultures (Melcangi et al. 1990a). In a first group of experiments trypsin-treated brain minces from adult male rats were dissociated by passage through calibrated screens. Neurons, astrocytes and oligodendrocytes were then isolated by ultracentrifugation. In a second group of experiments, cultures of neurons and of glial cell obtained respectively from fetal and newborn rats were utilized.

In the case of freshly isolated cells, the purity of the cellular preparations was evaluated by electron microscopy. Purity was 80-90 % for the neurons, 90-95 % for the oligodendrocytes and 60-70 % for the astrocytes. In the cell culture experiments, purity of the neuronal cultures appeared to be more than 90%, as evaluated by monoclonal antibodies against a 160kd neurofilament polypeptide and microtubule-associated protein (MAP-2), two established markers for neuronal cells. When immunostained with monoclonal antibodies against glial fibrillary acidic protein (GFAP), the glial cell cultures consisted of more than 90% GFAP-positive cells (Celotti et al. 1992).

Fig. 3 shows that neurons convert testosterone to DHT in significantly greater amounts than oligodendrocytes and astrocytes. Astrocytes appear to possess a lower 5α -reductase activity than oligodendrocytes; however, the difference in the ability to convert testosterone between the two types of glial cells is not statistically significant. In this experiment, the formation of 3 α -diol was not evaluated.

The results obtained with cell cultures derived from fetal or neonatal animals confirmed that neurons possess a significantly higher

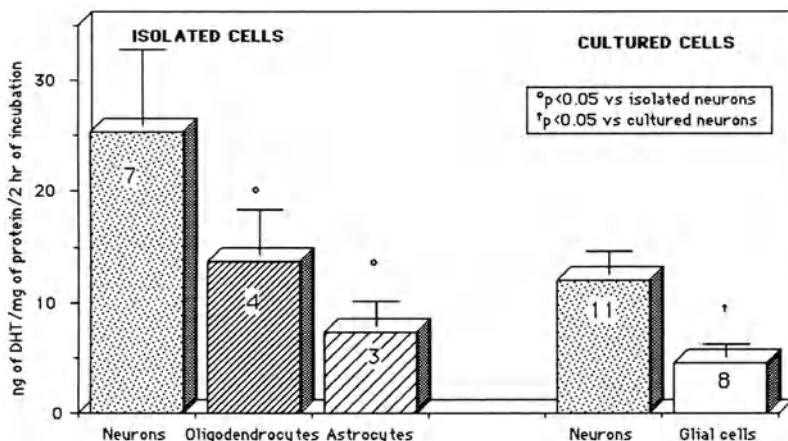


Fig. 3. Formation of DHT in isolated and cultured cells from rat brain. In the experiments with isolated cell numbers, inside columns represent the numbers of experiments. In the experiments with cultured cell numbers, inside columns represent the number of Petri dishes evaluated (from Celotti et al. 1991 with permission)

ability to form DHT than glial cells (Fig. 3) (Melcangi et al. 1990a). In parallel experiments, the presence of the aromatizing enzyme was also evaluated in neurons and glia, using labelled androstenedione as the substrate and the tritiated water method of Roselli et al. (1987b) as modified by Negri-Cesi et al. (1989). It was found that only cultured neurons possess the aromatizing enzyme; on the contrary, this was absent in mixed cultures of glial cells (Melcangi et al. 1990; Celotti et al. 1991).

The demonstration that the oligodendrocytes, the cells responsible for the process of myelination, possess significant 5 α -reductase activity, may explain the presence of the enzyme in the myelin (see above). In this connection, it is interesting to recall that in the oligodendrocytes of developing animals the 5 α -reductase activity appears to peak just before the beginning of the process of myelination (Melcangi et al. 1988a), suggesting a possible correlation between this enzymatic activity and the formation of myelin.

The present findings, showing that neurons are the cell population in which both 5 α -reductase and aromatase are present in higher quan-

ties, indicates that probably the most important effects of androgens take place in these cells. This is also confirmed by the fact that the majority of the uptake and receptor studies performed in the CNS, using either testosterone or DHT as the ligand, have shown the neurons are the more important target for these androgens (Sar and Stumpf 1975). There are few other reports on the localization of the 5α -reductase in the different cell populations present in the CNS, and no other study has analyzed simultaneously the distribution of 5α -reductase activity in the major cell types. Canick et al. (1986) suggested that the 5α -reductase activity is confined primarily to non-neuronal cells, but they measured the 5α -reductase activity in a mixed primary culture of fetal rat hypothalamus in which the neuronal component had been decreased by means of kainic acid. However, kainic acid neurotoxicity appears specific for some type of neurons and not for others (Seil et al. 1979). Weidenfeld et al. (1982, 1984) reported in two abstracts that oligodendrocytes isolated from calf and male rat brains possess some 5α -reductase activity. Moreover, the formation of 5α -reduced metabolites of progesterone was shown to occur in mixed glial cell cultures enriched in oligodendrocytes and incubated with labelled delta-5-pregnenolone (Jung-Testas et al. 1989). Finally, testosterone uptake has been shown to occur in isolated oligodendrocytes (Ovaida et al. 1984), and receptors for corticosteroids (a possible substrate for the 5α -reductase) are present in oligodendrocytes (Meyer 1985).

The absence of the aromatizing enzyme in the glial cell is surprising, especially in view of the fact that estrogen receptors have recently been shown to be present in these cells (Langub and Watson 1992). Perhaps estrogen formed in neurons or transported by the blood might influence glial cell function via paracrine or endocrine mechanisms respectively.

6 Modulation of 5α -Reductase in Brain Cells

Recently, some experiments were performed to investigate the possible effects of second messenger systems in modulating the 5α -reductase activity in cultured mixed glial cells. Phorbol esters were used to activate Protein Kinase C (PKC) and 8 br-cAMP to mimic the effects of increased adenylatecyclase activity (Celotti et al. 1992).

Fig. 4 summarizes the results of the experiments performed with a biologically active phorbol ester, phorbol 12-myristate 13-acetate (TPA), and with an inactive compound, 4 α -phorbol 12,13-didecanoate (4 α -Ph), utilized as control. Both drugs were added to the cultures dissolved in dimethyl sulfoxide (0.12 % final dilution) at a concentration 10^{-7} M. After 3, 6, 12 and 24 h the formation of DHT was evaluated, incubating the cultures with ^{14} C-testosterone for 2 h. The results indicate that the formation of DHT is not modified by the addition of either phorbol esters.

Fig. 5 presents the results of a similar experiment performed adding 8 br-cAMP to the culture media. The compound was dissolved in the medium of the cultures in a concentration 10^{-3} M. After 1.5, 3, 6, 12 and 24 h, the formation of DHT was evaluated incubating the cultures with ^{14} C-testosterone for 2 h. There is a statistically significant increase in formation of DHT over control levels after 6, 12 and 24 hours of incubation.

The effect of cAMP on glial cells was not unexpected, since it had already been reported that exposure to cAMP or to drugs which in-

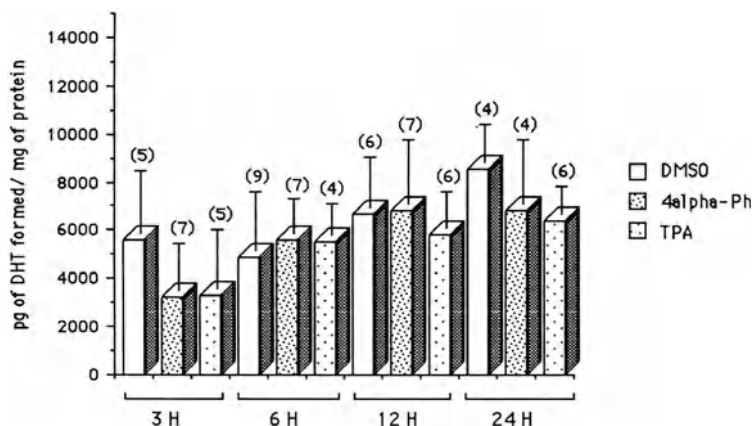


Fig. 4. Formation of DHT in cultures of mixed glial cells incubated for different times in presence of phorbol 12-myristate 13-acetate (TPA) or an inactive control compound, 4 α -phorbol 12,13-didecanoate (4 α -Ph). Numbers above columns represent the number of Petri dishes evaluated. Data were analyzed by ANOVA

crease intracellular stores of cAMP change the morphology of glial cells, possibly by an interference with cytoskeleton proteins (Goldman and Abramson 1990). Moreover, there is abundant evidence indicating that the glia receives adrenergic inputs through α -2 and β -receptors, whose second messenger is cAMP (Hansson 1989).

The present data apparently indicate that PKC is not involved in the intracellular signalling system controlling 5α -reductase, whereas cAMP is. Callard (1981) has found that dibutyryl cAMP and a phosphodiesterase inhibitor increase estrogen yields from ^3H testosterone in a dose and time-dependent manner when added to primary cultures of adult turtle (*Chysemys picta*) brain. In studies germane to the present ones, Baulieu and Robel (1990) have suggested that the addition of cAMP to glial cell cultures composed particularly of oligodendrocytes and astrocytes may increase the synthesis of delta-5-pregnenolone and 20α -pregnenolone from mevanolactone.

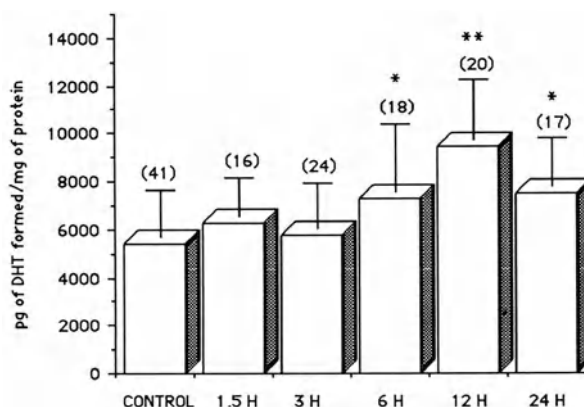


Fig. 5. Formation of DHT in cultures of mixed glial cells incubated for different times in presence of 8 br-cAMP. Numbers above columns represent the number of Petri dishes evaluated. Data were analyzed by ANOVA and Tukey HSD for multiple comparisons. ** $p < .01$ versus control; * $p < .05$ versus control

7 Conclusions

The data presented here confirm that the CNS is the site of intensive metabolism of androgens. In the brain, the 5α -reductase and the aromatase pathways are both present. The distribution of the enzymes which characterize each pathway, however, is different not only with regard to the brain structures in which each pathway predominates (limbic system and medial hypothalamus for the aromatase; lateral hypothalamus, central and peripheral white matter, myelin etc., for 5α -reductase), but also with regard to the type of cells in which the corresponding enzymes are present (5α -reductase and aromatase are both present in neurons, while only 5α -reductase is present in glial cells). The metabolism of androgens occurring in the brain may serve different physiological purposes at different times of life. The aromatization of androgens may be crucial during fetal and neonatal life ("organization" of the brain towards a male pattern of gonadotropin secretion and sexual behavior, control of the processes of synaptogenesis and of neuronal sprouting, etc.). On the other hand, the process of 5α -reduction may be important at later stages not only for the feedback regulation of LH secretion, but also for the control of some aspects of myelination. Only further work will clarify the physiological significance of the presence of 5α -reductase in the myelin and the modalities through which the enzyme is delivered from the oligodendrocytes to this tissue.

The demonstration that testosterone is 5α -reduced in the glia as well as in neurons indicates that glial cells may play a role in processes previously believed to be restricted to neurons. One should not forget that the synthesis of hormonal steroid precursors (dehydroepiandrosterone, delta 5-pregnenolone and their sulfates) also occurs in the glia.

As mentioned throughout the paper, the majority of the data have been obtained so far using testosterone as the substrate. Since the 5α -reductase is active on all steroid possessing a delta-4, 3-keto configuration (e.g., testosterone, progesterone, corticosterone, cortisol, etc.) (Martini 1982) the possibility exists that, in addition to androgens, progestagens and corticoids might also be physiological substrates for the enzyme. This is an important area for future work, since very little is

known on the physiological effects of 5α -reduced progestagens and/or corticoids. With regard to 5α -reduced progesterone derivatives it has been recently shown that they may bind to the GABA_A receptor. This obviously opens the field of the possible interrelationships between hormonal steroids on one side and cerebral neurotransmitters on the other. It is also possible that steroid metabolites formed in the brain might not act through the classical intracellular receptorial system; only further study will clarify their effects on possible membrane receptors, ion transport, etc. These phenomena may help in understanding some of the neurophysiological data, in which steroids have been shown to be able to elicit responses in so short a time to exclude the possibility of genomic effects.

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The Ovarian and Testicular IGF-I System: A Comparative Analysis

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1 Introduction

As the significance of putative intraovarian regulators becomes increasingly recognized, much of the attention centers on insulin-like growth factors (IGFs). Indeed, a large body of evidence now suggests the existence of an intraovarian IGF system complete with ligands, re-

ceptors, and binding proteins (Adashi et al. 1985c). More importantly, IGFs have been shown to exert a variety of significant effects in murine (Adashi et al. 1985a,b,d; 1986b; Bicsak et al. 1986; Cara and Rosenfeld 1988; Davoren et al. 1985; Magoffin et al. 1990; Zhiwen et al. 1987), porcine (Baranao et al. 1984; Maruo et al. 1988; May et al. 1988; Veldhuis and Demers 1985; Veldhuis and Furlanetto 1985, Veldhuis et al. 1986a, b; Veldhuis et al. 1987; Veldhuis and Rodgers, 1987; Veldhuis and Gwynne 1989), and human (Erickson et al. 1989, 1990; Steinkampf et al. 1988) somatic ovarian cells, thereby raising the possibility of a meaningful *in vivo* role.

The above notwithstanding, there is at this time no compelling evidence to indicate that IGFs (or for that matter any other putative intraovarian regulators) are indispensable to ovarian function. However, a large body of a somewhat indirect nature strongly suggests such a possibility. Clearly, much additional work would be required before unequivocal conclusions could be reached about the *in vivo* relevance of IGFs to ovarian physiology. Indeed, limitations imposed by current experimental approaches may well require that definite proof awaits more sophisticated experimental paradigms. In particular, it is to be hoped that developments in transgenic technology will eventually allow selective ablation of IGFs at the level of the ovary so that possible alterations in reproductive function in the experimental group may be compared with intact controls.

2 The Ovarian IGF-I System

2.1 Ovarian IGF Production

Ovarian production of IGF-I was initially suggested by studies revealing that the immunoreactive (i) IGF-I content of porcine follicular fluid substantially exceeded that encountered in serum. That the ovary is in fact a site of (hormonally-regulated) IGF-I gene expression has subsequently been clearly established in several species including the murine (Carlsson et al. 1989; Hansson et al. 1988; Hernandez et al. 1989; Murphy et al. 1987), porcine (Echternkamp et al. 1990; Hammond 1981; Hammond et al. 1988), and human (Barreca et al. 1990;

Eden et al. 1988; Geisthoevel et al. 1989a; Jesionowska et al. 1990; Rabinovici et al. 1990; Ramasharma et al. 1986). Significantly, of all adult *rat* organs tested (Murphy et al. 1987), the ovary displays the third highest level of IGF-1 gene expression, the uterus and liver being the most active in this regard.

To study ovarian IGF-I gene expression, we have recently begun assessing the relative ovarian abundance of IGF-I transcripts with alternative 5'-untranslated (UT) regions and their hormonal regulation (Hernandez et al. 1989). To this end, a solution hybridization/RNase protection assay was employed wherein total rat ovarian RNA was hybridized with a 404-base ³²P-labelled rat IGF-I riboprobe corresponding to the Class A 5'UT variant. As in the liver, three protected bands [322 (Class A), 297 (Class B), and 242 (Class C) bases long] were noted, in keeping with established alternative 5'UT transcripts. The ovarian (as the hepatic) Class C variant proved the most abundant. The ovarian Class B variant was barely detectable. Treatment of immature (21-23 days old) hypophysectomized rats with a diethylstilbestrol (DES)-containing subcutaneous silastic implant for a total of 5 days resulted in a 2-fold increase in the (densitometrically quantified) abundance of ovarian IGF-I transcripts, a diametrically opposed effect (2.6-fold decrease) being noted in the liver. Whereas treatment of hypophysectomized rats only with oGH (150mg, qd, sc x5 days) resulted in a 5-fold increase in hepatic IGF-I gene expression, a limited, albeit distinct inhibitory effect was observed on the steady-state levels of ovarian IGF-I mRNA. In contrast, combined treatment with oGH and DES yielded a 3-fold increase in the abundance of ovarian IGF-I transcripts, there being no net alteration in hepatic IGF-I gene expression. Taken together, these findings reveal ovarian expression of the 3 known 5'-UT IGF-I mRNA variants document the granulosa cell as the main somatic ovarian cell of IGF-I mRNA generation, and indicate that hepatic and ovarian IGF-I gene expression are differentially regulated in diametrically opposed directions. IGF-II gene expression, while clearly evident, appears much less pronounced (Hernandez et al. 1990).

Cellular localization studies revealed the murine (Hansson et al. 1988; Hernandez et al. 1989; Oliver et al. 1989) and porcine (Hammond et al. 1985; Hsu and Hammond 1987; Mondschein et al. 1988a, b)

granulosa cell to be the major intraovarian cell concerned with IGF-I gene expression. In the porcine ovary, granulosa cell-derived IGF-I is cycloheximide-inhibitable, gonadotropin-, estradiol-, and growth hormone-dependent iIGF-I in serum-free media conditioned by cultured *porcine* granulosa cells (Hammond et al. 1981; Hsu and Hammond 1987; Mondschein et al. 1988a,b). Although the *rat* ovarian content of IGF-I appears to be growth hormone-dependent (Davoren and Hsueh 1986), a direct effect of growth hormone (on IGF-I biosynthesis) on rat granulosa cell remains to be demonstrated. In contrast, murine ovarian IGF-II gene expression proved virtually theca-interstitial cell-exclusive (Hernandez et al. 1990). In the human ovary, the granulosa cell may be a site of IGF-II rather than IGF-I gene expression (Geisthövel et al. 1989b; Hernandez et al. in press; Ramasharma and Li 1987; Voutilainen et al. 1987), thereby suggesting possible species specificity. Moreover, preliminary data suggests that the human theca-interstitial cell compartment is in fact a site of IGF-I gene expression (Hernandez et al. in press). Thus, at least in the murine model and in humans, IGF gene expression is highly compartmentalized with two IGF species being expressed by two distinct somatic cell types. It is further presumed that granulosa cell-derived IGF is capable of traversing the cellular plasma membrane into the extracellular fluid wherein it may bind to specific cell membrane IGF receptors on its very cell of origin or else on adjacent cells. In so doing, granulosa cell-derived IGF may exert autocrine or paracrine effects, respectively. These concepts are indeed at the very heart of the intraovarian IGF hypothesis. Stated differently, it has been presumed that locally-generated IGF would in fact play a meaningful role *in vivo* by regulating its cell of origin or else immediately adjacent cell types. Although an attractive possibility, this hypothesis has (thus far) not received rigorous experimental verification.

2.2 Ovarian IGF Reception

Murine (Adashi et al. 1986a, 1988a,b), porcine (Baranao et al. 1984; Veldhuis and Furanetto 1985), ovine (Monget 1989) and human (Gates et al. 1987) granulosa cells have now been shown to display high affinity, low capacity binding sites for IGF-I. In our hands (Adashi et al.

1986b), binding of [^{125} I]IGF-I to FSH-primed rat granulosa cells proved time-, temperature-, and pH-dependent, optimal steady state conditions were being achieved following an 8h incubation at 15°C and a pH of 8.0. Although subject to regulation by the cellular density of plating, the binding of [^{125} I]IGF-I to its receptor proved saturable (apparent $K_d = 3.3 \times 10^{-9}$ M) as well as reversible, complete or partial tracer displacement being effected by competitive inhibition and dilution, respectively. Scatchard and Hill analyses yielded linear plots consistent with a single class of non-interacting binding sites. Specificity studies revealed the competition for [^{125} I]IGF-I binding to follow a rank order of potency of IGF-I MSA insulin, a pattern compatible with a Type I IGF receptor. Limited or no displacement was observed for a series of chemically-related and unrelated polypeptides as well as by a human insulin receptor antiserum. Using affinity cross-linking, we have also been able to observe that whole ovarian membranes of untreated (or FSH-treated) immature hypophysectomized, DES-treated rats are endowed with specific Type-I IGF receptors. Similar results have later been obtained with isolated granulosa cells from the same experimental model (unpublished).

We have also reported FSH and LH to be capable of upregulating rat granulosa cell IGF-I binding, an effect further augmented by growth hormone but not prolactin (Adashi et al. 1986a). Specifically, we were able to show that FSH is capable of upregulating IGF-I binding in a time- and dose-dependent fashion and that cAMP, its purported intracellular second messenger, may play an intermediary role in this regard. Indeed, granulosa cell IGF-I binding was enhanced following elevation of the intracellular cAMP content by a series of cAMP-generating agonists, inhibition of cAMP-phosphodiesterase activity, or the provision of non-degradable cAMP analogs. High dose forskolin (10^{-5} M), like FSH, proved capable of augmenting IGF-I binding by itself, while an essentially inert dose (10^{-7} M) synergized with FSH in this regard. Significantly, heterologous receptor upregulation was not limited to FSH, similar increments being observed for luteotropic, B₂-adrenergic, but not lactogenic granulosa cell agonists. Related *in vivo* studies using immature, hypophysectomized, DES-treated rats revealed that the ability of FSH to upregulate granulosa cell IGF-I binding a) is not strictly an *in vitro* phenomenon and that it can

be fully reproduced *in vivo*, b) is due to enhancement of IGF-I binding capacity rather than affinity, c) may be subject to diametrically opposed modulation by somatogenic and GnRH-like granulosa cell agonists (up- and downregulation, respectively), and d) is best maintained by gonadotropins but not prolactin (Adashi et al. 1988b). Inasmuch as gonadotropin-dependence constitutes a unique attribute of the ovarian granulosa cell, our findings further indicate that the granulosa cell IGF-I receptor may have thoroughly adapted to its unique environment, providing the first example of a cell type for which the complement of IGF-I receptors may be cAMP-dependent. Given the pivotal role of FSH in the induction of granulosa cell receptors for luteotropic and lactogenic ligands, this finding strongly suggests that the acquisition of IGF-I responsiveness may be part and parcel of granulosa cell ontogeny. Accordingly, gonadotropins may condition the cell to respond optimally to IGF-I, thereby conferring selective advantage upon follicles so endowed.

Both IGF-I and IGF-II have been shown to promote rat granulosa cell differentiation and proliferation (Davoren et al. 1986). While both Type I and Type II IGF receptors have been observed in rat granulosa cells (Adashi et al. 1990c; Davoren et al. 1986), the identity of the IGF receptor type(s) mediating IGF action cannot be completely evaluated at this time due to the lack of specific reagents. The availability of antibodies specific for the rat Type II IGF receptor (R-II-PAB1) has made studies of this receptor type possible. To validate the utility of the R-II-PAB1 antiserum at the level of the rat granulosa cell, its ability to immunoneutralize the granulosa cell Type II IGF receptor was examined (Adashi et al. 1990c). Significantly, R-II-PAB1 (10-100mg/ml) proved a potent inhibitor of [^{125} I]IGF-II (but not [^{125} I]IGF-I), binding to granulosa cell membrane preparations. Substantial, albeit finite R-II-PAB1-mediated inhibition of the cross-linking of [^{125} I]IGF-II was also observed. Moreover, R-II-PAB1 proved highly potent in immunoprecipitating the rat granulosa cell Type II IGF receptor.

In light of these observations, we have proceeded to use R-II-PAB1 to assess the functional role of the rat granulosa cell Type II IGF receptor in IGF-I and IGF-II hormonal action. To this end, FSH (20ng/ml)-primed granulosa cells were cultured for 72h in the absence or presence of IGF-I or IGF-II (50ng/ml), with or without increasing (re-

ceptor-active) concentrations of R-II-PAB1 (10-100mg/ml). Control incubations were carried out with an ammonium sulfate precipitate of non-immune rabbit serum dialyzed against phosphate-buffered saline. Significantly, both R-II-PAB1 and non-immune rabbit serum were without effect on the cytodifferentiative action of either IGF-I or IGF-II. Subject to limitations inherent to the immunoneutralizing potency of R-II-PAB1, these findings are in keeping with the notion that (inasmuch as the conventional cytodifferentiative process is concerned) the granulosa cell Type II IGF receptor does not appear to participate in transmembrane IGF signalling. By inference, these findings also suggest that IGF-I and IGF-II hormonal action at the level of the granulosa cell may be exerted largely, if not exclusively, via the Type I IGF receptor. Thus, the potential relevance and the functional role(s), if any, of the granulosa cell Type II IGF receptor remain to be determined. It is of note that murine (Cara et al. 1990; Hernandez et al. 1988) as well as human (Poretsky et al. 1985, 1990) theca-interstitial cells possess functional type I IGF receptors coupled to the promotion of ovarian androgen biosynthesis.

2.3 The Granulosa Cell as a Site of IGF Action

Studies carried out in the last several years have clearly established the granulosa cell as a site of IGF-I action. IGF-I action at the level of the rat (Adashi et al. 1985a,b,d,1986b; Bicsak et al. 1986; Davoren et al. 1985; Zhiwen et al. 1987), but not porcine (Baranao et al. 1984; Maruo et al. 1988; Veldhuis and Furlanetto 1985; Veldhuis et al. 1986a, b; Veldhuis and Rodgers 1987) granulosa cell appears largely (but not exclusively) contingent upon its ability to synergize with pituitary gonadotropins. These effects are unaccounted for by enhanced cellular viability, plating efficiency or DNA synthesis. Thus, this ability of IGF-I to augment differentiated phenotypic expression of the developing granulosa cell may be distinct from its well-established growth-promoting property and was thus considered a novel biologic effect of this polypeptide. In this connection, it has been shown that IGF-I is capable of augmenting FSH-supported (but not basal) progesterone and estrogen biosynthesis as well as the FSH-mediated acquisition of LH receptors.

More recently, IGF-I was also found to augment basal as well as FSH-supported proteoglycan biosynthesis (Adashi et al. 1986b). In this respect, IGF-I appeared to exert its classic "sulfation factor" activity at the level of the granulosa cell, the very chondrotropic effect which led to its discovery. Fractionation of the major extracellular proteoglycan species revealed FSH to favor the exclusive production of dermatan sulfate, whereas IGF-I supported the simultaneous biosynthesis of both heparan and dermatan sulfate.

These findings suggest that IGF-I may effect marked quantitative as well as qualitative alterations in proteoglycan economy. Given the possible role of proteoglycans in follicular antrum formation and follicular atresia, these findings raised the possibility that IGF-I of granulosa cell origin may partake in the growth as well as demise of the developing ovarian follicle.

2.4 The Granulosa Cell as a Site of IGF-Binding Protein Generation

IGF binding protein(s) (IGFBPs) are multifunctional proteins that regulate not only the transport of IGFs but also their presentation to cell surface receptors (Baxter et al. 1988; Binoux et al. 1986). The latter function is thought to be subserved by low molecular weight IGFBPs capable of binding IGFs (but no insulin) with affinities in the 10^{-10} - 10^{-9} M range. Although the precise role(s) of tissue-derived IGFBPs remains a matter of study, both stimulation (Clemmons et al. 1986; De Vroede et al. 1986; Veldhuis and Demers 1985) and inhibition (Frauman et al. 1989; Han et al. 1988; Ritvos et al. 1988; Rutanen et al. 1988) of IGF binding and action have been reported. Thus, the synthesis and secretion of IGFBPs may play a major role in the regulation of IGF hormonal action at the target cell level even at a time when the extracellular concentrations of the IGFs remain constant. This development adds a new level of complexity to the interaction of IGFs at the cellular level.

Although the relevance of IGFBPs to ovarian physiology remains unknown, acid-chromatographed whole ovarian extracts of murine origin (Davoren and Hsueh 1986), media conditioned by porcine gra-

nulosa cells (Hammond et al. 1985; Hsu and Hammond 1987), and human follicular fluid (Seppala et al. 1984), were all found to contain low molecular weight IGF-I binding activity. One such species, IGFBP-1, was localized to luteinized granulosa cells by immunohistochemistry (Seppala et al. 1984), immunoisolation (Suikkari et al. 1989), and radioimmunoassay (Jalkanen et al. 1989).

To explore the possibility that the granulosa cell is also capable of hormonally-regulatable elaboration of IGFBPs, granulosa cells from immature, diethylstilbestrol-primed rats were cultured for up to 72h under serum-free conditions in the absence or presence of FSH. Media conditioned by untreated granulosa cells reveal constitutively released polyethylene glycol (PEG)-precipitable [^{125} I]IGF-I binding activity, the daily elaboration of which proved constant throughout the 72h experimental period. IGFBP release by cultured rat granulosa cells proved cell density-dependent, displaying a minimal inoculum requirement of $\geq 3 \times 10^5$ cells/culture. Daily elaboration of IGF-I binding activity appeared constant throughout the 72h experimental period, the overall time-dependent accumulation of binding activity (over the same time period) proving virtually additive. Scatchard analysis of detailed competition studies with IGF-I suggests that the latter ligand binds to granulosa cell-derived IGFBPs with an apparent affinity of 3×10^{-10} M. Qualitatively similar results were obtained when using [^{125} I]IGF-II, suggesting that the IGFBPs in question are not IGF-I-selective. In fact, specificity studies using the [^{125} I]IGF-I or [^{125} I]IGF-II revealed a rank order of competitive potencies compatible with that observed in other tissues so studied (IGF-II > IGF-I >> insulin). The proteinaceous nature of the acid-stable IGF binding activity under study was indicated by its sensitivity to relatively low concentrations of cycloheximide, its apparent deactivation following repeated cycles of freezing and thawing, and its virtual elimination when subjected to boiling or trypsin treatment (Adashi et al. 1990a). Cycloheximide-induced blockade of protein biosynthesis also revealed that the IGF binding activity is subject to measurable turnover thereby suggesting that its accumulation represents the balance struck between synthetic and degradative processes.

Treatment of rat granulosa cells with FSH resulted in dramatic inhibition of the accumulation of IGF-I binding activity (89% at the

100ng/ml dose level). Systemic provision of FSH (10mg/rat/day for 2 days) revealed that this gonadotropic action is not strictly an *in vitro* phenomenon but that it can be fully reproduced under *in vivo* circumstances (Adashi et al. 1990b, 1991a). Western ligand blotting of SDS-PAGE-fractionated media conditioned by untreated granulosa cells revealed three IGFBP species comprising a major band doublet (28-29kDa) as well as a single minor band (23kDa). Treatment with FSH virtually eliminated the 23kDa species and substantially reduced the relative representation of the 28 and 29kDa IGFBP species (82 and 74% inhibition, respectively). Taken together, these observations disclose the multiplicity of granulosa cell-derived IGFBPs and reveal the striking ability of FSH to suppress their constitutive release under both *in vitro* and *in vivo* circumstances. This FSH action is all the more noteworthy in light of the generally stimulatory effect exerted by FSH at the level of the granulosa cell. Inasmuch as FSH may be concerned with the promotion of granulosa cell development, its ability to attenuate the release of (presumptively inhibitory) IGFBPs may enhance the access of endogenously-produced IGF-I to its cognate cell surface receptors and hence its cellular hormonal action.

More recently, detailed studies were undertaken to characterize the modulatory effect(s) exerted by FSH on the elaboration of IGFBPs by granulosa cells from immature, estrogen-primed rats (Adashi et al. 1991a). The ability of FSH to regulate the elaboration of granulosa cell-derived IGFBPs proved dose-dependent but biphasic in nature. Specifically, FSH concentrations in the range of 1-3ng/ml applied for 72h produced a significant (P) increase in PEG-precipitable [¹²⁵I]IGF-I binding activity corresponding to all IGFBP species detectable by ligand blotting. In contrast, treatment with higher concentrations (≥ 10 ng/ml) of FSH resulted in progressive dose-dependent inhibition of the constitutive release of IGF-I binding activity (80% inhibition at the 100ng/ml dose level) and the virtual elimination of all detectable IGFBP species. Time course studies disclosed a significant (P < 0.05) initial (apparent at the 24h time point) stimulatory effect of a high dose of FSH (100ng/ml) corresponding mostly (but not solely) to the single minor (23kDa) IGFBP band. In contrast, more prolonged exposure to FSH (≥ 48 h) produced progressive time-dependent decrements in IGF-I binding activity, an effect associated with a decrease in

the relative representation of the major band doublet (28-29kDa), the 23kDa species being all but eliminated under these experimental circumstances. Hypophysectomy produced significant ($P < 0.05$) inhibition of the subsequent *in vitro* release of granulosa cell-derived precipitable IGF-I binding activity strongly suggesting that (presumptively stimulatory) pituitary principles other than FSH are likely to be involved in the regulation of granulosa cell IGFBP release and that the trophic influence of these putative agents appears to outweigh the potential inhibition of FSH hormonal action.

Taken together, these findings indicate that the pituitary regulation of granulosa cell-derived IGFBPs is complex and is not FSH-exclusive. Our findings further indicate that the ability of FSH to regulate granulosa cell-derived IGFBPs is dose- and time-dependent but biphasic in nature, an effect characterized by early low-dose stimulatory effect and a late high-dose inhibitory effect. Although the physiologic significance of the above observations remains uncertain, physiologic considerations support the speculation that the biphasic nature of FSH hormonal action may play a role in determining the pace of follicular development and possibly the establishment of follicular dominance.

2.5 The Intraovarian IGF System as a Mediator of Gonadotropin Hormonal Action

A body of information now supports the hypothesis that the role of the intraovarian IGF system may be the amplification of gonadotropin hormonal action. To further test this hypothesis, we have undertaken to re-examine the role of granulosa-derived IGFs in the FSH-supported differentiation of this type (Adashi et al. 1991b). To this end, use was made of functionally-validated human (amniotic fluid-derived) IGFBP-1, the ability of which to bind (and thus sequester) IGFs with high affinity is well established. Implicit in this experimental approach is the prediction that IGFBP-1-associated diminution or abolition of FSH hormonal action may be construed as evidence supporting the intermediacy of endogenously-generated IGFs, whereas the lack of IGFBP-1 effect would render such possibility unlikely. Ovarian granulosa cells from immature, diethylstilbestrol-primed rats were cultured

for 72h under serum-free conditions in the absence or presence of FSH (100ng/ml), with or without increasing concentrations (10-1,000ng/ml) of IGFBP-1. Although without effect on accumulation of progesterone when applied by itself, IGFBP-1 produced dose-dependent inhibition of the FSH-induced accumulation of progesterone, an effect characterized by a median inhibitory dose of 380 ± 21 ng/ml and a maximal inhibitory effect of 65%. Comparable concentrations of denatured IGFBP-1 or of an unrelated amniotic fluid-derived protein (alpha fetoprotein) proved without effect. Importantly, intact IGFBP-1 was rendered ineffective when concurrently applied with excess exogenous IGF-I or IGF-II or in the presence of a functionally-validated monoclonal antibody capable of blocking its IGF binding site. A monoclonal antibody directed against a non-binding site epitope of IGFBP-1 did not reverse its antigonadotropic action. Taken together, these findings revealed the antigonadotropic potential of IGFBP-1 and its apparent dependence on an intact IGF binding site. These findings are in keeping with the notion that optimal FSH hormonal action is contingent upon the bioavailability of granulosa cell-derived IGFs and the consequent amplification of the gonadotropic signal. Similar observations were made by other authors (Bicsak et al. 1990; Shimasaki et al. 1990; Ui et al. 1989) using other IGFBPs.

3 The Intratesticular IGF-I System

As is the case for the ovary, the testis harbors a complete IGF-I system replete with ligand, receptor, and binding proteins (Skinner 1991; Saez et al. 1989; Chatelain et al. 1987a, b; Saez et al. 1988).

3.1 The Testis as a Site of IGF-I Production

As early as 1983, Ritzen reported on the ability of Sertoli cells to secrete a somatomedin-like compound for which a role in the regulation of the germ cell compartment has been proposed (Ritzen 1983). Shortly thereafter, D'Ercole and associates reported on the presence of immunoreactive IGF-I in testicular homogenates, the control of which

proved GH-dependent (D'Ercole et al. 1984). No more than a year later, Handelsman et al. identified immunoreactive IGF-I in testicular extracts, an activity unaccounted for by serum contamination (Handelsman et al. 1985). This work was followed in turn by the recognition of IGF-I immunoreactivity in media conditioned by primary cultures of Sertoli and testicular peritubular cells (Tres et al. 1986). Moreover, immunoreactive IGF-I proved associated with pachytene spermatocytes but not with spermatogonia or early meiotic prophase spermatocytes, suggesting a possible role for this growth factor in the meiotic process (Tres et al. 1986). Later, Smith and associates confirmed the ability of cultured rat Sertoli cells to secrete a peptide shown to be the rat equivalent of human IGF-I (Smith et al. 1987). Similar observations were made by other groups using cultured immature porcine Sertoli cells (Benahmed et al. 1987; Chatelain et al. 1987; Morera et al. 1987), wherein production was increased by bFGF and EGF but not FSH or GH (Tres et al. 1987; Chatelain et al. 1987; Naville et al. 1990). Interestingly, cells pretreated for 24h with EGF or bFGF became sensitive to the stimulatory effect of FSH. However, these observations proved discrepant with subsequent reports wherein note was made of the ability of FSH (Cailleau et al. 1990), thyroid hormone (Palmero et al. 1990), or cAMP- generating agonists (Cailleau et al. 1990), to stimulate the production of IGF-I, by cultured immature rat Sertoli cells. Concurrently, it became increasingly apparent that Leydig as well as Sertoli cells (of diverse species) secrete IGF-I (Vannelli et al. 1988; Hansson et al. 1989) and that IGF-I secretion is stimulated by the corresponding gonadotropin (Naville et al. 1990; Cailleau et al. 1990) bFGF (Naville et al. 1990) and by insulin (Naville et al. 1990). Importantly, Leydig cells express transcripts corresponding to IGF-I the regulation of which could be GH-dependent (Lin et al. 1990). Other *in vivo* studies suggest testicular IGF-I to be GH (Closset et al. 1989; Spiteri-Grech et al. 1991a, b), retinol (Bartlett et al. 1990), LH (Closset et al. 1989) FSH (Closset et al. 1989) and prolactin (Closset et al. 1989) dependent. Note must also be made of the fact that Leydig-Sertoli interactions may play a role in the control of IGF-I production, further supporting the contention that this growth factor may play an important role in the paracrine and autocrine control of testicular function (Naville et al. 1990; Cailleau et al. 1990). Indeed, the pro-

duction of IGF-I by Leydig-Sertoli cocultures clearly exceeds that expected from the monocultures (Naville et al. 1990; Cailleau et al. 1990).

3.2 The Testis as a Site of IGF-I Reception

Receptor affinity labelling studies using rat Sertoli cell membranes and whole Sertoli cells demonstrated these cells to possess abundant type I IGF receptors with high affinity for IGF-I (Borland et al. 1984). These observations were subsequently confirmed by Oonk and Groote-goe (1988) who demonstrated that the binding of IGF-I per testis was increased 6-fold between the age of 21 days and adult age. Not long thereafter, Handelsman et al. (1985) demonstrated the presence of specific type I IGF receptors in testicular homogenates and isolated Leydig cells. No more than a year later, Lin and associates reported on the presence of specific insulin and type I IGF receptors in adult rat Leydig cells (Lin et al. 1986a, b;). Others followed suit (Kasson and Hsueh 1987; Rigaudiere et al. 1988). Confirmatory observations were made by Perrard-Sapori and associates (1987) who reported on the documentation of specific type I IGF receptors using conventional radioligand receptor assays and cross-linking methodology in cultured porcine Leydig cells. Interestingly, several investigators observed that LH/hCG (less so FSH or GH) is capable of upregulating Leydig cell type I IGF receptor binding capacity but not affinity (Perrard-Sapori et al. 1987; Lin et al. 1987a, 1988). These observations suggested that the steroidogenic and growth-promoting effects of LH/hCG (or other pituitary hormones) at the level of the Leydig cell may be mediated by increased type I IGF receptors.

3.3 The Testis as a Site of IGF-I Action

As early as 1984-85, Borland and associates demonstrated the ability of IGF-I to promote thymidine incorporation (Borland et al. 1984) and glucose transport (Mita et al. 1985) at the level of the cultured rat Sertoli cell. Confirmatory evidence was provided by Oonk and associates

who demonstrated IGF-I to stimulate hexose transport as well as lactate production by cultured Sertoli cells (Oonk et al. 1989). In addition, IGF-I was reported to stimulate Sertoli cell DNA synthesis and proliferation (Jaillard et al. 1987). The effects of IGF-I and EGF in this regard were additive but those of IGF-I and bFGF were synergistic. Interestingly, IGF-I alone had no effect on the number of Sertoli cell FSH receptors or on the FSH-induced generation of cAMP (Jaillard et al. 1987). Concurrently, several investigators reported on the ability of IGF-I to significantly stimulate the basal as well as hCG-supported cAMP and testosterone formation by cultured Leydig cells of murine (Lin et al. 1986a, b; Kasson and Hsueh 1987; De Mellow et al. 1987, Horikawa et al. 1989; Moger et al.) or porcine (Smith et al. 1987; Bernier et al. 1986; Chatelain et al. 1988) origin. cAMP action was equally enhanced (Lin et al. 1986a, b; Bernier et al. 1986). As such, these findings suggested that IGF-I may potentiate LH/hCG hormonal action at sites both proximal and distal to cAMP generation. Others reported that IGF-I increased porcine Leydig cells steroidogenic responsiveness to hCG by increasing hCG receptor number (Morera et al. 1987; Perrard-Sappori et al. 1987; Bernier et al. 1986). Stimulation of 3 β -hydroxysteroid dehydrogenase activity was also noted (Lin et al. 1987b). Interestingly, IGF-I has also been noted to modulate aromatase activity of adult rat Leydig cells (Rigaudiere et al. 1989). *In vivo* studies proved supportive of the cytodifferentiative actions of IGF-I (Chatelain et al. 1991).

3.4 The Testis as a Site of IGF-I Binding Protein Generation

Analysis of IGFBP-3 and IGFBP-4 transcripts in rat tissues revealed (for both) a single 2.6kb mRNA in the rat testis (Shimasaki et al. 1989; 1990; Albiston and Herington 1992). Testicular expression of IGFBP-5 was documented as well (Shimasaki et al. 1991). Cellular localization studies revealed peritubular cells to synthesize primarily IGFBP-2 as assessed by ligand blot analysis and RNA blot hybridization (Smith et al. 1990). In contrast, IGFBP-3 is predominantly synthesized by the Sertoli cell as assessed by ligand blot, RNA blot hybridization, and deglycosylation (Smith et al. 1990). Treatment with FSH markedly reduced Sertoli cell IGFBP-3 release (Smith et al. 1990). Similar effects

were noted for isoproterenol, Bt_2 cAMP, and cholera toxin. In contrast, IGF-I was shown to increase Sertoli release of IGFBP-3 (Smith et al. 1990). As such, these observations revealed that IGFBPs are expressed in a cell type-specific manner. Whereas FSH as well as other cAMP-generating agonists have been shown to inhibit IGFBP release by cultured Sertoli cells, LH and cAMP appear to stimulate IGFBP secretion by cultured Leydig cells (Cailleau et al. 1990).

4 Comparative Summary

As is evident from the preceding information, the testis, just like the ovary, is a site of IGF-I production. Cellular localization studies reveal both the Leydig and Sertoli cell to be involved. In this respect, the testis differs from the ovary in that only the granulosa cell appears to be a site of IGF-I gene expression. Future studies would be required to determine whether or not IGF-II is in fact expressed at the level of the testis, an observation previously made at the level of the ovary. As is the case for the ovary, testicular IGF-I appears to be subject to hormonal regulation. Unfortunately, no direct comparison can be made at this time except to note that LH and FSH, apparently stimulatory agents level for the testis, have not as yet been shown to play a similar role in the ovary. If anything, FSH may prove inhibitory at the ovarian level. Similarly, GH may well play a role in the regulation of testicular IGF-I production. This observation runs counter to the limited information available for the ovary, wherein IGF-I gene expression, if anything, is subject to inhibition by GH.

Very much like the ovary, both the Leydig and the Sertoli cell are sites of IGF-I reception. The analogy is further extended in that LH/hCG has been shown to increase Leydig cell type I-IGF receptor complement. Likewise, similar observations were made for FSH at the level of the ovarian granulosa cell.

Perhaps the greatest degree of similarity between the testis and the ovary may be noted concerning IGF-I action. Indeed, IGF-I, for the most part, appears to exert a stimulatory effect on testicular function along similar lines previously observed in the ovary. These effects of IGF-I may involve potentiation of LH/hCG hormonal action at sites

both proximal and distal to cAMP-generation as previously demonstrated for FSH action in the ovarian granulosa cells. Interestingly, IGF-I has been shown to increase the number of LH/hCG receptors in the Leydig cell. In contrast, no such effect was noted for IGF-I on the granulosa cell FSH receptor. More recently, it has become apparent that the testis, not unlike the ovary, is a site of IGFBP generation. Not unexpectedly, it is now clear that IGFBPs are expressed in a cell type-specific manner, an observation previously made for the ovary. The analogy can be further extended in that FSH has recently been shown to inhibit Sertoli cell IGFBP release, a phenomenon not unlike that observed at the level of the granulosa cell.

All told, there is little doubt but that the intratesticular and intraovarian IGF-I systems are highly homologous but not identical. It is tempting to speculate that the variations noted may well reflect specific needs unique to the gonad in question, requiring subtle albeit meaningful modifications of this regulatory network.

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Inhibin, Activin, and Growth Factors: Paracrine Regulators of Testicular Function

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1 Introduction

In the last decade there have been rapid advances in our understanding of the importance of paracrine factors in regulating testicular function (Mather 1984; Bergh 1983; Parvinen et al. 1986; Jegou et al. 1988; Skinner 1991). These factors include substances known only through their effects on the gonads, such as Müllerian inhibiting substance (MIS), seminiferous growth factor and P-Mod-S; and those more widely implicated in cell-cell communication including: fibroblast growth factor, nerve growth factor, insulin-like growth factors, TGF- α , TGF- β and interleukins. Due to their nature, paracrine interactions are difficult to identify and verify *in vivo*. Thus, the majority of support for the hypothesis that these factors play an important paracrine role in regulating testicular function comes from *in vitro* experiments or circumstantial evidence (e.g. the presence of mRNA in a cell). The use of established cell lines both as assay systems and as producers of growth factors has been valuable in elucidating the factors which may play a role in paracrine regulation of the testis (Mather et al. 1982a; Zheng et al. 1990).

Ideally, production of mRNA, and bioactive protein from a specific testicular cell type together with evidence for actions on other (or the same) cells in the testis and localization of specific receptors would be required to define a molecule as a paracrine regulator of testicular function. Evidence from *in vivo* experiments supporting the presumed action of a given paracrine factor would then be final confirmation. Unfortunately, data of this nature for most of the factors discussed below is incomplete due to their local range of action and dispersed sites of production. It is hoped that transgenic animals overexpressing a protein in a tissue-specific fashion, or animals made with the deletion of a growth factor gene will begin to allow us to approach these questions *in vivo*. We will first discuss inhibin and activin as prototypic paracrine regulators of the testis followed by recent data which supports the hypothesis that other growth factors and lymphokines have important local actions in regulating testicular function.

2 Inhibin and Activin as Paracrine Factors in the Testis

Inhibin is a dimer composed of an alpha and one of two beta (βA or βB) subunits. Activins are homodimers of the two beta subunits ($\beta A\beta A$, $\beta A\beta B$, or $\beta B\beta B$) (for review see de Jong et al. 1990). Messenger RNA for all three subunits ($\alpha > \beta B >> \beta A$) has been demonstrated in the testis of embryonic, prepubertal and adult rats using either measurements of mRNA levels in testicular homogenates or by *in situ* hybridization. These observations suggest that the testis may produce both bioactive inhibin and activin throughout life.

Inhibin and activins are produced in, and act at, multiple sites in the testis. In addition, we and others have recently demonstrated specific binding of inhibin and activin in the testis. These observations, which are reviewed below, lend additional support to the hypothesis that a significant, perhaps the major, role of activins and inhibins in the male reproductive system is that of paracrine and/or autocrine regulators.

2.1 The Prepubertal and Adult Testis, Sertoli Cell Production of Inhibin and Activin

Sertoli cells were thought to be the sole source of inhibin production in the testis for several decades based on evidence of bioactivity derived from cultured Sertoli cells (deJong et al. 1990). Soon after the purification and cloning of the inhibin subunits, Sertoli cells derived from immature rats were shown to express mRNA for the α and β subunits of inhibin *in vitro* (Lee et al. 1988, 1989; Keinan et al 1989; Feng et al 1989; Toebosch et al. 1988). Immunohistochemical localization of α and βB subunit protein (Rivier et al. 1988; Shaha 1989) and *in situ* identification of α and β mRNA (Roberts et al. 1989) in the seminiferous epithelium of mature rats also predicts inhibin production by Sertoli cells in the adult testis.

Inhibin protein has been identified by immunoactivity and bioactivity in conditioned medium from Sertoli cell cultures derived from immature rats (Bicsak et al. 1987; Risbridger et al. 1990) and from seminiferous tubule cultures from mature rats. Most conclusively, inhibin has been purified from conditioned medium of cultured Sertoli cells

from 21 day-old rats and been shown to be 32kDa inhibin B (Grootenhuys et al. 1990). Sertoli cells contain the mRNA for both the α and β subunits and thus should be capable of secreting both activin and inhibin. Recently, a 25 kDa protein which increases FSH release from pituitary cell cultures has been partially purified from medium conditioned by Sertoli cells derived from 22 day-old animals (Grootenhuys et al. 1989). This strongly suggests that, at least in immature rats, Sertoli cells secrete both activin and inhibin.

FSH has been shown to be a major regulator of both immunoreactive and bioactive inhibin secretion and steady state levels of inhibin α (but not β B) subunit mRNA in the seminiferous epithelium of immature rats *in vitro* (Toebosch et al. 1988; Bicsak et al. 1987; Risbridger et al. 1990). A regulatory role for FSH in controlling testicular production of inhibin α subunit mRNA in adult animals has also been demonstrated *in vivo* (Keinan et al. 1989; Krummen et al. 1989). These data correlate well with reports that seminiferous tubules from adult animals release bioactive and immunoreactive inhibin into the media in response to FSH (Risbridger et al. 1990). Other factors which have been implicated in the control of Sertoli-cell inhibin secretion include epidermal growth factor (EGF), insulin (Gonzales et al. 1989a; 1989b) and opioids (Morris et al. 1987).

Identification of stage-specific expression of inhibin α and β B subunit mRNAs (Bhasin et al. 1989) and secretion of immunoreactive inhibin protein (Risbridger et al. 1990) represented the first observations that expression of inhibin by the seminiferous epithelium of adult rats was regulated in a paracrine manner by cell to cell interactions with, or secretory factors from, the germinal compartment. More recently, Allenby et al. (1991) have shown that depletion of late spermatids using a single dose of methoxy acetic acid is associated with a marked decrease of circulating levels of inhibin *in vivo*. Finally, the secretion of inhibin by Sertoli cell cultures is increased by the addition of spermatids or spermatid-conditioned medium (Pineau et al. 1990). Taken together, these data suggest that inhibin expression and secretion may be regulated by germ cells or products secreted by these cells and may play a role in the paracrine regulation of spermatogenesis.

2.2 Leydig Cell Production of Inhibin and Activin

Initially, the Leydig cell was not considered as a possible site of production of activin or inhibin. However, a Leydig cell line, TM3, derived from normal immature mouse testis was shown to express only the β subunits of inhibin, suggesting that this cell line produced activin (Lee et al. 1988). It was confirmed that conditioned medium from this line, and from primary cultures of interstitial cells from immature rats and pigs, contained an activity capable of stimulating FSH secretion by pituitary cell cultures which reacted with an antiserum that recognized both inhibin and activin (Lee et al. 1989). This activity was increased by the addition of forskolin or pregnant mare's serum gonadotropin to the cultures, suggesting that activin secretion is under gonadotropin regulation. More recently, work from our laboratory (Krummen et al. 1992) has confirmed that immunoactive and bioactive activin, but not inhibin, is made by an interstitial cell population from immature rat testes. Activin production decreases between 19 and 21 days of age and is not detectable either by bio- or immunoassay in the adult rat interstitium. These data suggest that activin derived from the Leydig cell may play a paracrine role in regulation of the immature, but not the adult testis. The identification of β subunit mRNAs in fetal testis also suggests a role for activin in early gonadal development (Roberts and Vale 1991).

Ogawa et al. (1991), using histochemical localization, have detected β A and β B, but not α , subunit protein in rat Leydig cells of adult as well as immature animals. However, other groups have detected only bioactive inhibin, immunoreactive inhibin- α , or inhibin α subunit mRNA in purified Leydig cells prepared from adult rats (Risbridger et al. 1990; Sharpe et al. 1988). In contrast to the TM3 cell line derived from normal immature mouse Leydig cells, two different rat Leydig tumor-derived cell lines (I10A, RC2) contain mRNA for the α , but not the β subunits (Lee and Mather, unpublished data). Immunoreactive α subunit protein has also been detected by immunohistochemical staining in a human Leydig cell tumor (Bergh and Cajander 1990).

Stimulation of Leydig cells, prepared by percoll gradient enrichment from adult rat interstitium, with LH results in a marked increase in the expression of inhibin α subunit mRNA as well as secretion of

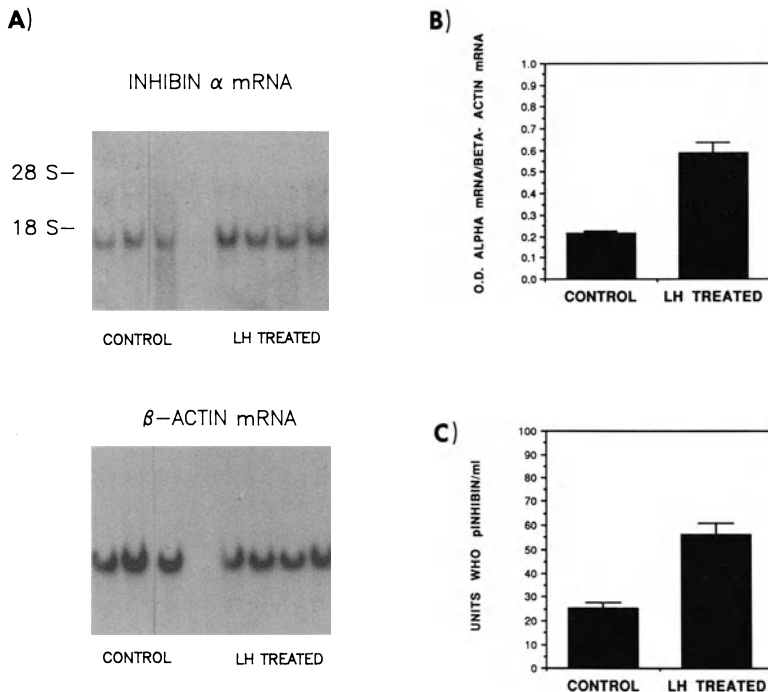


Fig. 1 A-C. Inhibin α -subunit mRNA and immunoreactivity in cultured Leydig cells incubated with or without 100 ng/ml oLH for 24h. (A) Northern analysis of 20 μ g total RNA/lane, hybridized to [^{32} P]-inhibin α cDNA (*top panel*) or [^{32} P] β -actin cDNA. Filters shown in (A) were subjected to densitometry. The results of that analysis are shown in (B). (C) Immunoreactive inhibin secretion into the media (U/ml/24h)

immunoreactive inhibin detected by an assay which measures both bioactive inhibin as well as free alpha subunits (Fig. 1). Expression of mRNA for the β B subunit of inhibin was not detected in these cells. Drummond et al. (1989) reported that hCG stimulation had no effect on adult Leydig cell secretion of inhibin bioactivity, although inhibin immunoreactivity was increased. Taken together, the preponderance of available data are consistent with the hypothesis that interstitial cells

from the immature testis produce activin, whereas Leydig cells from the adult interstitium produce primarily free α subunits. The question of whether inhibin or α -derived proteins from Leydig cells play a significant paracrine role in the adult testis remains to be answered.

2.3 Receptors for Inhibin and Activin in the Testis

We have used fluorescein labeling in combination with fluorescence-activated cell sorting to identify specific binding sites for both inhibin and activin on Leydig and Sertoli cells isolated from immature rats (Woodruff, Rice and Mather, unpublished data). In addition, studies using co-cultures of Sertoli and germ cells have allowed identification of activin binding to spermatogonia, late pachytene spermatocytes and spermatids. No binding was seen, however, to leptotene/zygotene spermatocytes. All germ cell types specifically bind inhibin (Woodruff et al. 1992). Recently, an activin receptor has been cloned from the AtT20 mouse pituitary tumor-derived cell line (Mathews and Vale 1991). Messenger RNA encoding the receptor has been detected in mouse brain, testis, intestine, liver and kidney. More work is needed to determine the relationship of the cloned receptor and the receptors detected by the binding studies mentioned above. However, these studies further strengthen the hypothesis that activin and inhibin have a significant paracrine role in the testis.

2.4 Response of Testicular Cells to Activin and Inhibin

A number of investigators have studied the local effects of activin and inhibin on various testicular cell types. In mixed testis cell cultures from neonatal or adult rats inhibin increased, and activin decreased, LH-stimulated testosterone production. Neither hormone affected basal testosterone in these cultures (Hsueh et al. 1987). In contrast, other studies using more purified rat Leydig cell cultures have shown that activin stimulates testosterone release (Lin et al. 1989). A more recent study on purified immature porcine Leydig cell cultures showed that activin A reduced hCG-stimulated dehydroepiandrosterone

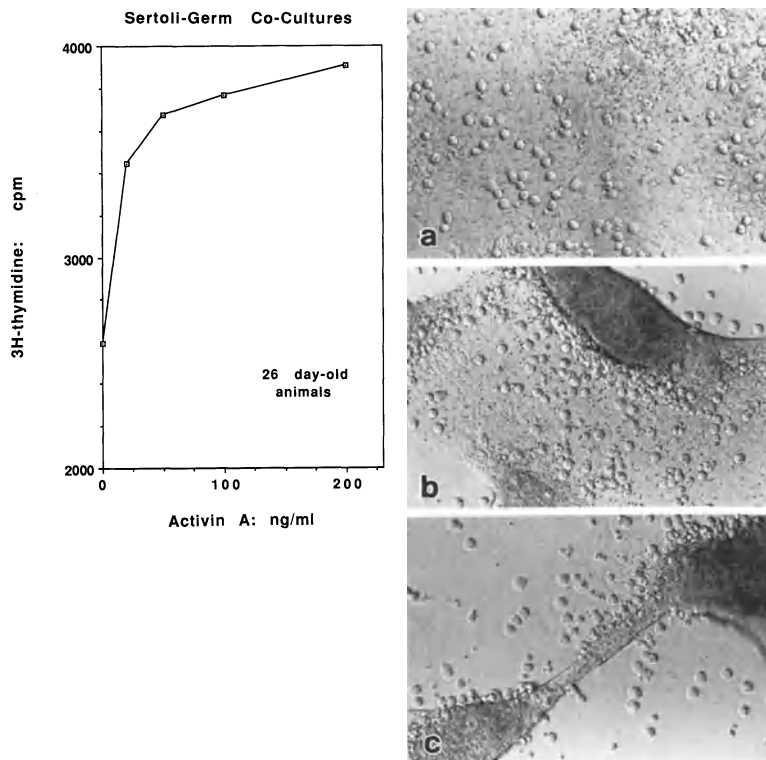


Fig. 2 a-c. The effects of activin on spermatogonial proliferation and Sertoli-germ cell monolayer remodeling. Left panel shows [^3H]thymidine incorporation into Sertoli-germ cell cultures in the presence of increasing doses of rh activin A. Right panel shows the effect of activin on monolayer remodeling (**a**, control; **b** and **c**, 100 ng/ml rh activin A)

(DHEA) accumulation but increased the conversion of pregnenolone and DHEA to testosterone (Maudit et al. 1991). While further investigation is needed to determine the exact mechanism of action, it seems clear that activin, and perhaps inhibin, can act directly on the Leydig cell. It is unclear whether this is an autocrine (eg. activin produced by the Leydig cell) or a paracrine (eg. Sertoli cell-produced ac-

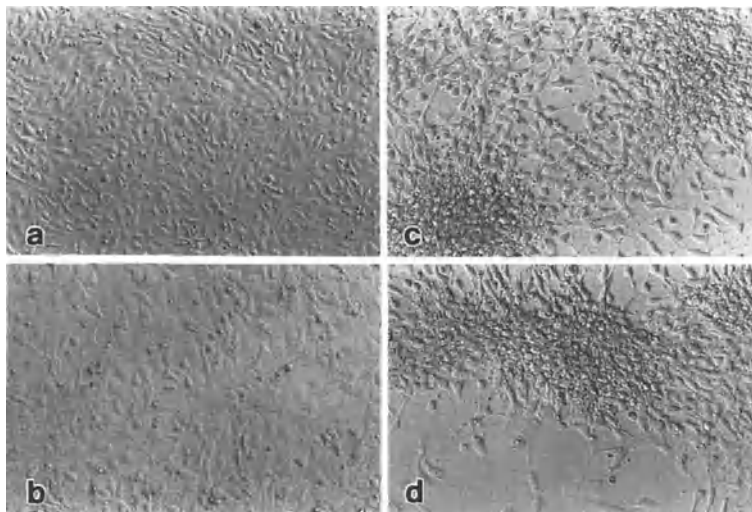


Fig. 3 a-d. The effects of activin on monolayer remodeling in TM-4 cells. Cells were incubated in the absence (a) or presence of 1 (b), 10 (c) and 50 ng/ml (d) for 24h

activin) effect. This may vary with the developmental stage of the animal.

The stage-specific secretion of inhibin by the seminiferous tubules suggests that Sertoli cell derived inhibin may, in turn, regulate the development of the germ cells. This hypothesis is supported by studies which demonstrate an effect of locally administered inhibin on hamster testis. Inhibin-injected testes showed a decrease in spermatogonial numbers (van Dissel Emiliani et al. 1989).

In contrast, *in vitro* data suggests that activin can stimulate spermatogonial proliferation in co-cultures of Sertoli and germ cells prepared from 17-26 day-old rats in a time and dose-dependent fashion (Fig. 2) (Mather et al. 1990). Activin also has a marked effect on the morphology of the Sertoli-germ cell cultures, causing a rapid re-aggregation of the monolayers into tubule-like structures (Fig. 2) This effect was also seen on the TM4 Sertoli cell line, indicating that this is a direct effect on Sertoli cells (Fig. 3). Inhibin had no effect in this *in vitro* system.

Thus, if inhibin inhibits, and activin stimulates spermatogenesis, they seem to act on different cell types or at different stages of germinal cell development. The existence of receptors for both proteins on germ cells (Woodruff et al. 1992) would allow for a direct action of activin and inhibin on these cells, although neither the *in vivo* studies nor the current *in vitro* co-culture models provide definitive proof of a direct action.

The data summarized above lend strong support to the hypothesis that activin and inhibin play a major role in the paracrine and autocrine regulation of testicular function. The specific type of activin or inhibin produced and the net effect locally may vary as a function of the sexual development of the organism or the stages of germ cell development. Finally, an activin binding protein, follistatin, has been described which is produced locally in the gonads and pituitary and which inactivates activin (dePaolo 1991). Follistatin will undoubtedly prove to be an important player in the paracrine regulation of activin function in the testis. A more complete understanding of these complex relationships will develop with advances in our ability to measure the proteins, their binding proteins, and cell surface receptors.

3 Growth Factors as Paracrine Factors in the Testis

3.1 Transforming Growth Factor- β and Müllerian Inhibiting Substance (MIS)

The transforming growth factor family currently contains five distinct dimeric proteins with related sequences and activities. These proteins are, in turn part of a larger superfamily of structurally related proteins which contains inhibin, activin, and MIS. TGF- β activity was demonstrated in conditioned medium from cultures of both immature porcine and rat Sertoli and peritubular cells. These cells secrete an active factor which binds to TGF- β receptors and inhibits the growth of a cell line sensitive to TGF- β . Additionally, Sertoli cells and peritubular cells contain mRNA which hybridized with the TGF- β 1 cDNA (see Morera et al. 1990; Skinner 1991). More recently, both TGF- β 1 and TGF- β 3 message have been detected in adult and embryonic mouse testis (Wa-

trin et al. 1991). Using mice genetically deficient in germ cells, this study showed that TGF- β 3 and TGF- β 1 mRNA was expressed in somatic cells of the testis. A smaller TGF- β 1 transcript was also selectively expressed in the germ cells.

TGF- β has been shown to influence peritubular cell migration (Skinner 1991), elicit shape changes and increase contractility in peritubular cells *in vitro* (Ailenberg et al. 1990). In addition, TGF- β 1 has been shown to decrease mRNA for tissue plasminogen activator in cultured Sertoli cells and increase mRNA coding for plasminogen activator inhibitor in peritubular myoid cells. TGF- β may thus play an important role in regulating net protease levels in the tubule and motility, both of which could be important in tissue remodeling during movement of developing germ cells into the luminal compartment of the tubule (Nargolwalla et al. 1990). TGF- β has been shown to affect both lactate production and aromatase activity in cultures of immature Sertoli cells (Morera et al. 1990). Regulation of Sertoli-derived germ cell nutrients (eg lactate) suggests that this growth factor may also play a role in regulating Sertoli-germ cell communication.

In addition to its effects on tubular cells, TGF- β has been reported to inhibit hCG-stimulated steroidogenesis in primary cultures of rat Leydig cells, but may stimulate some aspects of basal steroidogenesis. TGF- β binding has been demonstrated on porcine Leydig cells (Avallet et al. 1987; Morera et al. 1990). Thus TGF- β s may be involved in the modulation of the gonadotropin responsiveness of Leydig cells, germ-Sertoli cell communication, and tissue remodeling in the seminiferous tubule.

Müllerian inhibiting substance (MIS), another member of the TGF- β /activin superfamily, is a 140 KDa homodimeric glycoprotein that is expressed during early gonadal differentiation in several species. As its name implies, this factor is responsible for causing Müllerian duct regression in the male embryo (see Donahoe et al. 1987 for review). More recently, a role for this factor in maintaining spermatogonia in early developmental arrest has also been hypothesized (Taketo et al. 1991). Immunohistochemical data predicts localization of MIS to the Sertoli cells in neonatal testis (Hayashi et al. 1986). Levels of this factor in the testis of all species tested are highest during the perinatal period, then fall to low or undetectable levels shortly after birth (Hut-

son et al. 1981; Ueno et al. 1989; Kuroda et al. 1990). Decreases in the level of MIS mRNA parallel the decrease in MIS bioactivity in postnatal testis and are accelerated by *in utero* or immediate postnatal injection of FSH, but not LH or testosterone (Bercu et al. 1979; Kuroda et al. 1990; 1991). These data indicate that elevated serum levels of FSH in the newborn may restrict the production of MIS by the testis, allowing the differentiation of spermatogonia from the pool of undifferentiated gonocytes.

3.2 Epidermal Growth Factor and Transforming Growth Factor α

Epidermal growth factor (EGF) and transforming growth factor alpha (TGF- α) are distinct peptides (having 30% homology) which can both bind to the EGF receptor and have similar activities in many cells. We will therefore discuss the data concerning these two factors together. An activity which binds to the EGF receptor, but is distinct from EGF and TGF- α , has been isolated from Sertoli cell conditioned medium (Holmes et al. 1986). More recently TGF- α , but not EGF, mRNA was detected in cultured peritubular and Sertoli cells (Skinner 1991). Teerds et al. (1990) have located TGF- α in rat testis using immunocytochemistry with an antibody which does not cross-react with EGF. While these authors confirmed the presence of TGF- α in peritubular cells, intense staining was also seen in Leydig cells of 21-day and adult rats. Little or no immunoreactivity was detected in the seminiferous tubules. It is tempting to attribute all of the reported *in vitro* effects of EGF to paracrine actions of TGF- α . However, rat EGF has been shown to be present in the testis of vitamin-A synchronized rats with maximal levels seen at stages IX-II of the seminiferous cycle, suggesting that EGF levels in the testis are regulated and may be involved in spermatogonial division (Bartlett et al. 1990).

EGF was first reported to stimulate the growth of both a Sertoli-derived cell line (TM4) (Bottenstein et al. 1979) and a Leydig-derived cell line (TM3) (Mather 1980). Subsequently, primary cultures of immature porcine Leydig cells were shown to respond to EGF with an increase in PGF_{2a} and hCG-stimulated testosterone (T) production (Mather et al. 1982b). It should be noted that EGF causes cellular

hypertrophy both in primary porcine Leydig cells (Mather and Haour, unpublished data) and in other cells (Mather et al. 1981). As a result, EGF is seen to stimulate T production when data are expressed on a per cell basis, but not when expressed as T/protein. The stimulation of T production by EGF has been confirmed for both mouse (Murphy and Moger 1982) and human (Syed et al. 1991) primary Leydig cell cultures. Moreover, EGF has also been shown to be capable of regulating the hCG receptor on porcine Leydig cell cultures (Mather et al. 1982b), suggesting that the effects of this factor on hCG-stimulated T production may be regulated in part by effects on LH receptor number. Studies on the effects of EGF on cells derived from the seminiferous tubule indicate that EGF can increase transferrin secretion from primary cultures of rat Sertoli cells (Perez-Infante et al. 1986). In addition, EGF was also shown to stimulate the differentiation of spermatogonia in the absence of FSH in organ culture, but inhibited FSH-induced differentiation (Haneji et al. 1991).

Specific EGF binding has been reported in human testicular tissue from patients with prostatic cancer and localized to the interstitial tissue (Stubbs et al. 1990). EGF receptors (EGFR) have also been localized on the I10A (Leydig tumor derived) and TM4 cell lines (Suarez-Quian and Niklinski 1990) and in primary rat and monkey Sertoli cell cultures using an antiserum specific for EGFR (Suarez-Quian et al. 1989).

It thus seems clear that EGF and TGF- α are involved in regulating Leydig cell and tubular cell responsiveness. While data for the Sertoli cell are more controversial, it seems likely that this cell is a site of production of TGF- α and probably a target of TGF- α /EGF. However, it should be noted that, based on available data, the potential for paracrine or autocrine effects of TGF- α *in vivo* cannot be clearly differentiated from possible responses to circulating EGF. In light of evidence that removal of the salivary glands adversely affects spermatogenesis (Tsutsumi et al. 1986) it seems likely that both locally produced TGF- α and circulating EGF play important roles in regulating testicular function.

3.3 Insulin-Like Growth Factors (IGF-I, IGF-II) and Insulin

Early work showed insulin to have important effects on both the proliferation of testicular cell lines such as TM4 and TM3 and on the survival and function of primary cultures of Sertoli and Leydig cells in defined culture conditions (Mather 1980; Mather et al. 1982b; Rich et al. 1983). Ritzen's (1983) demonstration the Sertoli cells could produce an IGF-I like factor led to increased interest in the possible role of this growth factor as a paracrine regulator in the testis. IGF-I has similar, but not identical effects to insulin in a number of systems including stimulation of the proliferation of the TR1 and TM4 but not TM3 testicular cell lines (Mather and Furlanetto 1983), and the stimulation of androgen synthesis in primary cultures of Leydig cells (Bernier et al. 1986). IGF-I protein has been shown to be present in conditioned medium from Sertoli, peritubular myoid and Leydig cell cultures (Tres et al. 1986; Cailleau et al. 1990). IGF-I mRNA was demonstrated in purified rat Leydig cells (Lin et al. 1990).

Some disagreement exists as to the exact site of immunohistochemical localization of IGF-I in the testis. Vanelli et al. (1988) demonstrated localization of IGF-I in human testis primarily in Sertoli cells with some staining in primary spermatocytes and a few positive cells in the interstitium. Hansson et al. (1989) found staining for IGF-I primarily in spermatocytes of adult rat testis, although some Sertoli and Leydig cells were stained in the testis of immature animals.

Tres et al. (1986) demonstrated binding sites for IGF-I on rat Sertoli cells and pachytene spermatocytes. Receptors for human IGF-I were detected by immunocytochemistry in secondary spermatocytes and early spermatids, with faint staining in Sertoli cells and intense staining in some Leydig cells (Vannelli et al. 1988). Receptors for IGF-II have been shown to be present in high levels in rat testis but have not been localized to specific cell types (Taylor et al. 1987). Finally, insulin binding sites have been characterized from rat testicular membranes. These testicular binding sites increase 14-fold between 21 days of age and the adult (Loubassou et al. 1989), suggesting an important role for insulin in maintaining spermatogenesis. As with EGF/TGF- α , it seems likely that both insulin and IGFs are important in regulating testicular function.

There are currently four known binding proteins for IGF-I, all present in the testis and differentially regulated by hormones (Mather, unpublished data). The binding proteins are regulated differently from the IGFs (Cailleau et al. 1990) and undoubtedly play an important role in regulating the availability and activity of IGF-I in the testis.

3.4 Fibroblast Growth Factors

Acidic and basic fibroblast growth factors (aFGF and bFGF) are homologous molecules which exert similar effects in a broad range of tissues. Despite the differences in the pI of these two molecules, both factors bind to the same receptor, and are therefore considered to have similar biological activity (see Gospodarowicz et al. 1987). Both molecules are also known to exhibit a high affinity for heparin and heparin-sulfate molecules *in vitro* and *in vivo*. Thus, heparin sulfate-containing extracellular matrix proteins may form a reservoir for FGFs, and restrict their activity to certain cell types. Conversely, when ECM is broken down in the course of tissue remodeling, FGF may be released to act at more distant sites.

Basic FGF was initially isolated from bovine testes (Ueno et al. 1987) and has since been identified in testis of humans, mice and rats (Smith et al. 1989; Mayerhofer et al. 1991). At present, there is no evidence to suggest that aFGF is produced in the testis. During embryonic development in the rat, immunoreactive bFGF has been shown to be localized primarily in Leydig and peritubular cells, and in the basement membrane surrounding the sex cords (Gonzalez et al. 1990). In contrast, in day-5 postnatal rat testis, the greatest intensity of staining was found in the cytoplasm of the gonocytes (Mayerhofer et al. 1991), while in testis from adult rats and mice staining was restricted to pachytene spermatocytes present in stage IV-IX seminiferous tubules and to type A spermatogonia. A bFGF-like activity has also been reported in conditioned media of Sertoli cell cultures prepared from immature rats (Smith et al. 1989). These data indicate that the cellular expression, as well as the role, of bFGF in the testis may change as a function of development.

Several *in vitro* studies indicate that bFGF may be an important regulator of Leydig cell steroidogenesis and Sertoli cell proliferation and differentiation *in vivo*. Basic FGF has been shown to decrease both LH- and cAMP-stimulated testosterone secretion in whole testis cell cultures prepared from neonatal rats, by interfering with p⁴⁵⁰17 α activity (Fauser et al. 1988). These results were supported by Muroso et al. (1990) who reported that, in addition to inhibiting testosterone secretion, bFGF also inhibits 5 α -reductase activity immature Leydig cell cultures. In contrast, Sordollet (1988) found that bFGF had an acute stimulatory effect on basal and hCG-stimulated testosterone production by immature porcine Leydig cells. Although clearly more information is required on the activity of bFGF on Leydig cells derived from animals of various ages and species, these results strongly suggest that bFGF may be an important regulator of the various levels of steroidogenic enzymes expressed during testicular maturation. The effects of this factor on Leydig cell function in the adult animal remain to be determined.

Basic FGF may also be a potent regulator of Sertoli cell proliferation and function *in vivo*. Jaillard et al. (1987) showed that bFGF increased [³H]-thymidine incorporation, Sertoli cell number, specific [¹²⁵I]-FSH binding per cell, and plasminogen activator secretion in cultures prepared from immature rat and porcine testes. Moreover, the effects of bFGF were potentiated by the presence of insulin and IGF-1. Interestingly, however, concomitant addition of FSH to these cultures abolished the effects of bFGF. In another study (Boockfor and Schwartz 1990), addition of bFGF to cultures of immature rat Sertoli cells resulted in an increased rate of transferrin secretion and increased Sertoli cell number in cultures isolated from 8, 10, 13, 15 and 20 day old rats. However, FGF-stimulated proliferation in cultures prepared from older animals was inhibited by concomitant addition of FSH. These studies indicate that interactions between FSH, bFGF and other growth factors may play a role in controlling Sertoli cell proliferation *in vivo*. In this same regard, it is important to note that TGF- β has been shown to modulate the bioactivity of FGF in several systems (see Gospodarowicz et al. 1987). Local production of this growth factor in the testis could therefore be important in modulating the paracrine activity of FGF.

The presence of specific, high affinity binding sites for FGF have been shown in whole testis cell cultures from neonatal animals (Fauser et al. 1988). These observations are consistent with the identity of these factors as paracrine/autocrine regulators.

3.5 Seminiferous Growth Factor

Seminiferous growth factor (SGF) was initially isolated from seminiferous tubules based on its ability to stimulate Balb-3T3 cell growth. SGF is an acidic heparin-binding protein of 15,000-16,500 MW (Bellve and Zheng 1989). This growth factor has properties similar to the fibroblast growth factor family but seems to be distinct from both acidic and basic FGF based on its immunoreactivity and its effects on TM4 (Sertoli) and TM3 (Leydig) cell line growth and protein secretion (Zheng et al. 1990). SGF stimulates the growth of both primary Sertoli cell cultures from immature animals and TM4 cells, and can induce proliferation of capillary endothelial cells. These properties suggest that SGF may act as a mitogen, a regulator of cell-specific function, and as an angiogenic factor. SGF is produced by Sertoli cells but multiple cell types including Sertoli and germ cells and Sertoli and Leydig-derived cell lines show a response to SGF. It is not known at this time whether SGF production is unique to the testis or more widespread.

3.6 P-Mod-S

Peritubular cells are thought to contribute to regulation of the differentiated responses of Sertoli cells, and to serve as mediators of androgen action in testis. For many years it has been known that androgens regulate peritubular cell development and contractile function (Fritz and Tung 1986). More recently, a non-mitogenic androgen-regulated activity was found to be present in culture media conditioned by peritubular cells which stimulated Sertoli secretion of androgen-binding protein, transferrin and inhibin (see Skinner 1991). This activity, P-mod-S, has been purified, and found to be contained in two polypeptides of 56 and

59 Kd, which differ in terms of their behavior during RP-HPLC but exhibit similar activities. In Sertoli cell cultures prepared from both immature and late prepubertal animals, the effect of P-mod-S on transferrin secretion and cellular transferrin mRNA levels is greater than that of FSH alone, and equivalent to or greater than that elicited by a mixture of FSH, insulin and retinol. In addition, the effects of P-mod-S and FSH on Sertoli cell function appear to be additive, and regulated through different intracellular transducing systems (cAMP for FSH, cGMP for P-mod-S) (Anthony et al. 1991; Skinner 1991). These observations indicate that P-mod-S may act in concert with FSH to promote and maintain Sertoli cell function *in vivo*. Interestingly, an androgen-responsive activity with similar biochemical and biological properties has also recently been isolated from prostatic stromal cell-conditioned media (Swinnen et al. 1989), suggesting that P-mod-S like activity may not be an exclusive product of the testis, but a widespread mediator of mesenchymal:epithelial cell interactions in androgen target tissues.

3.7 Nerve Growth Factor (NGF)

Nerve growth factor is present in significant amounts in seminal plasma and the male accessory sex glands including the vas deferens, epididymis and seminal vesicles of several species (Ayer-Lelievre et al. 1988; Seidl and Holstein 1990a). The presence of mRNA, immunostaining for NGF protein, and NGF bioactivity in spermatocytes in the testis conclusively demonstrates testicular production of this growth factor (Olsen et al. 1987). Evidence that NGF may act as a paracrine factor in the testis was provided by the demonstration that NGF receptor (NGFR) mRNA is expressed in rat Sertoli cells (Persson et al. 1990). This message is expressed only in Sertoli cells associated with spermatocytes in stage VI to VIII of the seminiferous cycle. Hypophysectomy or treatment with EDS led to a 50 fold increase in NGFR mRNA and to a loss of the stage-dependency of expression. Treatment with testosterone restored the normal NGFR mRNA level and stage-specific expression pattern. Therefore expression of NGFR mRNA appears to be strongly negatively regulated by testosterone. Seidl and

Holstein (1990b) demonstrated NGFR on sympathetic nerve fibers and peritubular myoid cells in the human testis. Using organ culture of human seminiferous tubules, these authors found that NGF maintained Sertoli and myoid cell morphology and prevented thickening of the tubules wall.

Thus, the NGF/NGFR system provides an example of a paracrine loop involving three, and perhaps four, cell types of the testis. NGF is produced in the spermatocytes. The target cells are the Sertoli cells and perhaps the myoid cells. The ability of the Sertoli cell to respond to NGF, however, depends on the presence of the NGFR which can be downregulated by testosterone produced by the Leydig cell. This effect of testosterone on the Sertoli cell NGFR expression may be direct or may be modulated by myoid cell-produced factors (such as P-mod-S). These types of complex interrelationships are likely to be the rule in paracrine action of growth factors rather than the exception.

4 Immunoregulatory Factors

Interleukins and other immunoregulatory cytokines may have important actions on reproductive function in both health and disease. In the testis, these factors may be involved in regulation of steroidogenesis as well as DNA synthesis by developing germ cells. Cytokines may be produced in the testis by infiltrating lymphocytes or may be derived from the resident population of macrophages which comprise approximately 20% of all testicular interstitial cells. Although the role of the testicular macrophages remain unclear, these cells, like their peripheral counterparts, may provide a source of interleukin 1 (IL-1) and TNF α (Pollanen et al. 1990). In addition, at least some testicular cytokines may be derived directly from the tubular elements (Sertoli or germ cells) or interstitial Leydig cells.

4.1 Interleukin-1 (IL-1)

Testicular extracts from rats and humans contain an activity that stimulates *in vitro* mouse thymocyte proliferation in a manner similar to IL-1

derived from peripheral macrophages (Soder et al. 1988). Although the exact nature of testicular IL-1 (tIL-1) remains unclear, the molecular weight (15-20 Kd) and acidic pI of this activity ($\sim$ 6.0) is more similar to that of human macrophage-derived IL-1 α than the more basic (and more abundant form in humans) IL-1 β (see below). *In vitro* experiments have shown that this factor is secreted under basal conditions from seminiferous tubules isolated from both rat and human testis (Syed et al. 1988). Persistence of tIL-1 activity in the testes of animals after germ cell depletion by experimental cryptorchidism or prenatal testicular irradiation suggests that this factor is produced predominantly by Sertoli cells. Testicular macrophages do not contribute to the production of tIL-1 in the rat (Pollanen et al. 1990). Similarly, extracts of interstitial cells or culture media conditioned by either crude or Leydig- or peritubular myoid-enriched interstitial cells show little evidence of IL-1-like bioactivity. However, a recent report by Wang et al. (1991) demonstrated induction of IL-1 α mRNA expression in macrophage-free Leydig cell cultures exposed to increasing doses of IL-1 β . Thus, under certain conditions, Leydig as well as Sertoli cells may contribute to testicular production of IL-1 activity. Previous studies show that testicular production of IL-1 activity is reduced after hypophysectomy but can be restored by administration of hCG, but not FSH or testosterone (Soder et al. 1988; von Euler et al. 1989). It is unclear whether the hCG-dependent restoration of tIL-1 activity is due entirely to direct stimulation of Leydig cell IL-1 production, or to indirect effects on Sertoli cell IL-1 production.

A role for tIL-1 in the regulation of germ cell proliferation and differentiation in the testis is suggested by the observation that testicular expression of IL-1-like bioactivity is initiated during puberty and increases, reaching maximum levels coincident with the onset of sexual maturation in the rat (Syed et al. 1988). This hypothesis was further advanced by experiments which demonstrated that administration of recombinant human IL-1 α (rhIL-1 α) to hypophysectomized rats by intratesticular injection increased spermatogonial DNA synthesis. (Pollanen et al. 1989). More recently, Soder et al. (1991) demonstrated that the presence of endogenous IL-1 activity in the various stages of the seminiferous epithelium correlates well with the levels of active DNA synthesis. In an *in vitro* model wherein tubule segments from various

stages of the cycle are allowed to continue differentiation over a 72 hr period, tIL-1 activity was again shown to be initially highest in cultures initiated from segments with high initial rates of DNA synthesis (Parvinen et al. 1991). After 48h in culture, IL-1 activity was found to increase in cultures which would have been expected to show initiation of pre-meiotic DNA synthesis. Thus, tIL-1, along with other factors discussed previously (activin, inhibin, SGF), may play a role determining the rate or extent of premitotic or premeiotic germ cell DNA synthesis.

Recombinant human IL-1 β (rhIL-1 β) has also been shown to specifically decrease FSH-stimulated aromatase activity and cAMP accumulation by Sertoli cells *in vitro* (Khan and Nieschlag 1991). RhIL-1a alone had no effect in these experiments, but augmented the effects of rhIL-1b. The onset of tIL-1 expression by the testis parallels peripubertal the decline in Sertoli sensitivity to FSH. Thus these data are consistent with a role for tIL-1 in decreasing Sertoli cell responsiveness to FSH *in vivo*.

A considerable amount of data also indicates that that IL-1 related factors have an important role in regulating testosterone production by Leydig cells in the interstitium of adult (Calkins et al. 1988; Moore and Moger 1991) and immature rats (Verhoeven et al. 1988; Fauser et al. 1989). Several studies agree that both rhIL- α and IL-1 β dramatically reduce hCG-stimulated testosterone release and cAMP formation in whole testis cell cultures prepared from neonatal rats or enriched Leydig cell cultures prepared from immature or adult rats. These agents were found to act by inhibiting LH receptor binding, and therefore cAMP accumulation, as well as by inhibition of p⁴⁵⁰_{17a} activity and induction of p⁴⁵⁰_{scc} mRNA levels by hCG (Lin et al. 1991). Taken together, these data indicate that the effects of IL-1 on Leydig cell steroid biosynthesis may be quite complex and exerted at multiple sites along the stimulus-secretion coupling cascade.

While both rhIL-1 α and IL-1 β have been shown to share biological activities in many systems, it now seems clear that the relative potency of IL- α compared to IL- β shows species specificity. Recent data suggest that both rhIL- β and murine IL-1 α are considerably more potent in regulating Leydig cell testosterone production than rhIL- α when compared in the same experiment (Calkins et al. 1990a). Likewise,

binding sites for IL-1 have been demonstrated in interstitial tissues of the adult mouse testis by binding of [^{125} I]-IL-1a (Takao et al. 1990). However, in both rat and guinea pig testis, binding of [^{125} I]-IL-1a and was negligible. These data suggest that multiple types of IL-1 receptors may exist in the testis and that receptor affinity for a given ligand may be highly species specific. Identification and localization of testicular receptors in various species may require further characterization and or purification of the various testicular IL-1 factors before meaningful conclusions can be reached.

4.2 Interleukin-2 (IL-2), and Interleukin-6 (IL-6), Tumor Necrosis Factor α and Interferon

There are no data presently available to confirm that interleukins, or cytokines, other than IL-1, are produced within the testis (Soder et al. 1990). However, given the presence of various types of immune cells, and the ability of IL-1 to induce the production of and receptors for other cytokines in many systems, it is likely that these molecules may be available for local interaction with Leydig and Sertoli cells. Exposure of the testis to these molecules during periods of inflammation via infiltration of lymphocytes from the periphery may impact upon normal endocrine and paracrine control of testicular function.

Potential effects of IL-2 on Leydig cell testosterone production have been investigated by Guo et al. (1990). Similar to reported actions of IL-1, these authors report that IL-2 decreased hCG-stimulated (but not basal) testosterone production and cAMP accumulation, as well as testosterone production stimulated by cAMP analogues or forskolin. These investigators further demonstrated that at least part of the activity of IL-2 is exerted at the levels of p ^{450} 17a. However, unlike the actions of IL-1, IL-2 had no significant effect on [^{125} I]-hCG binding to Leydig cell membranes.

The effects of IL-2, as well as IL-6 on Sertoli cell function have also been investigated *in vitro* (Boockfor and Schwarz 1991). Using Sertoli cell cultures derived from staged segments of seminiferous tubules, these authors found that short-term treatment of cultures prepared from stage IX-XIII tubules with IL-2 or IL-6 (< 8h) augmented

transferrin secretion, but had no effects on cultures from stage III-VII tubules. In addition, preincubation of cells with IL-6, but not IL-2, augmented the FSH-responsiveness of cultures derived from stage IX-XIII, without affecting FSH-responsiveness of cells derived from other stages. These data indicate that IL-2 and IL-6 may be important regulators of stage-dependent Sertoli cell function in the testis, as well as modulators of the effects of LH on Leydig cell function.

TNF- α has no effect on basal testosterone production by Leydig cell cultures prepared from adult rat testis (Calkins et al. 1990b). However, this agent augments the actions of IL-1 β in decreasing hCG-stimulated testosterone production and cAMP formation. In contrast, Warren et al. found that whole testis preparations from adult animals exhibited increased basal testosterone production, possibly due to effects of TNF α on other testicular cell types. TNF- α was also found to be capable of increasing transferrin secretion in staged cultures of tubules in a manner similar to that seen for IL-2 and IL-6. However, like IL-2, TNF- α had no effect on FSH-responsiveness of the various cultures (Boockfor and Schwarz 1991).

Similar to the activities of IL-2, human leucocyte interferon has been found to decrease hCG-stimulated testosterone production in primary cultures of immature porcine Leydig cells by decreasing accumulation of the steroidogenic enzymes P450_{17 α} and P450_{scc}.

In summary, immunoregulatory agents have marked effects on the regulation of reproductive function during various disease states as well as participating in the day to day regulation of testicular function. Because of the inter-related nature of cytokine production and action, the potential for the existence of such a strictly controlled network of locally acting mediators with specific effects on Leydig and Sertoli cell function indicates that these factors may play a complex, but integral role in paracrine regulation of testicular function.

5 Conclusions

Our current knowledge of growth factors in the testis represents something of an embarrassment of riches. We now have evidence that a number of growth factors are present in the testis and have demon-

strated effects on testicular cells *in vitro*. Since we have a limited number of endpoints to measure for cell specific function, however, we do not know if the effects seen represent the major role of the growth factor, a relatively minor effect, or a non-physiologic culture artifact. To add to the confusion, many of the growth factors described above (e.g. activins, TGF- β s) are present as related families of proteins with similar activities but different patterns of expression. Very little is known at this time about the significance of the differential control of these closely related species. Some factors have locally produced binding proteins which may be involved in regulating their function (eg. activin/follistatin or IGF-I/IGF-IBP3). Some of the related growth factors with similar activities such as insulin and IGF-I, or EGF and TGF- α represent a pair containing an endocrine factor with receptors and activities in the testis and a true paracrine factor. Attributing effects observed *in vitro* to one or the other *in vivo* will continue to be a challenge. Additionally, many of these growth factors form interrelated networks with levels of one growth factor regulated by levels of another, as well as by endocrine signals. Finally, the site of production and actions of many of these factors may change as a function of development. Thus, while most investigators in the field now accept the important role of growth factors in the paracrine regulation of testicular function, the unravelling of the true temporal and spatial interrelationships of the expression and action of these factors will be the challenge of the next decade.

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Expression of Proto-Oncogenes and Protein Kinases in the Testis

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1 Introduction

Specificity of expression is regarded as a strong indication that genes play a role in determining the phenotype or function of the cells in which they are expressed. A molecular genetic approach to understanding the control of male germ cell differentiation involves, in part,

identifying genes that are expressed in a spermatogenic cell type-specific manner. Previous observations from our laboratory and others have shown that some testis-specific genes are expressed in spermatogenic cells in a stage-specific manner. Similarly, more ubiquitously expressed genes can exhibit particularly abundant expression in germ cells, often of uniquely sized transcripts. Both patterns of expression suggest a role for these genes in spermatogenesis or subsequent function of male gametes (rev. in Willison and Ashworth 1987; Hecht 1990; Erickson 1990; Wolgemuth and Watrin 1991).

The fact that several of these genes are proto-oncogenes is of interest, especially given the potential role of proto-oncogenes in regulating cellular growth and differentiation in a wide variety of systems (Bishop 1983). Various proto-oncogenes have been examined for their expression during mammalian development (Adamson 1987). Although distinct patterns of expression have been described in various developing germ layers, tissues, organs, etc., the data are insufficient to determine their specific roles in development. It is clear, however, that virtually all proto-oncogenes examined are expressed during cellular differentiation as well as during proliferation.

Several proto-oncogenes exhibit protein kinase activity (Bishop 1983). Protein phosphorylation is believed to play a major role in the mechanisms by which cells receive and interpret extracellular signals as well as being critical for normal cellular metabolism. The potential protein kinase activity of several of the proto-oncogenes expressed in the testis and their pattern of expression in germ cells suggest that these genes could be important in regulating the metabolic activities and differentiation of spermatogenic cells and/or in initiating meiotic and mitotic events of gametogenesis.

In this paper, we present examples, from our laboratory as well as those of other groups, of proto-oncogenes which exhibit known or postulated protein kinase activity, and which have been reasonably well characterized with respect to their expression during mammalian gametogenesis and embryogenesis. We also discuss recent observations on the expression of other genes, a murine cyclin B gene and a new gene designated *meg1*, that may directly or indirectly be involved with phosphorylating activity during germ cell differentiation. The structure and pattern of expression of these genes suggest that they

could play a role in the regulation of mitotic, meiotic, or post-meiotic cell cycles of spermatogenesis by associating with other cellular proteins to form complexes with kinase activity or by serving as potential substrates for other protein kinases.

2 Proto-Oncogenes with Tyrosine Kinase Activity Expressed in the Testis

2.1 *C-abl* Gene Expression

C-abl, the cellular homologue of the transforming region of the Abelson murine leukemia virus, encodes a cytoplasmic-class tyrosine kinase and is expressed at low levels in all tissues and cell lines examined (Wang and Baltimore 1983), producing mRNAs of ~6 and 8 kb. An interesting exception to this pattern is observed in the adult mouse testis, where high levels of a 4.7 kb transcript are found (Muller et al. 1982). We have shown that the pattern of expression of this novel transcript is developmentally regulated and restricted to post-meiotic germ cells (Ponzetto and Wolgemuth 1985).

The predicted amino acid sequence obtained from cloning studies (Meijer et al. 1987; Oppi et al. 1987), from *in vitro* translation analysis (Meijer et al. 1987), as well as from our studies on testicular ABL proteins (Ponzetto et al. 1989), indicates that the 4.7 kb testicular *c-abl* mRNA would produce a 150 kDa ABL protein indistinguishable from the ABL protein produced from the somatic Type I *c-abl* mRNA (Ben-Neriah et al. 1986) on one dimensional SDS-PAGE. Observations from our laboratory, using immunoprecipitation and an *in vitro* kinase assay to detect the presence of ABL proteins, revealed the presence of autophosphorylated ABL proteins (P150) in mouse testicular tissue and in spermatogenic cells (Ponzetto et al. 1989). In addition, a protein exhibiting a relative molecular weight of ~74 kDa (P74) was labeled. The recovery of P74 protein was variable, depending most critically on whether one or two cycles of immunoprecipitation were used. This suggests that the P74 protein was probably co-precipitated by the anti-ABL antibodies by virtue of its close association with P150 in the cellular lysate, rather than by being precipitated since it was recognized

directly by the anti-ABL antibodies. Immunoprecipitates of cellular extracts revealed that P150 is found in testicular cell lysates from both immature and mature testes, whereas the P74 protein is present at detectable levels only in extracts of mature testes (Ponzetto et al. 1989).

We then determined whether P150 and P74 were present in spermatogenic cells. Enriched populations of meiotic prophase spermatocytes, haploid spermatids, and elongating spermatids along with residual bodies were analyzed by the *in vitro* kinase assay. P150 was shown to be present in all spermatogenic cells, whereas P74 was observed only in post-meiotic cells (Ponzetto et al. 1989).

Since the ABL kinase is tyrosine-specific, possible substrates should contain phosphotyrosine. Phosphoamino acid analysis revealed that while P74 contained mostly phosphoserine, equimolar amounts of phosphothreonine and phosphotyrosine were detected (Ponzetto et al. 1989; Wadewitz and Wolgemuth 1991). These results support the hypothesis that P74 may be a substrate of P150. However, the abundance of phosphoserine and phosphothreonine also suggested that an undefined associated serine/threonine protein kinase was present during the *in vitro* kinase assay.

Pendergast et al. (1987) have shown that treatment of NIH-3T3 cells with activators of the 74 kDa protein kinase C (PKC) results in a four to eight-fold increase of ABL activity. Further, purified PKC phosphorylated all ABL proteins at the same two serines as were phosphorylated *in vivo*. This suggests the intriguing possibility that the co-precipitating P74 may be PKC. Alternatively, P74 may be the product of the proto-oncogene *c-raf*, a serine/threonine protein kinase which co-precipitates with the tyrosine kinase platelet derived growth factor (PDGF) receptor (Morrison et al. 1989). This observation is of interest at several levels. Recent studies on *c-raf* and other members of the *raf* family have demonstrated high levels of expression in the testis and other reproductive tissues (see below). In addition, *Flt3*, a member of the PDGF receptor family has been identified in mouse testis (Rosnet et al. 1991). Testes express two testis-specific transcripts of 2.6 and 3.3 kb, but not the 3.7 kb transcript seen in most somatic tissues. Furthermore, expression of the two transcripts is developmentally regulated: the 3.3 kb transcript is present in testes from 15 day-old mice, but not in gonads from 10 day-old mice, suggesting that this transcript may be

expressed initially in pachytene spermatocytes. In contrast, the more abundantly expressed 2.6 kb transcript, representing a truncated form of the 2.8 kb open reading frame, is first expressed at 20 days of post-natal development, concurrent with the appearance of post-meiotic round spermatids. These results suggest that both cytoplasmic tyrosine kinases such as *c-abl* and membrane bound receptor tyrosine kinases may function in germ cell development. Furthermore, different forms of the same gene may regulate discrete stages of germ cell development.

2.2 *C-kit* and its Ligand *Steel*

Genetic, rather than molecular or biochemical, evidence has linked the activity of *c-kit*, another transmembrane receptor tyrosine kinase related to the PDGF-receptor (Besmer et al. 1986; Yarden et al. 1987; Qui et al. 1988), to germ cell development. *C-kit* is the product of the dominant *white spotted* (*W*) locus in the mouse (Geissler et al. 1988; Nocka et al. 1989). The pleiotrophic phenotypes of *W* mutants include white coat color, sterility in both sexes and anemia, and have been attributed to the failure of stem cells to migrate or proliferate during development (Mintz and Russell 1957). In *c-kit* mutations such as *W^v*, primordial germ cells migrate to the seminiferous tubules but fail to proliferate or differentiate beyond the spermatogonial stage (Coloumbre and Russell 1954), suggesting that the *c-kit* gene product may also play a role in the postnatal development of male germ cells.

Analysis of the expression of *c-kit* during embryonic and adult development has shown that the gene is expressed in a wide variety of tissues, including sites in which there is no obvious phenotype in animals bearing the mutant alleles (Manova et al. 1990; Orr-Urtreger et al. 1990; Keshet et al. 1991). *In situ* analysis revealed the expression of *c-kit* in the primordial germ cells during days 9-10 of fetal development, as they migrate from the yolk sac to the gonadal ridge (Orr-Urtreger et al. 1990). Perhaps somewhat unexpected was the observation of *c-kit* expression in the adult gonads as well. In the testis, the *in situ* analysis localized *c-kit* transcripts in both Type A spermatogonia and in Leydig cells (Manova et al. 1990). Germ cell expression of *c-kit* decreased in

intermediate and Type B spermatogonia, but still exhibited diffuse labeling in the preleptotene spermatogonia. Leptotene/zygotene cells were unlabeled, as were all subsequent stages of meiotic and post-meiotic germ cell development. Leydig cells remained heavily labeled in the adult mouse testis (Manova et al. 1990). However, subsequent Northern blot analysis of *c-kit* expression in gonads has modified this pattern somewhat (Sorrentino et al. 1991). An abundant 5.5 kb *c-kit* transcript was observed in total testis RNA, as well as apparently novel testis-specific transcripts of 3.5 and 2.3 kb. Analysis of enriched populations of germ cells indicated that the 5.5 kb transcript was most abundant in Type A spermatogonia, decreased through the pachytene spermatocyte stage and was absent in post-meiotic germ cells. In contrast, the 3.5 kb transcript first appears at low levels in late pachytene spermatocytes and is abundantly expressed in post-meiotic round spermatids, along with the 2.3 kb transcript. The 5.5 kb transcript is also expressed in some of the somatic tissues of the testis (Sorrentino et al. 1991). The apparent lack of *c-kit* expression in the post-meiotic cells observed by *in situ* analysis may have been due to the fact that the probe employed for the *in situ* hybridization studies included only that portion of the gene which codes for the transmembrane and extracellular domains of the protein, but which lacked the more highly conserved kinase domain. It may therefore have been unable to detect the truncated or alternatively spliced forms of the gene represented by the 3.5 and 2.3 kb transcripts present in the post-meiotic germ cells.

The *Steel* (*Sl*) gene has been identified as the ligand for the *c-kit* receptor (Copeland et al. 1990; Flanagan et al. 1990; Huang et al. 1990; Williams et al. 1990; Zsebo et al. 1990a; 1990b). *Sl* is expressed abundantly in the stromal cells of the mesoderm, along the migratory pathway of the primordial germ cells during the same period of fetal development in which the germ cells themselves express the *c-kit* gene; *Sl* is also expressed in the genital ridge (Matsui et al. 1990). However, it appears that the *Sl* product does not act as a chemotropic factor to guide the migration of the primordial germ cells, nor does it affect their proliferation. Rather, it seems to be required for maintaining the viability of the cells (Dolci et al. 1991; Godin et al. 1991). Recent studies on *Sl* expression in adult mouse tissues revealed that in adult gonads, *Sl* is

expressed in follicular cells of the ovary and in Sertoli cells of the testis (Motro et al. 1991; Tajima et al. 1991).

2.3 Identifying New Protein Tyrosine Kinases That May Function in the Testis

Several approaches have been employed to isolate new tyrosine kinases (some of which may fall into the category of proto-oncogenes). To apply these techniques to genes which might function in spermatogenesis, one simply focuses on genes represented in a testis cDNA library. In the first strategy, a known tyrosine kinase could be used as a probe to screen the cDNA library to isolate closely related genes. Alternatively, synthetic oligonucleotides generated against one of the several conserved amino acid regions that are characteristic of tyrosine kinases could be used as the probe in the screening. Finally, anti-phosphotyrosine antibodies could be used as probes to screen testicular cDNA expression libraries. The various approaches would be expected to yield overlapping collections of new tyrosine protein kinases. The latter two approaches have been used independently to isolate a gene now referred to as *ferT*, which exhibits putative tyrosine kinase activity and an expression pattern suggestive of a role during late meiotic and post-meiotic events (see below).

Anti-phosphotyrosine antibodies were used to identify clones producing phosphorylated proteins from a rat brain lambda gt11 cDNA expression library (Letwin et al. 1988). One clone, *elk*, shared homology to the *eph* proto-oncogene and demonstrated limited tissue-specific expression, being abundant in the brain and present at low levels in testes. Another clone shared homology to the *fps/fes* proto-oncogene and was originally designated *flk* (Letwin et al. 1988), now referred to as *fer* (Fischman et al. 1990). *Fer* was found to be expressed in a variety of tissues but most abundantly in adult testes. Testes contained two additional *fer* transcripts of 1.3 and 2.4 kb not seen in somatic tissues.

Concurrent studies using the degenerate oligonucleotide approach for isolating genes with putative tyrosine kinase domains, revealed that in fact these uniquely sized testicular transcripts represented, at least in

the mouse, the products of a second gene, designated *ferT* (Fischman et al. 1990). Structural analysis suggests that *ferT* evolved from a common precursor by alternative splicing, and codes for a 51 kDa protein, apparently a truncated form of the 94 kDa protein produced by the more ubiquitously expressed *fer* gene (Fischman et al. 1990). The transient accumulation of its 2.4 kb transcripts during mid and late pachytene spermatogenic stages (Keshet et al. 1990) suggests a possible role in germ cell development.

Finally, antibodies against specific, known tyrosine kinases may be used to determine directly the expression of such proteins in the testis. This method was used to demonstrate the expression of the 62 kDa product of *c-yes*, another proto-oncogene tyrosine kinase of the non-receptor type, in the acrosomal region of mature spermatozoa (Zhao et al. 1990). Given the proposed role of tyrosine kinase and phosphotyrosine phosphoproteins during the zona pellucida-induced acrosome reaction (Leyton and Saling 1989), it is possible that proto-oncogene gene products may be crucial not only for germ cell development, but also for the subsequent function of mature spermatozoa during fertilization.

3 Serine/Threonine Phosphorylating Proto-Oncogenes with Developmentally Regulated Testicular Expression

3.1 Members of the *Raf* Family

C-raf-1 is a member of the cytoplasmic serine/threonine protein kinase family and is expressed throughout spermatogenesis as a 3.1 kb transcript coding for a 74 kDa protein. *C-raf-1* mRNAs have been detected in germ cells from type A and B spermatogonia through the round spermatid stage, but not in residual bodies. The highest levels are observed at the pachytene spermatocyte stage (Wolfes et al. 1989). The adult ovary also abundantly expresses a 3.1 kb transcript (Sorrentino et al. 1988).

C-raf-1 belongs to a family of related genes, all of which are known to be expressed in the testis. *A-raf* is expressed at low levels in the mouse testis as a 2.6 kb transcript coding for a 68 kDa protein (Beck et

al. 1987). Highest levels of *A-raf* expression occur in the epididymis, as well as in seminal vesicle and prostate (Storm et al. 1990). These observations invite speculation that *A-raf* may be involved in regulating the hormonal responsiveness of these androgen-dependent tissues. *B-raf* was first identified in a human testis cDNA library (Ikawa et al. 1988). It appears in the testis as abundant, testis-specific transcripts of 2.6 and 4.5 kb, along with lower levels of the 10 and 13 kb transcripts expressed in somatic tissues (Storm et al. 1990). *B-raf* codes for a major 75 kDa protein in mouse testis, as well as a less abundant 77 kDa protein. While the 77 kDa protein appears to be authentic *B-raf*, it is unclear whether this represents a post-translational modification of the 75 kDa species or the product of an alternatively spliced gene (Sithanadam et al. 1990).

All three members of the *raf* family have been shown to have transforming activity and share a high degree of sequence homology at both the nucleotide and amino acid level (Sithanadam et al. 1990). Three regions in particular are highly conserved: a cysteine-rich, putative zinc-finger region, a serine/threonine rich domain which may serve as a site of autophosphorylation/activation, and the kinase domain itself. In contrast, the C- and N- termini of the genes are more highly divergent. Intriguingly, *B-raf*, which appears to be the form most highly expressed in testis, contains a sequence, preceding the putative zinc-finger region, which is potentially phosphorylatable by the *cdc2* kinase, suggesting it may play a crucial role in the regulation of the meiotic cell cycle (see below). Studies are currently underway in our laboratory to determine the cell- and stage-specificity of expression of all three *raf* genes in the mouse testis at both the mRNA and protein level.

The potential role of *c-raf* during germ cell development is particularly intriguing since *c-raf* kinase associates with the ligand-activated PDGF receptor, a tyrosine protein kinase. Morrison et al. (1989) have shown that baculoviral-expressed PDGF receptor kinase and *c-raf* kinase associate *in vitro*. Following co-incubation, *c-raf* kinase activity is stimulated 4 to 6-fold. Similarly, cells transformed with other tyrosine kinases such as *v-src*, *v-Ha-ras*, or *v-fms* show elevated *c-raf* kinase activity (Roberts et al. 1988). Increases in both *c-raf-1* kinase phosphorylation and kinase activity have been observed in cultured cells treated with insulin (Kovacina et al. 1990), erythropoietin (Carroll

et al. 1991), acidic fibroblast growth factor and epidermal growth factor (Morrison et al. 1988). Thus, *c-raf* may play a critical role in the signal transduction of growth factor receptor kinases expressed during spermatogenesis. The possible association of *c-raf* kinase with the *c-abl* gene product would indicate that cytoplasmic tyrosine kinases may also regulate RAF activity. The ultimate role of such regulatory cascades in gene expression has not been elucidated. However, both *c-raf* and *A-raf* have been shown to stimulate the PEA1 (AP1) transcription regulatory factor (Wasylyk et al. 1989).

3.2 *C-mos*

C-mos, the normal cellular homologue of transforming sequences in the Moloney murine sarcoma virus (Moloney 1966), encodes a cytoplasmic serine/threonine protein kinase. *C-mos* transcripts are detected most abundantly in the gonads of the adult mouse, and at very low levels in the adult epididymis, day 19 embryo (Propst and Vande Woude 1985), and in certain somatic tissues (Mutter and Wolgemuth 1987; Propst et al. 1987). The pattern of *c-mos* expression during gametogenesis and embryogenesis is intriguing. In the mouse testis, *c-mos* is most abundantly expressed as a testis-specific 1.7 kb transcript in early spermatids (Goldman et al. 1987; Mutter and Wolgemuth 1987; Propst et al. 1987). Surprisingly, antibodies specific for the *c-mos* gene product demonstrated highest levels of the 43 kDa protein in purified populations of pachytene spermatocytes, and lower levels in the post-meiotic stages of development (Herzog et al. 1989). Similar studies on rat testis also demonstrated highest levels of *c-mos* mRNA expression in round spermatids, while MOS protein expression was restricted to pachytene spermatocytes (van der Hoorn et al. 1991), suggesting that $p43^{mos}$ expression is regulated in a stage-specific fashion at the level of translation as well as transcription. MOS protein was also observed in germ-cell deficient testes, from a number of mutant strains, as well as cell culture lines derived from Sertoli and Leydig cells, indicating that the somatic cells of the testes also express *c-mos* (Herzog et al. 1989).

In the ovary, murine *c-mos* mRNA levels exceed testicular levels by several-fold (Goldman et al. 1987; Mutter and Wolgemuth 1987).

Northern blot and *in situ* hybridization analyses indicate that *c-mos* transcripts of 1.4 kb begin to accumulate in the cytoplasm of growing oocytes (Goldman et al. 1987; Mutter and Wolgemuth 1987) and undergo a slight increase in size (~250 nt) in the meiotically mature ova (Goldman et al. 1988; Mutter et al. 1988). The levels of *c-mos* transcripts appear to be stable as the oocytes resume meiosis, then drop precipitously at metaphase II and are not detected in two-cell or early pre-implantation embryos (Mutter et al. 1988).

The function of *c-mos* in murine oocytes has been investigated by injection of *c-mos* antisense oligodeoxynucleotides (oligos) (O'Keefe et al. 1989; Paules et al. 1989). Oocytes injected with *c-mos* oligos failed to arrest at metaphase II, and often cleaved into two cells. It was concluded that loss of *c-mos* function leads to premature reformation of a nucleus, essentially permitting ensuing DNA synthesis and cell cleavage. These results also implied that the *c-mos* product is needed for oocyte maturation. This suggests the possibility that *c-mos* may be important in subsequent stages of oocyte differentiation or in early embryogenesis. The importance of *c-mos* in regulating the cell cycle of the maturing oocyte and early zygote is further underscored in recent studies in which the presence of MOS protein was shown to be critical for stabilizing cyclin in the mouse oocyte (O'Keefe et al. 1991).

In marked contrast to the considerable amount of information concerning the possible function of *c-mos* in the vertebrate oocyte, studies on the function of *c-mos* during spermatogenesis are lacking. However, given the high levels of MOS protein expression in pachytene spermatocytes, it is likely that this kinase plays a role in male germ cell meiosis comparable to that in oocytes.

4 Other Genes with a Possible Role in Spermatogenesis

4.1 Cyclin

Cyclin was originally identified as a protein in sea urchin eggs whose synthesis is greatly increased upon fertilization and whose levels oscillate during the cell divisions of the early embryo (Evans et al. 1983). The level of cyclin protein increases during interphase, peaks at the

G2/M-phase transition, and drops precipitously at mitosis of each cell cycle. Cyclins have been highly conserved through evolution and have been identified in organisms ranging from yeast to man (rev. in Hunt 1991; Pines 1991). Cyclin has been found to be the only newly synthesized protein required for meiotic maturation of starfish oocytes (Standart et al. 1987) and for activated frog eggs to enter mitosis (Minshull et al. 1989a), thus demonstrating its role in both meiotically and mitotically dividing cells.

Cyclin associates with a serine/threonine protein kinase, the *S. pombe cdc2* gene product, p34^{cdc2}, or its homologues, to form a complex known as the maturation or M-phase promoting factor, MPF (Draetta et al. 1989). Cyclin functions as a regulatory subunit of MPF, targeting p34^{cdc2} for activation (Solomon et al. 1990; Meijer et al. 1991). Once activated, p34^{cdc2} phosphorylates cellular components, triggering entry of the cell into M-phase. Destruction of the cyclin component by selective proteolysis results in inactivation of the kinase and exit from M-phase (Glotzer et al. 1991). Therefore, synthesis of cyclin is required for cells to enter M-phase and its destruction is required for cells to exit from M-phase (Murray and Kirschner 1989; Murray et al. 1989).

Cyclins have been divided into three groups, G1, A and B, based on amino acid homology, timing of appearance during the cell cycle, and kinetics of degradation (rev. in Minshull et al. 1989b; Hunt 1991; Pines 1991). The B-type cyclins appear during interphase and function at the G2/M-phase transition to trigger entry of the cell into M-phase. The observation that cyclin B transcripts are localized to the pole cells during *Drosophila* embryogenesis and that cyclin B mRNA is abundantly expressed in the testes of *Drosophila* larvae (Whitfield et al. 1989), along with the fact that cyclins are required for both mitotic and meiotic cell division, led us to study cyclin B expression during germ cell development in the mouse. Using the human B1 and B2 cDNAs as probes, we have screened an adult mouse testis cDNA library and have isolated cDNAs encoding a B-type cyclin, designated mouse cyclin B1, *cycB1* (Chapman and Wolgemuth 1992).

The pattern of *cycB1* expression was examined by Northern blot and *in situ* hybridization analyses. Two major transcripts, 1.7 and 2.5 kb, were detected in RNA from various adult and embryonic mouse

RNA samples. The highest levels of hybridization in the adult tissues examined were in the testis and ovary. The 1.7 kb mRNA was the major testis transcript while the 2.5 kb species predominated in the ovary RNA. Northern blot and *in situ* hybridization analysis revealed that the prominent sites of *cycB1* expression were in growing oocytes in the ovary and in early spermatids in the testis, with low but readily detectable levels observed in some pachytene spermatocytes and elongating spermatids (Chapman and Wolgemuth 1992).

Localization of *cycB1* transcripts to post-meiotic germ cells in the testis was surprising. These cells have completed the mitotic and meiotic cell divisions that are normally associated with cyclin expression. In light of the relative patterns of mRNA and protein expression seen with *c-mos*, it will be interesting to examine the role of *cycB1* at the protein level in these post-meiotic germ cells (Westendorf et al. 1989). Although we have no protein data at this time, it is possible to propose some hypotheses concerning a function for the p34^{cdc2}/*cycB1* complex at this stage by examining the known substrates of the p34^{cdc2} kinase. Several structural proteins are phosphorylated by the p34^{cdc2} kinase, resulting in the physical reorganization of the cell and ultimately entry of the cell into M-phase. Some of the changes that take place during M-phase of mitotic as well as meiotic divisions may also be taking place in these post-meiotic germ cells. For instance, the chromatin of the sperm nucleus must undergo a complete reorganization, involving the exchange of somatic histones for transition proteins and finally for the highly basic protamines (Goldberg et al. 1977). In trout, this process involves phosphorylation of histones at serine residues which is believed to alter their ability to bind DNA, perhaps facilitating the exchange process (Sung and Dixon 1970). This is consistent with the histone H1 kinase activity of p34^{cdc2} (Arion et al. 1988; Labbe et al. 1989). Other cellular substrates of the p34^{cdc2}/cyclin complex have been reported (rev. in Moreno and Nurse 1990). Phosphorylation of microtubules in particular may be involved with the physical restructuring of the cell during M-phase (Verde et al. 1990), as well as with the restructuring of the round spermatid into the morphologically polarized spermatozoa.

Although the localization of *cycB1* transcripts to the meiotic and post-meiotic compartment of the ovary and testis, respectively, was at

first unexpected, the similarity between the expression pattern of *c-mos* and *cycB1* is striking. In mouse oocytes and in other species, phosphorylation of cyclin B by the *c-mos* protein product has been implicated in the stabilization of cyclin B (O'Keefe et al. 1991). It is interesting to speculate that *c-mos* could be performing the same function in the post-meiotic cells of the testis.

4.2 *Meg1*

Given the conservation of both the meiotic process itself and of several key molecules thus far identified, such as p34^{cdc2} and cyclins, we hypothesized that genes which regulate entry into meiosis, in species such as yeast, might have evolutionally conserved counterparts in mammals. We therefore attempted to identify murine genes structurally and/or functionally homologous to yeast meiosis regulating genes. Our initial efforts resulted in the identification of a novel gene, designated *meg1*, which exhibits an interesting pattern of restricted expression in male germ cells and which may function in phosphorylation events in spermatogenic cells (Don and Wolgemuth 1992).

Meg1 appears as an abundant transcript of 0.75 kb in total testis RNA from adult mice, but not in RNA from other adult tissues or in total RNA from mid-gestation embryos. Moreover, this transcript was not detected in testes of two germ cell deficient mutant strains, as assessed by *in situ* and Northern blot hybridization analysis, indicating that *meg1* expression is primarily germ cell-specific. Analysis of RNA from enriched populations of spermatogenic cells revealed that *meg1* was most abundant in meiotic cells. This meiotic predominant expression was confirmed by *in situ* analysis of d17 and d21 post-natal testis.

Two aspects of *meg1* expression are distinct from those observed for other genes, also expressed in spermatocytes, and support its potential association with meiotic events. First, Northern and *in situ* hybridization analysis showed that *meg1* mRNA begins to accumulate in developing testes which contain late leptotene and zygotene spermatocytes. Second, *meg1* mRNA levels appear to decrease in the haploid cells. This expression pattern would seem to be less charac-

teristic of genes whose mRNAs would be stored for post-meiotic translation and function, such as the phosphoglycerate kinase-2 mRNA or some acrosomal specific proteins (Hecht 1990; Wolgemuth and Watrin 1991).

Conceptual translation of the putative 264 bp ORF of *meg1* yields a protein of 88 amino acids with an estimated pI of 9.16 and a calculated molecular weight of 10.8 kDa (Don and Wolgemuth 1992). Comparison of p11^{*meg1*} with other germ cell-specific, size-related proteins with several common structural features might give a clue to the function of this protein. P11^{*meg1*} is rich in phosphorylable amino acids (~17%). It is reminiscent of the transition proteins *TP1* and *TP2*, which are small (54 and 117 amino acids, respectively), basic, germ cell-specific, DNA-associated proteins involved in DNA condensation (Hecht 1990). *TP1* and *TP2* also contain many phosphorylable residues, mainly serine and threonine (Kleene and Flynn 1987; Kleene et al. 1988). The transcriptional patterns of *TP1* and *TP2* are, however, different from that of *meg1*, since the *TP* genes are haploid expressed genes (Hecht 1990). An attractive speculation would be that the putative *meg1* protein product could resemble the transition proteins in its ability to interact with DNA, possibly facilitating meiotic chromosomal events. This interaction could be regulated by phosphorylation or dephosphorylation.

As mentioned above, p11^{*meg1*} is relatively rich in potential phosphoacceptor amino acids. The relatively high percentage of tyrosine residues in p11^{*meg1*} suggests that it might be phosphorylated on one or more tyrosine residues. The putative tyrosine kinase *ferT* noted above might be of special interest given its pattern of expression which closely parallels that of *meg1*. However, several other potential tyrosine kinase proto-oncogenes which are shown to be expressed during spermatogenesis, including *c-abl* (Ponzetto and Wolgemuth 1985), *c-kit* (Sorrentino et al. 1991), and *pim-1* (Sorrentino et al. 1988), could also be involved.

We also examined p11^{*meg1*} for consensus phosphorylation sequences, most frequently recognized by various serine/threonine protein kinases (Kennelly and Krebs 1991). Four protein kinases have the potential to phosphorylate the p11^{*meg1*} protein (Don and Wolgemuth 1992), including cAMP dependent protein kinase (cAMP-PK), cyclic

GMP dependent protein kinase, protein kinase C, and calcium/calmodulin dependent protein kinase II (CaM kinase II). Interestingly, two isozymes of cAMP-PK have been reported in mouse spermatogenic cells: type I is present in pachytene spermatocytes and in round spermatids, whereas type II is present in round and elongating spermatids (Conti et al. 1979; 1983).

5 Summary and Future Directions

Although there is relatively little information available on the function of the protein kinase-encoding proto-oncogenes in male germ cells, it is likely that such proteins exert significant effects on spermatogenic differentiation and function. Furthermore, the presence of novel transcripts or unique isoforms for several of these proto-oncogenes, in different stages of spermatogenic cells, suggests that there may be unique, stage-correlated and specialized functions of the products as well. For example, the *c-kit* gene appears to be necessary for normal migration and/or proliferation of primordial germ cells from the yolk sac to the embryonic gonad, and may also be important for spermatogonial proliferation in the adult gonad. The expression data further suggest that a unique form of *c-kit* may function at later stages of gametogenesis.

Proto-oncogenes, particularly those of the protein kinase class, may also play crucial roles during entry into meiosis. There is a complex network of protein phosphorylation and dephosphorylation involved in regulating entry of cells into mitosis and meiosis, and a major requirement for protein phosphorylation in the mitotic events of nuclear membrane breakdown, chromosome condensation, spindle formation, and activation of microtubule-organizing centers (discussed in Llamazares et al. 1991). Some of these phosphorylation events have been postulated to be mediated directly by the p34^{cdc2} kinase (Moreno and Nurse 1990). However, it is likely that such a diversity of substrates reflects a diversity of phosphorylating and dephosphorylating enzymes as well.

The cytoplasmic proto-oncogene serine/threonine kinase *c-mos* also has been implicated in the regulation of the cell cycle. As mentioned above, studies in both frogs and mice have shown that *c-mos* product, pp39^{mos}, is the cytostatic factor (CSF) responsible for arresting verte-

brate oocytes at the metaphase II stage of meiosis and may be involved in blocking the proteolytic degradation of cyclin, thereby stabilizing the *cdc2*/cyclin complex. In contrast to the information accumulating about possible functions of *c-mos* and the cyclins in the oocyte and early embryo, their function in male germ cells is still enigmatic.

Identification of possible substrates is crucial to addressing the potential function of protein kinases. Preliminary studies in our lab have indicated that a number of phosphotyrosine phosphoproteins are present in germ cells at different stages of development (Winer, Wade-witz and Wolgemuth, unpublished observations). Western blots probed with anti-phosphotyrosine antibodies revealed that adult mouse testes contained intensely labeled phosphotyrosine phosphoproteins of 40 and 87 kDa, a doublet of ~120 kDa, and less intensely labeled proteins of 45, 67 and 150 kDa, as well as a doublet of ~55 kDa. The proteins of 45 and 120 kDa did not appear in Western blots of proteins from pachytene spermatocytes but were present in early spermatids, suggesting their expression and/or their phosphorylation on tyrosine is haploid specific. In contrast, only the 87 and 120 kDa proteins were detectable in cytoplasmic fragments or residual bodies. It will be important to identify these potential substrates, possibly by employing the cDNA expression library screening procedures described above. Alternatively, it may be possible to identify substrates of some classes of tyrosine kinases by virtue of their interaction with the SH2 domain typical of receptor and certain cytoplasmic tyrosine kinases (Mayer et al. 1991), again in expression libraries.

Activation of proteins by phosphorylation may also play an important role during fertilization. A tyrosine phosphorylated 95 kDa protein has been localized to the acrosomal region of the mouse sperm head (Leyton and Saling 1989). This protein may be involved in sperm binding to the zona pellucida and subsequent induction of the binding-dependent acrosome reaction. The level of phosphorylation of the 95 kDa protein appears to be enhanced both by capacitation and the binding of specific zona pellucida glycoproteins. This suggests that the zona-pellucida receptor kinase is capable of autophosphorylation upon binding its ligand; it may also serve as a substrate for other intracellular tyrosine kinases. Interestingly, sperm-activating polypeptides derived from sea urchin eggs stimulate the receptor-mediated phospho-

rylation of three proteins in sea urchin sperm of 52, 75 and 100 kDa (Ribot et al. 1984; Kinsey 1984). While the amino acids on which phosphorylation occurred was not determined in those studies, the similarities in the molecular weights of the proteins to those phosphorylated during capacitation of mouse sperm suggest that highly conserved mechanisms involving tyrosine phosphorylation may function in fertilization.

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Y Chromosome Function in Spermatogenesis

Peter Vogt

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1 Introduction

In mammals a function of the Y chromosome during spermatogenesis has long been hidden by the sex determining function of the Y chromosome in this species class. In contrast to *Drosophila*, where a X,0-karyotype is compatible with a normal viable male, although sterile, in humans the same karyotype is related to a female with sterility and different phenotype abnormalities commonly known as Turner's syndrome (Turner 1938). This state of affairs persisted until 1976, when Tiepolo and Zuffardi (1976) presented clear evidence that the human Y chromosome also must have a function in spermatogenesis. Analyzing a terminal deletion in the Y chromosome of six sterile males with a normal phenotype but azoospermia, they suggested a male fertility gene complex called azoospermia factor (AZF), and located to the distal region of the euchromatic part of the long Y arm (Yq11).

An indirect function of the Y chromosome in spermatogenesis was discussed in the mouse. Y chromosomal effects on testis weight, androgen metabolism and a serologically detected male antigen were inferred (Zenzes et al. 1978; Stewart 1983; Jutley and Stewart 1985). A direct influence of the mouse Y chromosome on spermatogenesis was recognized only recently. Studying the developmental pathway of the male germ cells in chimeric X0-XY mice with X0 karyotype, it was observed that only germ cells with a XY constitution developed to spermatozoa (Levy and Bourgoyne 1986). An early disruption during the differentiation process of the T₁-prespermatogonia taking place in the X0 germ cells was seen by Sutcliffe and Bourgoyne (1989).

To date several genes of the mouse and human Y chromosome which are expressed during spermatogenesis have been cloned at the molecular level (Y353/B: Bishop and Hatat 1987; ZFY: Page et al. 1987; Zfy-1/2: Nagamine et al. 1989; TSPY: Arnemann et al. 1991; A1S9-Y: Kay et al. 1991; Sby: Mitchell et al. 1991). Unfortunately, however, strong evidence for a relation of their expression pattern to the genetically defined Y functions mentioned above is still lacking and mutations in the Y genes interfering with spermatogenesis are not yet known.

On the short arm of the mouse Y chromosome the position of the genetically defined spermatogenesis function, Spy, is close to that of

the molecularly isolated Y genes: Zfy-1/2, A1S9-Y and Sby, and the position of the genetically defined spermatogenesis function, Smy is, like the Y gene, Y353/B, on the long Y arm. Thus on the mouse Y chromosome it may be only a matter of time until a correlation between cloned Y gene and the Spy or Smy function is proved (for review see: Bourgoyne 1991). On the human Y chromosome the position of the cytogenetically defined spermatogenesis function, AZF, in Yq11 is not related to the positions of the molecularly isolated Y genes, ZFY and TSPY, which are both mapped in different regions on the short arm of the Y chromosome. Thus a correlation of the AZF function to the expression pattern of the ZFY and/or TSPY gene is not expected.

This review on Y chromosome function in spermatogenesis is focussed primarily on the human Y chromosome and its AZF function, since only Y chromosomal mutations, disrupting or deleting the AZF gene structure, can be unequivocally related to specific distortions in the development of the male germ cell. In addition, it is evident that AZF mutations in the Y chromosome of sterile men with azoospermia may be more frequent than previously thought (Vogt et al. 1991a, Vogt 1992).

For a basic introduction to the human Y chromosome, a physical description of its structure, its underlying DNA structure and gene content is presented first. Based on a general overview of the different Y chromosomal abnormalities known in sterile males, place and time of the function of AZF during spermatogenesis will then be elucidated.

We also will describe what is known about the molecular structure and expression pattern of the ZFY gene and TSPY gene in human testes, but since no mutation of these genes has yet been found in sterile males, and since in the testis many genes are known to be activated *de novo* with no apparent function (Willison and Ashworth 1987), it is premature to discuss whether the transcription of those Y genes is essential, like the AZF function, for the process of spermatogenesis in human males.

An aneuploidy of the X and Y chromosome can also interfere with normal spermatogenesis. Sterile males with a 47,XXY karyotype and the Klinefelter syndrome are the most frequent and best known examples (for review see: Bandmann and Breit 1984). 47,XYY males show a great variability in the expression of their fertility. Their sper-

matogenesis may be “normal”, or arrested at meiosis, or arrested at the spermatogonial stage (Speed et al. 1991). A disruption of the gene structure of AZF is not expected in these men. The potential influence of a wrong dosage effect of AZF in 47,XYY males seems to be variable, if present at all. It should be discussed only when the functional pathway of AZF is better understood. Sterile males with aneuploidy of their sex chromosomes will therefore not be discussed in this review.

2 Physical Characteristics of the Human Y Chromosome

2.1 Chromosome Structure

The human Y chromosome is normally the smallest chromosome in the human male karyotype. It is easily detectable by its prominent fluorescence of the distal part of its long arm following staining with quinacrine (Fig.1). This staining technique is frequently used to count the number of Y chromosomes in human interphase nuclei (Pearson et al. 1970) and spermatozoa (Kelberman et al. 1970). In population studies variation of the length of the Y chromosome stained with quinacrine is often observed (Fig.2; Mikelsaar et al.1973). Deficiencies or duplications of small segments in other chromosomes most often lead to severe phenotypic damages. However, no effect on the phenotype could be demonstrated for these length variations of the Y chromosome. This led to the conclusion that the quinacrine stained Y segment is genetically inactive, keeping its chromatin structure compact i.e. constitutively heterochromatic (Brown 1966). This conclusion was supported by the advent of *in-situ*-hybridization of highly repetitive DNA sequences (“satellite DNA”) to the same Y chromosome region (Evans et al. 1974).

The distal heterochromatin constitutes about one half to two thirds of the long arm in most Y chromosomes. A very small block of centromeric heterochromatin can also be demonstrated in very well stained preparations. This grossly divides the human Y chromosome into three regions: the euchromatic short arm (Yp11), the euchromatic proximal region of the long arm (Yq11) and its heterochromatic distal region (Yq12). The euchromatic Y regions are commonly considered



Fig. 1. Human chromosomes at metaphase, stained with quinacrine. The picture was kindly provided by Dr. H-D Hager (Inst. Humangenetics & Anthropology, Heidelberg)

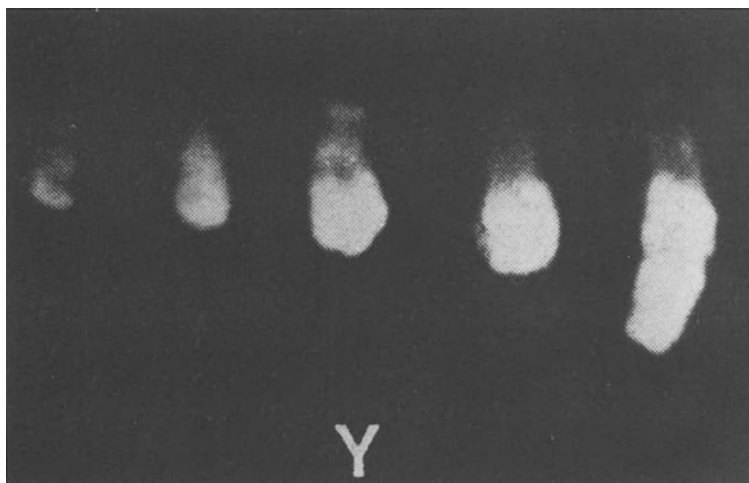


Fig. 2. In human populations the quinacrine stained block of the human Y chromosome in its long arm has a large length variation

invariable in size (Bobrow et al. 1971). This may be true for the short Y-arm but still is in question for the euchromatic part of the long Y-arm (Yq11; Soudek et al. 1973). Several studies have demonstrated that the distal heterochromatic band Yq12 is not homogeneous, but that it consists of a complex succession of distinct subbands. Thus a narrow gap can often be observed in the fluorescent heterochromatin block (Bühler et al. 1971; Sperling and Lackmann 1971). In the less condensed Y chromosome from C-banded prometaphases, up to five small bands could be distinguished (Jalal et al. 1974). A study of the G-band patterns in the Y chromosome from the late prophase stage to midmetaphase revealed that the distal heterochromatin in the Y long arm exhibits the most extended variability in length of any segment in the human karyotype and can be subdivided into up to 12 subbands in late prophase (Fig.3; Yunis et al. 1979). Today we know that this Y chromosome region is composed mainly of two highly repetitive DNA sequence families, DYZ1 and DYZ2, containing about 5000 and 2000 sequence copies, respectively (Smith et al. 1987).

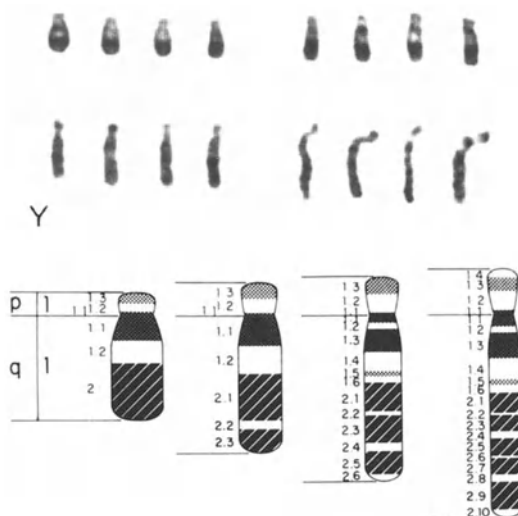


Fig. 3. Illustration and schematic representation of the human Y chromosome at various stages of condensation. *Top:* G-band pattern at mid-metaphase, early metaphase, prometaphase, and late prophase. *Bottom:* Same G-band patterns, schematically drawn and numbered (Yunis et al 1979)

By restriction with the endonuclease TaqI, the proximal part of the Y heterochromatin can be differentiated from the distal part, indicating a different chromosome structure in proximal Yq12 (Fernandez-Piqueras et al. 1991). Since the same chromosome region stains differentially with quinacrine plus Giemsa (FPG) after incorporation of 5-bromodeoxyuridine (Limon et al. 1979), it is obvious that the heterochromatic chromosome block of the human Y is not uniform, especially in its proximal region. Recent results of *in situ* hybridization with DNA probes of the distal part of the X chromosome reveal that at the distal end of Yq12 a DNA block homologous to the X chromosome is present (Pedicini et al. 1991). This homology can explain the association observed between the distal tips of Xq and Yq in about half of the early pachytene spermatocyte nuclei observed by Chandley et al. (1984) and may be important for the “chromosomal pairing” function

of the Y chromosome during the pachytene stage (see: Summary and outlook).

By replication banding with 5-bromodeoxyuridine (BrdU-banding), the human Y chromosome can be divided into four early replicating chromatin domains, the position of which corresponds quite well to its light G bands: Yp11.32, Yp11.2, Yq11.21, Yq11.23 (Schempp 1985). There is a nonvariable replication sequence found in all individuals examined: the distal segment Yp11.32 is the first to replicate, followed by the two bands Yq11.21, Yq11.23 in the proximal euchromatic part of the long Y arm. After the replication of the latest euchromatic band Yp11.2 is nearly completed, the heterochromatin block Yq12 starts to replicate at four different initiation points, indicating a distinct intraindividual variability (Schempp 1985).

Since the replication sequence in the short Y arm coincides to with distal part of the short arm of the X chromosome, the pairing of both sex chromosomes in this region seems to be regulated by its replication time.

2.2 DNA Structure

The human Y chromosome contains 50000kb of DNA, 60% of which are included in its heterochromatic region, Yq12. By pulse field gel electrophoresis the physical extension of the short Y arm is estimated to be about 13000kb of DNA (Müller and Lalande 1990). Hence, the euchromatic part of the long Y arm (Yq11) should not exceed 7000kb.

At present more than 200 cloned DNA sequences have been mapped on the Y chromosome (for review see the reports of the Human Gene Mapping conference (HGM): Weissenbach and Goodfellow 1990). According to the HGM-nomenclature, every human DNA probe, cloned and mapped on the Y chromosome, is given an official "DYSn" symbol which defines it as a specific Y-DNA-locus. The position of most cloned Y-DNA probes on the Y chromosome is mapped along a so-called Y-interval map (Vergnaud et al. 1986).

The Vergnaud interval map divides the Y chromosome into 7 intervals. Their extension is not physically determined but arbitrarily fixed by seven different hybridization patterns of a set of 11 Y-DNA sequences to the genomic DNA of patients with different deletions of

their Y chromosome. The short Y arm is divided into 4 intervals, distal to proximal. The distal tip of the short Y arm is highly homologous to the distal tip of the short X arm. It is therefore called "pseudautosomal" (Bourgoyne 1982). This region is excluded from the Y interval map. The euchromatic part of the long Y arm is divided into interval 5 and 6, proximal to distal. The heterochromatic part is defined as interval 7 (Vergnaud et al. 1986).

Unfortunately, the authors do not establish how their interval map, determined rather arbitrarily by the actual breakpoints in the Y chromosomes of their patients, relates to the more commonly used, cytogenetically defined, G-band pattern of the Y chromosome. Consequently, other Y interval maps published by other research groups (e.g. Ferguson-Smith et al. 1987; Oosthuizen et al. 1990) cannot be related to the Vergnaud map, since they all are based on other patients with other Y chromosomal breakpoints. Only Oosthuizen has tried to relate the position of his probes to the G-band pattern of the Y chromosome, but unfortunately limits it to his own cloned Y-DNA sequences, and does not incorporate the Y-DNA sequences of the Vergnaud and Ferguson-Smith map commonly used in diagnostic laboratories analyzing Y chromosomal breakpoints.

We have done this for the euchromatic part of the long Y arm and were able to define Y interval 6 as the Y chromosome region from the middle of the dark G-band Yq11.22 to the end of the light G-band Yq11.23 (Vogt et al. 1991a). Y interval 5 is then defined from Yq11.21 to the middle part of Yq11.22.

As a first step towards the molecular isolation of Y genes, every Y interval supposed to include a gene has now been subdivided into smaller segments called subintervals. For the long Y arm three subinterval-maps were published with the aim of enclosing the position of the AZF gene in Y interval 6 (Ferguson-Smith et al. 1987; Bardoni et al. 1991; Donlon and Müller 1991). Once again, unfortunately the subinterval-borders of the different maps cannot be related to each other since different probe sets are used. We recently have tried to overcome these shortcomings, at least for a detailed subinterval map of Y interval 6 (Ma et al. 1992). This map divides Y interval 6 into 14 subintervals (I-XIV) using 30 Y-DNA probes commonly used in different laboratories and now located for the first time in an order relative to each other between Y interval 6 (Table 1). The

physical extension of the various subintervals can now be determined by pulse-field-gel electrophoresis (PFGE).

It is intriguing that a lot of Y-DNA sequences are distributed in more than one Y interval (Vergnaud et al. 1986; Disteche et al. 1986b; Müller et al. 1989), often located between Y interval 3 and interval 6. This suggests duplication events of certain DNA blocks during the evolutionary history of the human Y chromosome. The presence of a family of DNA sequences in the proximal part of the pseudoautosomal region with homology to the euchromatic part of the long Y arm (Yq11; Fisher et al. 1990b) indicates that not only simple duplication events, but also complex rearrangements of yet unknown mechanisms must have created the present state of the DNA structure of the human Y chromosome. This is also documented by the sequence homology of various Y-DNA sequences to different parts of the X chromosome and different autosomes (for review see: Weissenbach et al. 1989). Most prominent is the relationship of a probably large block of Y-DNA of the proximal part of the short Y arm (Y interval 4) to the proximal part of the long X arm (Page et al. 1984) and the relation of a similar block of Y-DNA from the long Y arm (Yq11) to the distal part of the X short arm, outside the pseudoautosomal region in Xp22.3-22.1 (Ballabio et al. 1989; Knowlton et al. 1989).

2.3 Gene Content

Genetically, the most distal part of the short arms of both sex chromosomes behave as if they were autosomal, with no sex-linked inheritance pattern of the most distal loci (Rouyer et al. 1986). This chromosome region is therefore defined as "pseudoautosomal". Its physical extension is less than 3000kb (Rappold and Lehrach 1988).

Three genes have been mapped on this part of the Y chromosome: MIC2Y, a gene coding for a protein on the cell surface of various cells (Goodfellow et al. 1986); CSF2R, a gene encoding the receptor for the granulocyte-macrophage colony stimulating factor (Gough et al. 1990) and a gene controlling stature (Henke et al. 1991).

SRY, a sex-determining gene of the Y chromosome has been mapped immediately adjacent to the boundary of the X-Y homologous

Table 1. Relative order of 37 restriction fragments in 14 different subintervals (I-XIV) of Y interval 6

	M	8	J	B	W	8	L	L	F	8	8	8	F	G	G	G	
	1	7	7	O	I	S	6	G	1	R	6	D	6	M	7	D	F
	8	3	S	W	T	M	3	L	0	A	3	3	0	A	4	2	2
	0	2	F	A	R	1	8	2	7	B	7	M	8	N	7	A	W
	0		B	L	A	8	2	1	8	O	1	3	4				
	4					4	9	7		2		0	0			3	9
Probes	4							2									
50f2/b	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
50f2/A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
52d/B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
52d/C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
50f2/D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
pJA4402	-	+	+	+	+	+	+	+	N	+	+	+	+	+	+	N	-
12f3	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
pY6HP35	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
118e	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Y-253	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Y-198	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Y-221	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-
Y-216a/A	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
Y-294	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
pY6BS64	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
pY6BS65B	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
Y-202	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
pY6PHc54	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
pY6D14-1	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
pY6Ba34	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
Fr25-II/B	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
pY6BS65C	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
LLY22g/A	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-
50f2/E	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
52d/A	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
Y-367/A	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
GMGXY10	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-
GMGY18	N	-	-	-	-	N	-	N	-	-	N	N	N	+	+	N	N
Y-261a/B	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
FR25-II/A	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
pY6BS65D	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
LLY22g/B	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
p69/6	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
pY6HP52	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
50f2/C	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
p116/21	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+
pY6BS65E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Y-367-B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Fr15-II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
RBF8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
49f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
GMGY5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
GMGY1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
poxY1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
phY2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

-: no hybridization; +: correct fragment present; N: not tested. Patients and DNA probes are described in detail by Ma et al. (1992)

region, (Sinclair et al. 1990). Distal to the Y-specific SRY gene, three Y genes were isolated, surprisingly, again with homology to the X chromosome. Two of them are in the direct neighbourhood of the SRY gene: the ZFY gene with high homology to the ZFX gene on the short X arm (Schneider-Gädick et al. 1989) and a gene coding for an isoform of the ribosomal protein "S4", RPS4Y. The RPS4Y gene has a high homology to the RPS4X gene on the long X arm (Fisher et al. 1990a). The third gene is called amelogenin gene (AMGL). It codes for a protein in the enamel of the teeth and is located near the centromere of the Y chromosome and in the distal part of the short arm of the X chromosome (Nakahori et al. 1991). Whether the Y copy of the amelogenin gene (AMGL) is indeed actively expressed has not yet been proven.

In the proximal part of the short Y arm another gene, called TSPY, has been mapped (Arnmann et al. 1991). It is located in between or adjacent to the tandem repetitive sequence blocks of the DNA locus, DYZ5 (Tyler-Smith et al. 1988). This gene is expressed during spermatogenesis (see 3.2). The accumulation of molecularly isolated Y genes from the distal part of the short Y arm merely reflects the actual summary of the hunting game towards the molecular isolation of the sex-determining function of the human Y chromosome and reveals nothing about the total number of Y genes to be expected within the total Y chromosome.

In the euchromatic part of the long Y arm several Y genes have been mapped, genetically or cytogenetically, but have not yet been isolated molecularly. Beside the AZF gene, defining the Y spermatogenesis function of Tiepolo and Zuffardi (1976), the gene for a male specific cell-surface protein, commonly known as H-Y antigen (Billingham and Hings 1981) has also been mapped on Y interval 6 (Cantrell et al. 1989). The H-Y antigen has been discussed for a long time as the primary sex determination signal of the Y chromosome (Wachtel 1983). Since, however, the genetic loci for the H-Y antigen and testis determination have been separated on the human Y chromosome (Simpson et al. 1987) it is obvious that this protein must have another function, specifically expressed in the male. It is intriguing that the H-Y locus has been mapped in the neighbourhood of a male Y-fertility gene on the human and mouse Y chromosome. In the human it is

the AZF gene and in mouse it is the Spy gene (Bourgoyne 1991). Although the H-Y antigen is present in all cells except in prespermatogonia and erythrocytes, it is only actively secreted from Sertoli cells (Zenzes et al. 1978). Since receptor proteins binding to H-Y antigen are present only in the gonads, Ohno et al. (1979) have suggested a morphogenetic function of this protein during development of the testes tubules. Three different experimental test systems screening for the presence of the H-Y antigen protein are used today and it is an open question whether every system indeed detects the same epitope or protein (for discussion see: Wiberg, 1987). It is, therefore, too early to consider the expression of the H-Y antigen in mammalian males as a Y chromosomal spermatogenesis function.

A discrimination between Y interval 5 and 6 has not been yet published for the position of two other Y genes mapped on the euchromatic part of the long Y arm (Yq11). A gene coding for a growth factor (GCY) has been postulated by Alvesalo and de la Chapelle (1981) and a gene predisposing gonadoblastomas in dysgenetic gonads (GBY) has been postulated by Page (1987).

The GBY gene is discussed as an oncogene provoking tumors in dysgenetic gonads, which frequently occur when a mosaic karyotype such as 45,X,0/ 46,X,Y is present in the gonad cells of a male or female (Scully 1970). The author believes that in normal testes GBY may function prior to spermatogenesis (Page 1987).

A gene in Yq11, regulating the binding affinity of dihydrotestosterone to the androgen receptor has been postulated by Tyrkus et al. (1979). As spermatogenesis is dependent on the activity of testosterone, it may well be possible that the AZF gene is related to this gene or even identical with it. Since neither any gene product of the AZF factor nor the time of its genetic activity (most probably at the onset of the spermatogonial division cycles (see: Summary and outlook)) are known, the question arises whether the Tyrkus-gene, the GBY gene and H-Y antigen gene are actually three distinct genetic loci or only reflect pleiotropic manifestations of the AZF gene. Extensive molecular deletion mapping experiments will be necessary to differentiate the molecular position of these genes in Yq11, and to analyze their function separately.

In distinction to the short Y arm, genes in a "non-functional" state are also present in the euchromatic part of the long Y arm. They are

commonly defined as "pseudogenes". In the G-band Yq11.22 a pseudogene has been mapped, related to an autosomal gene transcribed in human testis (Leroy et al. 1987). The Y-pseudogene has been found in the Y-DNA sequence "12f" (HGM-symbol: DYS11). The location of the DYS11 locus in Yq11.22 marks the distal border line of Y interval 5 to interval 6 (Vergnaud et al. 1986).

A pseudogene related to the argininosuccinate synthetase gene (ASSP6; Daiger et al 1982) is located proximal to the position of the DYS11 locus in Y interval 5. This enzyme of the urea cycle is coded and actively expressed on the long arm of chromosome 9 (9q34-qter). In the same region a pseudogene related to a cytoskeletal gamma-actin gene (ACTGP2; Gomez-Pedrozo et al. 1987) and a pseudogene of the steroid sulfatase gene (STSY), located in the distal part of the short X arm has been mapped (Yen et al. 1988; Bardoni et al. 1991). The genomic structure of the STS-Y pseudogene spans over 100kb. Since sequenced exons of STS-Y show a series of base substitutions, small deletions and insertions, the authors define the Y gene as a non-functional pseudogene (Yen et al. 1988).

Distal to the position of the DYS11 locus in Y interval 6 a pseudogene has been mapped in the Y-specific DNA fragment "49f", which is a 2.8kb EcoRI fragment of the DYS1 DNA locus (Bishop et al. 1984). This pseudogene has been identified by crosshybridizing to a 4kb long messenger RNA transcribed specifically in human testes and coded from a gene located on chromosome 3 (Seboun et al. 1986). It is intriguing that the "49f" sequence is repetitive in Y interval 6. The copy number of the 49f-sequence family has not yet been determined, but may not be higher than 20-30, estimated from its genomic TaqI restriction pattern (Ngo et al. 1986). The 49f-sequence is embedded in a genomic Y region which is highly polymorphic (Lucotte and Ngo 1985). The polymorphic pattern of the genomic Y fragments is detected with TaqI and PvuII (Spurdle et al. 1989) but not with BamHI, HindIII, PstI, XbaI (Ngo et al. 1986). The number of the sequence copies is also variable. Thus the molecular structure of the 49f-Y-region, which may extend over at least 100kb of DNA, seems to be very dynamic in the human population. Highly polymorphic regions on the human Y chromosome are very rare (Malaspina et al.1990) and are concentrated mainly in the distal tip of the short Y arm, the pseudoau-

tosomal region. This raises the question whether the 49f-sequence region in Y interval 6 is not a similar hot spot for local recombination events with the X chromosome, as is the pseudoautosomal region of both sex chromosomes. The 49f sequence is deleted in a microdeletion of an azoospermic male (see 3.7) and so may be located in the close neighbourhood of the AZF gene.

3 Structural Anomalies of the Human Y Chromosome in Sterile Males

Before the era of chromosome banding it was difficult to detect structural anomalies of the Y chromosome at all. Due to its well-recognized length polymorphism in normal males it even seemed impossible to relate phenotypical abnormalities of the male, causally, to a detected “abnormality” of the Y chromosome. Very often “abnormal” Y chromosomes were found in combination with an 45,X cell line. Since a great variability exists in the phenotypes of these X,0/XY cases including sex, the question was raised whether phenotype variability is at all related to an observed anomaly of the Y chromosome and whether X,0 complement does not contribute primarily to the phenotypes observed (for review see: Pfeiffer et al. 1968). Different types of gonadal dysgenesis, including the development of intersexual gonads are observed in human males and females with a X0/XY mosaic karyotype (Sohval 1964). Thus it seems likely that a distortion in spermatogenesis is a secondary effect of the distortion of the development of the gonads. This is supported if the XY cells in the dysgenetic gonad cells develop a gonadoblastoma (Scully 1970). In mice with a X0/XY karyotype, the fate of X0 germ cells in the testes is short and does not survive the spermatogonial stage. The XY germ cells can develop to mature spermatozoa (Bourgoyne 1991).

Only after it became possible to mark the heterochromatic block in the long arm of the human Y chromosome by its brilliant quinacrin fluorescence was it recognized that the “normal looking” Y chromosome in sterile males with a mosaic X0/XY karyotype completely lack this heterochromatic block (Caspersson et al. 1971). Since the size of the Y chromosome was normal, they were defined as “Ynf” (non-fluores-

cent) chromosomes. Later, the "Ynf" chromosomes were recognized as dicentric isochromosomes with a breakpoint in Yq11 and an inactivation of the second centromere (Magenis and Donlon 1982).

At the same time, large deletions in the long Y-arm, including the distal part of the euchromatic Yq11 region, had been already found in various azoospermic males (Neu et al. 1973; Tiepolo and Zuffardi 1976; Yunis et al. 1977). This monocentric Yq anomalies had led Tiepolo and Zuffardi to postulate the AZF locus in distal Yq11. The sterility of males with a dicentric "Ynf" chromosome and a breakpoint in Yq11 could now be explained by an interruption of the functional gene structure of AZF.

Six different classes of abnormal karyotypes with a deletion of parts of Yq11, including the region of AZF, are known today in sterile males. In the following sections they will be illustrated and discussed separately.

3.1 Monocentric Deleted Yq-Chromosomes

The first male with a 46,X,Yq- karyotype and azoospermia was described by Neu et al. (1973). However, since fertile males with a 46,X,Yq- karyotype were also known, the authors first hesitated to correlate the Yq11-abnormality observed with the sterile phenotype. Only when Tiepolo and Zuffardi (1976) also described a partial deletion of the distal portion of the euchromatic Yq11 region in six azoospermic males, did it become evident that this region of the Y chromosome must carry genetic information important for the cell differentiation process of the male germ cell. They define this Y-information as a azoospermic factor (AZF). All patients had normal external genitalia but hypoplastic testicles. In a biopsy of testis tissue, it was revealed that one patient had neither germ cells nor Sertoli cells, and one patient had Sertoli cells but no germ cells. A severe example of the histopathologic appearance of the testis tissue is presented in Fig. 4. The AZF function should therefore be necessary at a very early stage during spermatogenesis. This view has been confirmed in a series of other sterile patients with a 46,X,Yq- karyotype (for review see: Hartung et al 1988; Beverstock et al. 1989). If the breakpoint in Yq11

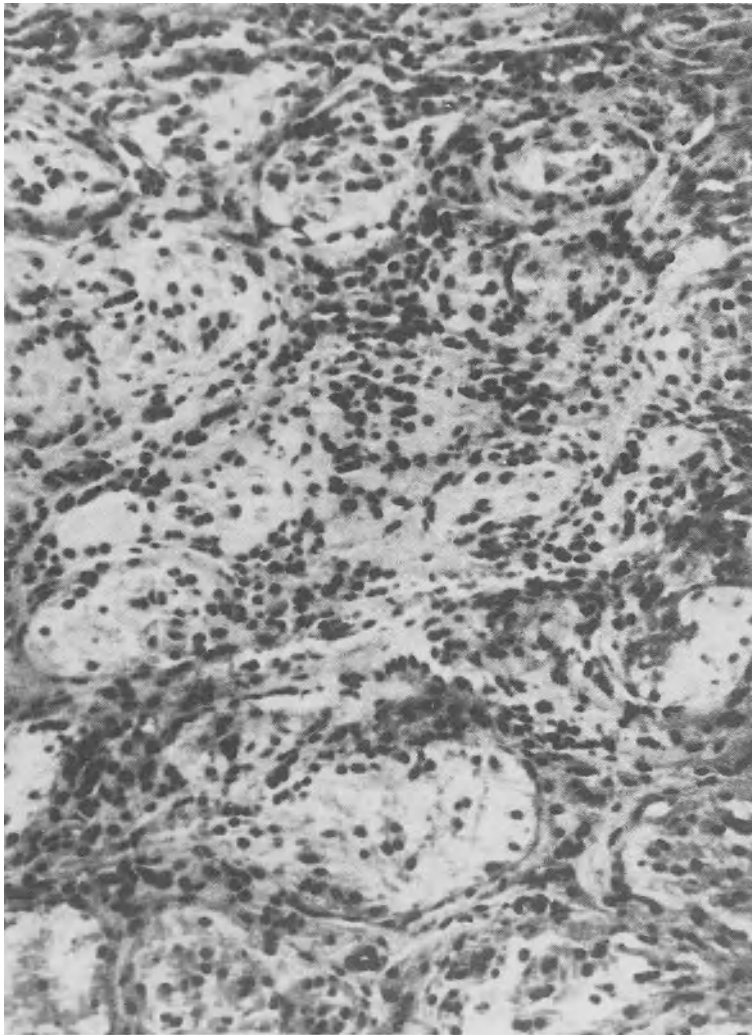


Fig. 4. Histological appearance of the testis tissue of a sterile male with a monocentric Yq abnormality, studied by Yunis et al. (1977)

is proximal, indicating a very large deletion of the long Y arm, a short stature of the azoospermic male has been consistently observed (Yunis et al. 1977; Kosztolanyi and Trixler 1983). The Y chromosomal factor controlling growth (GCH; (Alvesalo and de la Chapelle 1981)) has therefore been postulated proximal to the AZF factor.

3.2 Dicentric Yp Isochromosomes

A karyotype with a dicentric Yp isochromosome in a sterile male is nearly always associated with a 45,X cell line, the proportion of which is highly variable and often different in the patient's fibroblasts and lymphocytes (for review see: Daniel 1985). If the breakpoint and fusion point of the two associated Y chromosomes lie in the distal Yq11 region and the second centromere is inactivated, the Y chromosome has a normal size but cannot be stained with quinacrine (Caspersson et al. 1971). They were therefore called "nonfluorescent" Y chromosomes (Ynf). It is interesting to note that the mosaic karyotype sometimes includes more than two cell lines with "different looking" Y chromosomes, suggesting that the dicentric "Ynf" chromosome is unstable *in vivo* or at least *in vitro* (Chandley et al. 1986; Fryns et al. 1978). From molecular analysis of the breakpoint in Yq11, it is obvious that the function of AZF must be distorted (see e.g.: Ferguson-Smith et al. 1987). The external genitalia of Ynf males are often ambiguous but can also be normal, probably depending on the variable number of X,0 cells present during early development of the gonads (Daniel 1985). The testes of such men are small and soft and often slight gynecomastia and a small penis are observed. Unfortunately, in most cases a biopsy of testis tissue has not been performed. In the few cases available, an early block in spermatogenesis has been observed such as is seen in sterile males with monocentric Yq-chromosomes (Chandley et al. 1986; Giraud et al. 1977).

It is interesting to note that sterile male patients with a Ynf chromosome do not develop gonadoblastoma in their dysgenetic gonad cells, in distinction to men with a normal fluorescent Y chromosome and a mosaic X,0 cell line in their karyotype (Lukusa et al. 1986). It is intriguing that these males are positive for the H-Y antigen. Since we know

that a gene with oncogenic activity (GBY) and the gene for the expression of the H-Y antigen are both mapped on the distal part of Yq11 as is AZF (see above), sterile males with a "Ynf" chromosome developing a gonadoblastoma may be very helpful for mapping the GBY locus next to the AZF locus and probably the H-Y antigen locus.

3.3 Ring-Y Chromosomes

Sterile males with a ring-Y chromosome are phenotypically often quite normal, but azoospermic (for review see: Daniel 1985). This is surprising since in most cases the karyotype of those males is a mosaic containing the 45,X cell line in lymphocyte cultures. Moreover, sterile males with a nonmosaic ring-Y karyotype have been described (Wilson et al. 1976). Since *in vitro* cultures of ring chromosomes are known to generate 45 chromosomes minus a ring because of sister chromatid exchanges induced by the culture media, the question arises whether a mosaic karyotype with a ring-Y chromosome exist *in vivo* at all (Daniel 1985; see also: Steinbach et al. 1979).

To date no one has really seen a ring form in the small dotlike Y chromosomes of a metaphase. The ring constitution of a Y chromosome has only been described in a meiotic chromosome study (Chandley and Edmond 1971). A ring form suggests a fusion between a breakpoint in the short and long Y arm. The molecular analysis of a ring-Y chromosome of a sterile male with short stature indicates that this may indeed be true (Pohlschmidt et al. 1991). Beside a deletion of most of the euchromatic part of the long Y arm, this patient showed also a deletion of the distal part of the short Y arm. An unequivocal test for the formation of a Y ring is, however, only possible if the junction fragment between the short and long Y arm can be analyzed.

The analysis of testis tissue of sterile males with a ring-Y chromosome indicates an early arrest of spermatogenesis with the complete absence of germ cells, as is the case in males with a Ynf or Yq- chromosome (Daniel 1985). In one case, however, a block in spermatogenesis occurs later, during the premeiotic stage of spermatocyte maturation (Chandley and Edmond 1971). It is easy to imagine that generally a ring in the Y chromosome will interfere with its pairing with the X

chromosome during the meiotic prophase. A second stage of the function of AZF, in the case of Chandley and Edmond, cannot however be ruled out. It would therefore be interesting to map the breakpoint in Yq11 of this patient in comparison to another patient with a ring-Y and the complete absence of germ cells.

3.4 Y/Y Translocation Chromosomes

Translocations between two Y chromosomes are difficult to examine and can be only differentiated from Yq-isochromosomes by different lengths of both Y arms. The seven cases described are azoospermic or oligospermic (Wahlström 1985). Their testicles are small and soft. An analysis of the testis histology has been performed only in one case. A maturation arrest at the primary spermatocyte stage suggests that the Y/Y translocation chromosome in this male interfered with a normal pairing complex of the Y to the X chromosome during the pachytene stage. Since no studies have been done to determine whether one Y breakpoint is located in Yq the question remains whether the gene structure of AZF may be distorted in one of the rearranged Y chromosomes and whether perhaps a double dose of AZF is present in those males. Often their karyotype is a mosaic with a 45,X,0 cell line.

3.5 Translocations of the Y Chromosome to the X Chromosome

Cytogenetically not visible, X;Y translocations are frequently observed in sterile males with 46,XX karyotypes (Petit et al. 1987). This type of Y rearrangement is actually an abnormal interchange between parts of the short X and Y arm. In their general masculine appearance XX males resemble sterile males with a 47,XXY karyotype and Klinefelter syndrome (de la Chapelle 1981). Smaller stature, smaller tooth size and the general absence of mild mental retardation can, however, differentiate them from the latter. Like the Klinefelter patients, however, XX males have small testes, weak or abnormal secondary sexual characteristics and normal to low androgen levels. Only Sertoli cells and no

germ cells are found in their testes. Since the long Y arm is totally deleted, AZF is certainly also deleted.

Sterile males with cytogenetically visible X;Y translocation are rare. If the breakpoint in the Y chromosome is in Yq11, the distal part of the long Y arm is translocated to the X chromosome and the karyotype additionally includes a normal Y chromosome: 46,Y,t(X;Y). If the breakpoint in the Y chromosome is in Yp11, the distal part of the short Y arm is translocated to the X chromosome and the karyotype is: 46,X,t(X;Y). Phenotypically, sterile males with the 46,X,t(X;Y) karyotype resemble those with the 46,XX karyotype mentioned above. In three cases the breakpoint of the X chromosome is described as being in Xp22.3 (Bernstein 1985). All had short stature, small testes, and, in one case, severe mental retardation. In one patient with azoospermia the testicular histology showed Leydig cell hypertrophy and nodular hyperplasia. Seminiferous tubules were reduced in number and size, showing a marked tubular hyalinization, involuting Sertoli cells and the absence of germ cells. These features were comparable to testicular findings in sterile males with Klinefelter syndrome. Since the total long arm of the Y chromosome is expected to be deleted in such sterile males, the abnormalities observed in their testis tissue again suggested the involvement of the AZF locus during early germ cell development.

In comparison to the testicular histology described for sterile males with a Yq-, Ynf or Y-ring chromosome, however, (see 3.1-3) the distortion of the early testis development seems to be more severe. One may therefore assume that the extent of genetic activity of the X chromosome, especially of genes in the neighbourhood of the Xp22.3 breakpoint, also influences early testis development. The inactivation of the X chromosome is nonrandom in those males, suggesting a delicate balance in their X inactivation process, which is known to be essential during the early development of the male germ cell to the stage of spermatogonia. It is easy to imagine that the "normal" process of inactivating X chromosomal genes at this stage may be hampered by the breakpoint in Xp and fusion to the short Y arm in those patients. Another possibility, supporting the same view, may be that the 46,X,t(X;Y) karyotype is only present in fibroblasts or lymphocytes and is derived from a 47,XXY karyotype in the germ line. Since a mosaic karyotype between 46,X,t(X;Y) and 47,XXY has been described

in one case, it indeed seems possible that at least in some cases the 46,X,t(X;Y) cell line can be created via a nondisjunction, translocation process of the second X chromosome to the Y chromosome (Bernstein 1985).

All sterile males with a 46,Y,t(X;Y) karyotype had inherited their translocation chromosome from the mother (Bernstein 1985). The breakpoint in the short X arm seems to be in the Xp22 region. Recent molecular analysis of different X DNA probes located in the Xp22 region and Yq11 region revealed that the X-Y homologous DNA blocks in those chromosome regions may be involved in the etiology of these Y;X translocation events (Ballabio et al. 1989).

Although additionally a complete normal Y chromosome is present, anomalies in the sexual development of 46,Y,t(Y;X)-males are described. Phenotypically, these males had a small penis, small testes and a hypoplastic scrotum. In two adult brothers with azoospermia the histology of their testes revealed reduced spermatocytes reaching diakinesis and an absence of secondary spermatocytes and sperm (Yamada et al. 1982). In a similar case, it has been shown recently that the spermatogenic failure in those males is, most likely, not due to a dysfunction of the AZF locus, broken by the Yq11 breakpoint in the X-Y translocation chromosome, but to a distortion in the pairing structure between the tips of the short arms of both sex chromosomes (Gabriel-Robez et al. 1990).

3.6 Autosomal Translocations of the Y Chromosome

In most familial cases with a balanced Y;A translocation the distal heterochromatic part of the Y chromosome is translocated to the short arm of an acrocentric chromosome (Smith et al. 1979). This chromosomal preference may be explained by the observed nonrandom association of those chromosomes to the XY complex in the sex vesicle during the early pachytene stage (Stahl et al. 1984). If the breakpoint in the Y chromosome is in the heterochromatic Yq12 region, the male is fertile with no anomalous phenotypical features. The Y heterochromatin, attached to the autosome, can be easily detected by fluorescent staining with quinacrine. The free Yq12-chromosome is nonfluores-

cent, but shorter than the "Ynf" chromosomes, described earlier (see 3.2).

The same Y;A translocation also has been observed in sterile males. This indicates that the breakpoint in Yq12 can be variable and able to shift to the distal part of the euchromatic Yq11 region, disrupting the functional gene structure of the AZF locus. Whether this shift is a secondary following a primary breakage event in Yq12 and occurring during the formation of a new Yq telomere, or whether breakage in the Y long arm can simply take place at different sites is difficult to distinguish. The first possibility may be indicated by a family with fertile and sterile male carriers with the same cytogenetic Y;A translocation (Reitalu 1973). Sterile males with this type of a Y;A translocation chromosome can have normal external genitalia or hypogonadism. Their testicular histology may be variable. Mostly an arrest of spermatogenesis is observed before meiosis (Fryns et al. 1985).

The second type of a Y;A translocation karyotype found in sterile males is an unbalanced translocation of the Yq12- chromosome part to an autosome, losing the acentric heterochromatic Yq12 chromosome part. These males are rare and were described earlier with a 45,X karyotype, including a rare mosaic with a 46,XY cell line (de la Chapelle et al. 1986). Molecular analysis of the presence of Y intervals in those males had revealed, however, that probably in most cases a Y chromosome is present, translocated to an autosome. The breakpoint is in the Yq11 region (Disteche et al. 1986a; Gal et al. 1987; Andersson et al. 1988a). Phenotypically, their testis can be normal but also hypoplastic or small or hypoplastic and hidden in the inguinal canal. Testis histology reveals an early disruption of germ cell development, only showing Sertoli cells and immature seminiferous tubules.

The third type of a Y;A translocation chromosome has been described in an azoospermic male with normal external genitalia and secondary sex characteristics but only Sertoli cells in his normally developed seminiferous tubules (Andersson et al. 1988b). The breakpoint in the Y chromosome was not in Yq11 but in distal Yp11 between the pseudoautosomal region and Y interval 4. Since the distal part of the long arm of the autosome (chromosome 4) is shown to be translocated to the tip of Yp, the translocation seems to be balanced. At the moment it is not known whether this chromosome rearrangement distorts the

Table 2. Most common clinical features of sterile males with an anomaly in Yq11

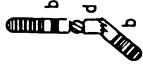
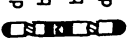
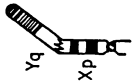
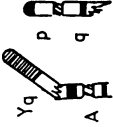
Karyotype	Yq11-Anomaly	Genitalia	Testicular Histology	Diverse Somatic Anomalies
46,X,Yq-		hypoplastic testicles	a: no germ cells and no Sertoli cells b: no germ cells, only Sertoli cells c: spermatogonia and rare spermatocytes	short stature gynectomastia mental retardation
46,XYnf 45,X0		ambiguous genitalia possible hypoplastic and soft testicles hypospadias cryptorchidism small penis	a: no germ cells, only Sertoli cells b: spermatids and rare spermatozoa Leydig cells hyperplasia atrophic tubules	slight gynectomastia short stature
46,X,r(Y) (45,X0)		hypoplastic testicles hypospadias cryptorchidism	a: no germ cells, only Sertoli cells b: arrest at premiotic spermatocyte st.	short stature
46,X,t (Yp11;YQ11) (45,X0)		hypoplastic and soft testicles hypospadias	a: arrest at premiotic spermatocyte st. b: spermatids and rare spermatozoa	short stature

Table 2. (cont.)

Karyotype	Yq11-Anomaly	Genitalia	Testicular Histology	Diverse Somatic Anomalies
46, Y,t (Xp22;Yq11)		hypoplastic testicles small penis	arrest at premeiotic spermatocyte st.	mental retardation craniofacial dysmorphism digital anomalies
46, X,Yq-,t (Yq11-qter;A)		normal or hypogonadism	arrest at premeiotic spermatocyte st.	
46, X,t (Yq11-pter;A)		hypoplastic or normal soft testicles hypospadias cryptorchidism	immature seminiferous tubules no germ cells, only Sertoli cells	

function of a fertility factor on the short arm of the Y chromosome or in the distal part of the long arm of chromosome 4. Since two Y genes in Yp11, ZFY and TSPY, transcribed during spermatogenesis, have already been isolated molecularly (Page et al. 1987; Arnemann et al. 1991) it should be easy to analyze their possible disruption in this patient. In table 2 the most common clinical features of sterile males with a breakpoint in Yq11, in the different karyotypes discussed, is summarized.

3.7 Interstitial Microdeletions in Yq11

From all Y chromosomal aberrations of sterile males described above, it is obvious that the functional gene structure of AZF in distal Yq11 must be deleted or interrupted in those males. A critical evaluation of the dysfunction of their AZF gene at the molecular level is, however, generally hampered by the fact that the deletions in Yq11, observed cytogenetically, are certainly larger than the extension of the AZF locus and so may also include the deletion or distortion of the other Y genes located in Yq11 and probably involved in spermatogenesis like the Tyrkus-gene, GBY and HY-antigen locus, mentioned above. Another problem is the mosaic with a 45,X cell line, mostly present in sterile males with a "Ynf" chromosome. In those patients it is inherently difficult to discriminate the specific contribution of the Yq11 abnormality from the potential contribution of the X,O cell line in an observed disruption of spermatogenesis.

Therefore, in order to differentiate the genetic effects of the AZF gene, beside the Tyrkus gene, GBY and H-Y antigen locus on spermatogenesis, it is necessary to screen the Y chromosome of azoospermic males, in Yq11, not for large but for small interstitial deletions or even point mutations. Point mutations are difficult to map in a DNA region of about 5 million nucleotides, as long as sequence information of the gene is not available. Small interstitial deletions ("microdeletions") in Yq11 can be, however, easily detected with the aid of molecular genetic screening tools.

The first interstitial microdeletion in Yq11 was described by Johnson et al. (1989) in a sterile male with germ cell aplasia. Unfortunately, this study includes only one DNA probe of Y interval 6, 50f2

(DYS7). In addition, the deletion of this probe in one of their azoospermic patients cannot be correlated to a microdeletion in AZF, since the same sequence copy is shown to be polymorphic and also deleted in a fertile Finnish male (Disteche et al 1986b). Microdeletion observed in Yq11 in sterile patient must therefore always be proved by the presence of this probe in the Y chromosome of the patient's father.

This was first performed in an extensive molecular screening program on the Y chromosome of 19 azoospermic males with a "normal" 46,X,Y karyotype by our research group in Heidelberg, in collaboration with the Dr. A. Chandley's group in Edinburgh (Vogt et al.1991b). For this screen 30 Y-DNA probes of Y interval 6 were used, located in 14 different subintervals (Table 1; Ma et al. 1992). The primary screening probe is the repetitive pY6BS65 probe. This DNA-sequence is spread over 5 subintervals of Y interval 6 (Vogt et al. 1991c). Thus this probe can score five different Y-regions for their potential deletion in an azoospermic male in only one experiment (Fig. 5). The 19 sterile males were selected for their high FSH level occurring with normal LH and testosterone levels. This combination should differentiate them from the class of sterile males with an androgen resistance syndrome characterized by high LH and testosterone levels. Early disruption of spermatogenesis, similar to the cases described in sections 3.1-3.6 should of course be expected from their testicular histology.

According to these rather crude clinical diagnostic criteria, 19 sterile males were selected for our first screening experiment. Using the 30 Y-DNA probes of shown in Table 1 as our screening tool, we were indeed able to detect microdeletion in Y interval 6 in two different males (Vogt et al. 1991b). These deletions were detected using Y-DNA probes of the pY6H sequence family, which has a distinct homology to a Y fertility gene sequence from *Drosophila hydei* (Vogt et al. 1991a).

According to the subinterval map presented by Ma et al. (1992) and its correlation with the cytogenetically defined G-band pattern (Vogt et al. 1992), one microdeletion is mapped on the distal part of Yq11.22, including, most likely, the proximal part of Yq11.23 and the second microdeletion on the distal part of Yq11.23 (Fig.6).

Since the second microdeletion does not overlap the first, we also found molecular evidence for what is probably a second spermatogen-

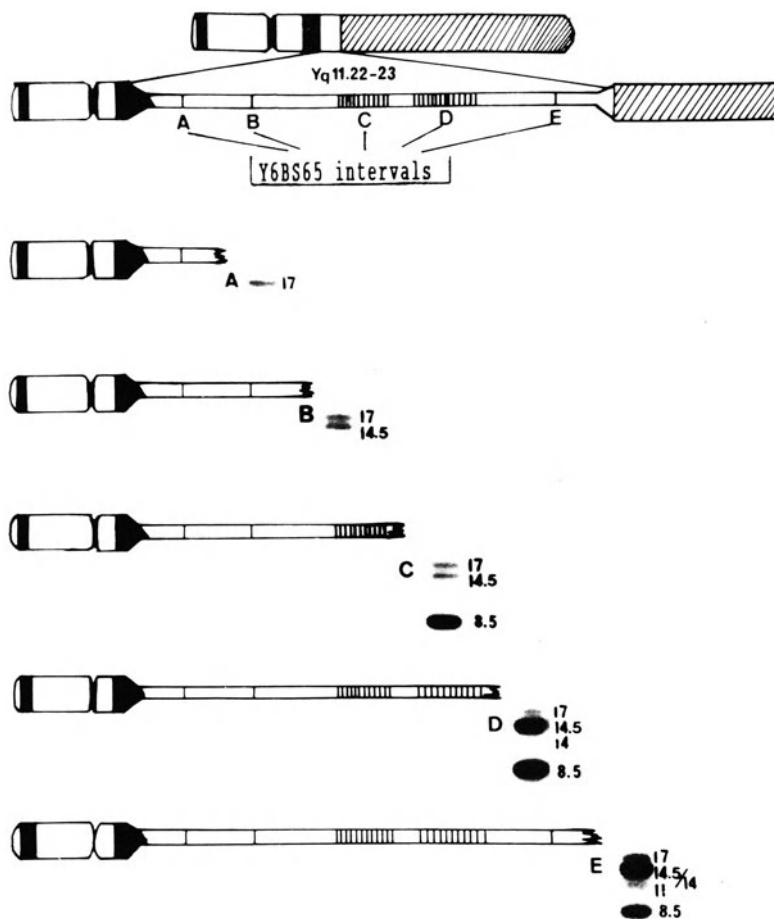


Fig. 5. Depending on the position of the molecular Y-breakpoint in Yq11.22-23, the genomic DNA-blot hybridization with the pY6BS65 probe shows, from proximal to distal, the A, B, C, D, E pattern (from Vogt et al.1991c)

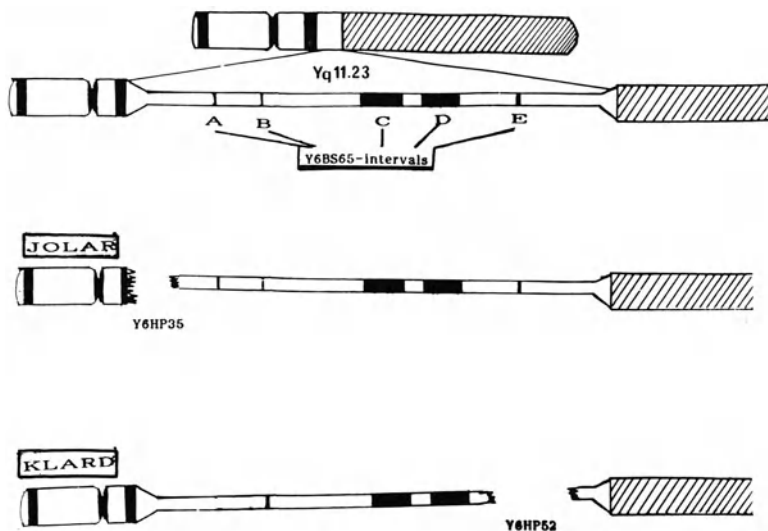


Fig. 6. Schematically drawn position of the interstitial microdeletion in Yq11.22-23 of two azoospermic patients. The microdeletion of JOLAR is detected with the probe Y6HP35 and the microdeletion of KLARD with Y6HP52 (from Vogt et al. 1992)

esis gene in the distal Yq11.23 region. This may be the first indication for a molecular separation of the Tyrkus-gene or H-Y antigen locus or GBY locus from the AZF locus. (In this context it would be interesting to analyze the potential development of a gonadoblastoma in one of these patients or the expression of the HY-antigen).

It is worth noting that two microdeletions have been detected in only 19 sterile azoospermic males. This may have been fortuitous or may indicate that a microdeletion in Yq11.22-23 can be expected in about 10% of sterile males with idiopathic azoospermia. The second possibility would suggest that abnormalities in the euchromatic part, Yq11, of the human Y chromosome, are more frequent than had been imagined earlier (Vogt 1992). In our research group, an extensive screening program is in progress to test this fascinating working hypothesis.

4 Potential Functions of Genes on the Short Arm of the Human Y Chromosome Transcribed During Spermatogenesis

In addition to the genetically and cytogenetically well documented spermatogenesis function “AZF” of the human Y chromosome in Yq11.22-23, a second and third spermatogenesis function of the Y chromosome may be located on the short Y arm. Two genes, ZFY, TSPY, have been isolated molecularly from this Y region, which are known to be transcribed in testis. Since, however, no mutations of these genes have yet been described in sterile males, it is too early to claim a specific function of these genes for spermatogenesis. Moreover, many genes in the testis are known to be activated *de novo* without any apparent function (Willison and Ashworth 1987).

4.1 ZFY Gene

The ZFY gene has been found in subinterval A2 of Y interval 1 (Page et al. 1987), according to the map of Vergnaud et al. (1986). This gene on the Y chromosome first becomes well known as a “candidate” for the TDF locus, the sex determining gene of the Y chromosome (Page et al. 1987). When it was shown that ZFY was also present in 46,X,Y females, it became obvious that this could not be true. The ZFY gene has a very conserved gene structure coding for a protein with 13 so called zinc fingers, known from other proteins as being indicative for a DNA binding domain. In addition, it contains a nuclear localization signal and a 5' terminal acidic region, known also to be present in transcription activation factors. The ZFY protein is therefore discussed as a transcription factor located in the nucleus and stimulating the transcription of downstream genes. There is a highly homologous gene on the short arm of the X chromosome called ZFX. The ZFY gene, like the ZFX gene, is expressed in all human tissues (transcripts ZFY: 5,7 kb; ZFX: 6.7kb; 8kb). However, a highly conserved ZFY transcript of a specific length of 3 kb is only found in adult testis, providing additional evidence for a testis-specific function of the ZFY gene. The ZFX gene expresses a 5 kb transcript in testis (for review see Koopman et

al. 1991). At present there is no indication at what stage of spermatogenesis the ZFY and ZFX gene are transcribed.

There is experimental evidence that the tissue-specific expression of the ZFY and ZFX gene is regulated posttranscriptionally by the use of alternative polyadenylation sites and by the amount of conserved RNA control elements in the untranslated 3' region, regulating the stability of the ZFY transcript (North et al. 1991). The ZFY protein contains 801 amino acids and has a molecular weight of 90.6 kda (Lau and Chan 1989).

Taking into account that the ZFY gene, like many other zinc finger genes, is transcribed in many tissues, a general function of its protein during the developmental pathway of different cells seems to be more likely than a specific function during the development of the germ cell.

4.2 TSPY Gene

The second Y gene in Yp11 transcribed during spermatogenesis has been mapped on Y interval 3 in the proximal part of the human Y chromosome (Arnemmann et al. 1991). It is called "TSPY" for it is expressed in the spermatid nuclei with a RNA of 1.1 kb. Its expression seems to be specific for this tissue and it contains at least two copies, one of which is discussed as an pseudogene, for it could not be found in a poly A⁺ cDNA library. TSPY codes for a membrane protein with 258 amino acids and has a molecular weight around 28 kda. Whether this protein is integrated in any morphological structure of the developing germ cell or the seminiferous tubules is not known.

5 Summary and Outlook

In summary, it is obvious that the human Y chromosome is essential, not only for sex determination but also for spermatogenesis. Most evidence for this genetic Y-function can be collected from mutations in the distal region of the euchromatic part of the long Y arm in sterile males with azoospermia. The corresponding gene locus has therefore been defined as "azoospermia factor" (AZF; Tiepolo and Zuffardi 1976).

A molecular product or functional pathway of AZF during spermatogenesis is not yet known. Most patients with an AZF deletion are detected by their hypergonadotropic hypogonadism. Especially high levels of FSH, in addition to quite normal levels of testosterone and LH, differentiate the AZF-patient group from those with an anomaly of the androgen receptor. Unfortunately, the medical workup of sterile males only rarely includes a testicular biopsy. Since it was established that the absence of germ cells can be diagnosed indirectly by high serum levels of FSH (de Kretser et al. 1974) there is rarely a medical indication for a diagnostic investigation of testis tissue sections.

When testicular histology of sterile patients with a deletion in Yq11 is available (chapter 2.1-2.7), the early disruption of spermatogenesis observed suggests a function of AZF during the development of spermatogonia. This functional picture of AZF is similar to that of the *Spy* gene on the short Y arm of the mouse (Bourgoyne 1991). Experiments for selecting functional AZF gene sequences with the aid of potential *Spy* gene sequences (Mitchel et al. 1991) are therefore in progress (Vogt and Bishop, unpublished results).

In some patients with a deletion in Yq11, spermatogenesis becomes arrested later during the premeiotic stage of spermatocyte development (e.g. Chandley and Edmond 1971). If AZF is functional during the spermatogonial stage, it can be postulated that in Yq11⁻ patients with a later arrest in spermatogenesis, the function of AZF is expressed normally. When we estimated the breakpoint in Yq11 for these patients with the aid of our subinterval map (Table 1) a proximal location (subinterval III-IV) was usually found (Vogt, unpublished). The AZF gene should then be located in the distal part of Yq11.22 or proximal part of Yq11.23. Support for this mapping position is given by the proximal microdeletion found in our sterile male with germ cell aplasia (Vogt et al. 1992; Fig.6) Unfortunately, a testis biopsy was rejected by our second patient with a deletion in the distal part of Yq11.23 (Fig.6), and we cannot yet prove whether our mapping hypothesis for AZF is correct and whether a second spermatogenesis gene in Yq11.23 is functioning during the spermatocyte stage.

During the leptotene phase the Y chromosome starts to pair with the X chromosome at the tip of their short arms, where both chromosomes have a homologous DNA region, called "pseudoautosomal"

(Bourgoyne 1982). Although this region only includes about 20% of the short Y arm, the pairing structure between both sex chromosomes becomes distinctly larger at the early pachytene stage, following a time gradient (Solari 1980). A pairing of the Y chromosome along the total length of its euchromatic region including Yq11 has been observed by Chandley et al. (1984). Studies of human spermatocytes in fertile and sterile males suggested that the time gradient of the X-Y pairing structure is an important parameter for the regular premeiotic development of the male germ cell (Speed and Chandley 1990). This raises the question of how this Y-pairing behaviour is regulated. There is distinct evidence that its initiation is determined by an intact DNA structure in the pseudoautosomal region of both chromosomes (Chandley 1989; Gabriel-Robez et al. 1990). Since a homologous DNA region also exists at the tip of the long arm of both sex chromosomes (Pedicini et al. 1991), one may speculate that the second function of AZF, now differentiated molecularly from the first one by the distal microdeletion in Yq11.23, can mediate the gradient of the pairing structure of the Y chromosome to the X chromosome like a "collochore", discussed earlier for the sex chromosomes of *Drosophila* (Coyne 1985). This hypothesis can be easily proved or disproved by the analysis of the X-Y pairing structure in sterile males with a distal deletion in Yq11.23.

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Spatial and Quantitative Aspects of Spermtogenetic Tissue in Primates

Wolfgang Schulze and Andrea Salzbrunn

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1 Introduction

In recent years ever more sophisticated experimental procedures such as studies of Sertoli cell cultures in bicameral chamber systems (Byers et al. 1986; Janecki and Steinberger 1986), or techniques of molecular biology, have led to numerous new insights into the control of testicular function. In most of these advances, based largely on animal experiments, the molecular control mechanisms are adapted towards a particular organizational pattern in the germinal eipthelium. Such a pattern may vary considerably between different species. Especially at the evolutionary level of the non-human primates and man, there are notable structural and quantitative modifications which presumably demand a special adaptation in functional regulation. In order to establish a broader reference base for clinical and basic research, new data of spermatogenetic efficiency in non-human primates and in man are

presented. These extend the comprehensive data published by Russell et al. (1990).

2 On the Organization of Spermatogenetic Tissue in Primates

The development of the germ cells in clonal cell clusters, as well as the symbiotic interaction of the germ cells with somatic supporting cells (in mammals Sertoli cells) are characteristics of spermatogenesis shared by almost all species (Roosen-Runge 1974). In mammals, spermatogenesis occurs within seminiferous tubules. Here it is subject to "germinal epithelium kinetics" which are expressed morphologically in spermatogenetic stages, as well as in a serial continuum of the various stages along the length of the tubule, the so-called spermatogenetic wave (Roosen-Runge 1977). Whereas the pattern of the stages can easily be explained as resulting from certain kinetic rules (Clermont 1972), its origin and its physiological significance remain largely unclear. Since the waves are frequently interrupted by modulations (transient reversals of the stage number within a sequence) or inversions (changes in complete sequence orientation), the original interpretation by Regaud (1901) that "l'onde est dans l'espace ce que le cycle est dans le temps" appears to be contravened (Perey et al. 1961; Courrot et al. 1970).

Information about how stages and waves are interrelated derives from studies on the organization of the germinal epithelium in the human testis. Originally, the human germinal epithelium had been regarded as irregular (Roosen-Runge and Barlow 1953), since the stages appeared to be much smaller than in other species and frequently exhibit variability in cell composition (Clermont 1963). In addition, a wave-like arrangement of stages was at first not recognized (Heller and Clermont 1964; Leidl and Waschke 1970). However, a much more comprehensive appraisal of the microstructural architecture of the human germinal epithelium was obtained using distribution analysis of spermatocyte populations in the seminiferous tubule. With the help of this method, a complex scheme became evident which could be described in terms of helical geometry (Schulze and Rehder 1984; Schulze et al. 1986). The geometric properties of this scheme are illus-

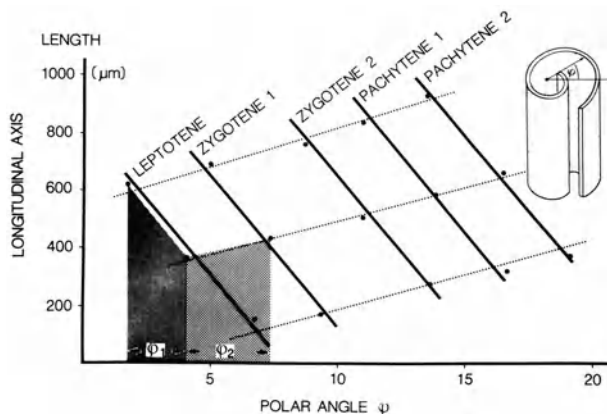
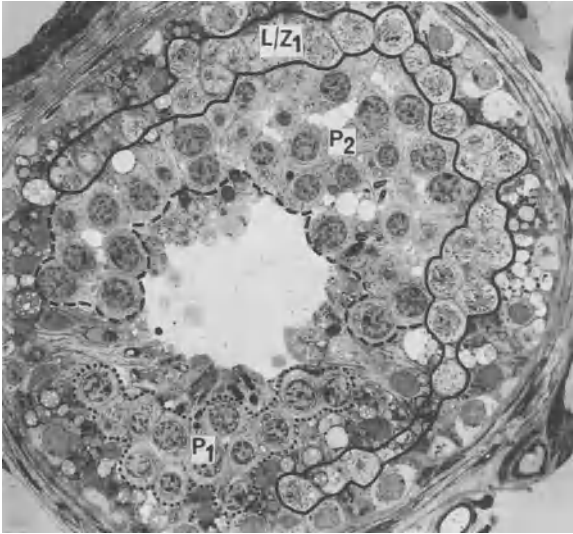
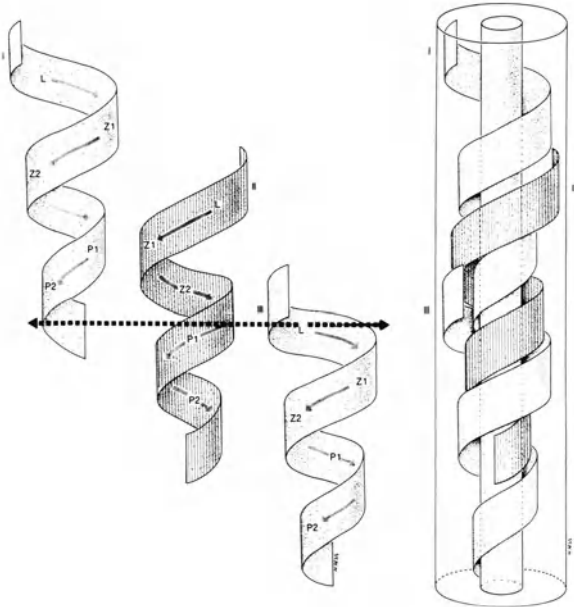


Fig. 1. Two-dimensional representation of the distributional pattern of primary spermatocytes in human seminiferous tubules. The representation has to be imagined as if the tubule had been unwound according to an Archimedean spiral. The points characterize the centers of gravity of the various populations. The dotted lines record populations of subsequent developmental stages. The centers of gravity diverge continuously $173.8^\circ \pm 32.4^\circ$ ($\approx \varphi 2 = 3.03 \pm 0.57$ radian). The solid lines record populations of the same developmental stages. Here, the centers of gravity diverge $142.6^\circ \pm 14.2^\circ$ ($\approx \varphi 1 = 2.49 \pm 0.25$ radian) (from: Schulze and Rehder 1984)

trated in detail in Figs. 1 and 2. The geometry shows surprising similarities to pattern formations occurring in a variety of growth and differentiation processes and are evidently ubiquitous. The formation of such spiral patterns can be accounted for mathematically by relatively simple prerequisites, for example, the production of a local inhibitory substance by the successively developing elements of a growing organism (Richter and Schraner 1978). Today such control of morphogenesis would be considered as a paracrine regulatory system. That paracrine mechanisms exist in the germinal epithelium was neatly shown by Djakiew and Dym (1988) using Sertoli cell cultures: peptides released from pachytene spermatocytes induced specific Sertoli cell secretion. Although this does not prove that morphogenesis of the germinal epithelium is also regulated in a paracrine manner, the geometric characteristics we have identified clearly point in this direc-



tion, and encourage the hypothesis of autoregulated (paracrine), coordinated kinetics of germinal epithelium along the length of the tubule. This hypothesis is supported by other morphological and quantitative data. Thus, Miething et al. (1987) showed that clones of a special degenerative form of primary spermatocytes often observed with aging, so-called megalospermatocytes, confirm this underlying organizational principle. Moreover, an investigation of so-called tubule diverticula showed that the fundamental organization of the germinal epithelium is retained in spite of marked changes in form and volume of the seminiferous tubule (Schulze 1989). These results demonstrate the essential biological purpose of this morphological principle: wherever germ cells degenerate, normal spermatogenic tissue always regenerates. This applies both when germ cells degenerate physiologically at quite a high frequency (Roosen-Runge 1955; Barr et al. 1971; Johnson et al. 1983; Breucker 1981) or, in the extreme situation, when there are marked changes of shape of the whole tubule.

The question arises whether this organizational scheme is compatible with what is observed in the germinal epithelia of other species, that is, whether this concept is generally valid across species boundaries. This question will only be answered by demonstrating morphogenetically active regulatory systems. A comparative anatomical study of testicular tissue from crab-eating macaques (*M. fascicularis*) indicates that an intermediate form in the organization of the germinal epithelium exists (Dietrich et al. 1986). On the one hand, spermatogenetic stages can be seen in this old world monkey which are reminiscent of those seen the rat, mouse guinea pig or also in new world monkeys, such as *Saimiri sciureus* (1968). On the other hand, there are also tubular segments where the stages occupy only small areas of the spermatogenetic tissue. In these regions a clear helical arrangement of the wave pattern is obvious. Together with the frequent occurrence of pat-

Fig. 2. Three-dimensional model of the arrangement of primary spermatocytes in the human seminiferous tubule. The construction of the model is based upon the data shown in Fig. 1. The micrograph (bottom) shows a cross section of a human seminiferous tubule. The arrangement of the primary spermatocytes can be explained by a section through the model at the level of the dotted arrow (from: Schulze and Rehder 1984)

tern reversals (modulations), these features are typical of human spermatogenetic tissue. Such intermediate patterns are probably also present in the baboon (*Paio anubis*, see illustration in the article by Chowdhury and Marshall 1980).

The reasons for these changes in the aspect of the seminiferous epithelium in old world monkeys and in man are as yet poorly understood. Leidl (1968) hypothesized that this may reflect a regression of the reproductive capacity during hominoid evolution. In order to test this hypothesis, efficiency of spermatogenesis in different primate species was investigated.

3 The Elongate Spermatid – Sertoli Cell Ratio as a Parameter of Spermatogenetic Efficiency

The Sertoli cell serves as a standard for quantifying the different germ cell types in numerous studies (Rowley and Heller 1971; Skakkebaek and Heller 1973; Wing and Christensen 1982; Russell and Peterson 1984; Johnson 1986). Since, however, most immature germ cells reside in the spaces between Sertoli cells, it is often difficult to define the germ cell cohort served by a single Sertoli cell. This is not the case at the developmental level of the elongate spermatids. These cells are no longer associated with the outer lateral surface of the Sertoli cell, but are embedded in deep cylindrical recesses at the cell apex (Russell and Peterson 1984). This topography permits precise allocation of a germ cell cluster, which generally represents part of a clone, to a particular Sertoli cell (Fig. 3).

It has been suggested that the ratio of elongate spermatids to Sertoli cells (es-Sc-ratio) is a prime factor determining daily sperm production (Russell and Peterson 1984). In this regard, cluster analysis would appear to be a suitable instrument for comparing spermatogenetic efficiency in different species. Russell and Peterson (1984) have used the es-Sc-ratio for just such an analysis in a wide range of mammals (rat, mouse, hamster, gerbil, guinea pig, rabbit, vole, opossum and crab-eating macaque). They found that the ratios in most species are quite similar (from $9.75 \pm$ in the vole to 12.17 ± 1.99 in the rabbit). The monkey, however, has a significantly lower ratio (5.94 ± 1.39).

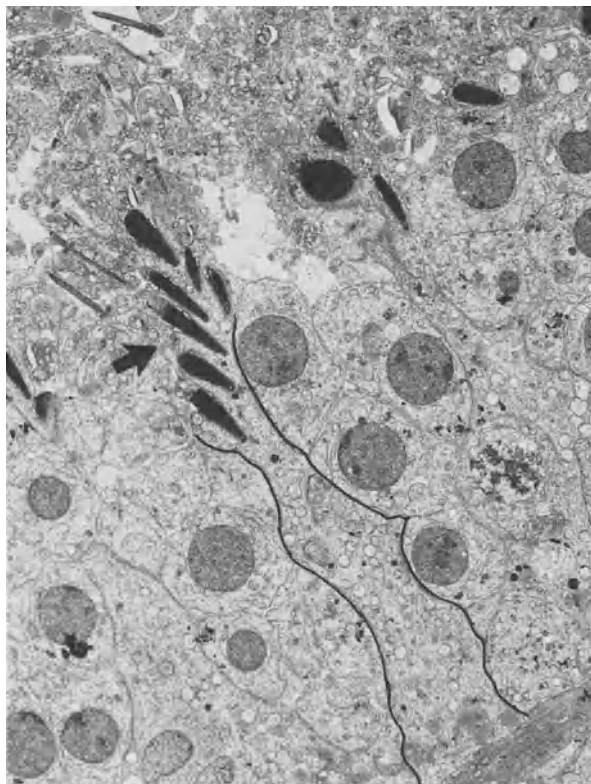


Fig. 3. Electron micrograph of a Sertoli cell in the germinal epithelium of a crab-eating macaque (macaque I). Towards the tubular lumen a cluster of several elongated spermatids (arrow) is embedded in apical recesses of the Sertoli cell

We have followed up this observation by investigating two monkeys from the same species (*M. cynomolgus*), an orang-outang as well as four men aged 23, 33, 37 and 47 years.

Three of the human samples derived from testicular biopsies of vasectomized patients wishing to regain fertility. The 23 year-old man

had been vasectomized four years before, the 33 and 37 year-old patients seven years prior. The subjects were in good health and hormone analysis indicated values for FSH, LH, prolactin and testosterone all within the reference range. The testis biopsies were investigated using the semithin section technique. All biopsies presented normal morphological appearance and the vasectomy reversal operation was successful in all patients. The post-operative control spermiograms showed sperm concentrations of at least 80 Mio/ml ejaculate (total sperm number per ejaculate consistently over 160 Mio). The 47 year-old patient was a sexual delinquent undergoing voluntary castration. Unfortunately, no spermiogram data were available, though the histological analysis of the testicular tissue indicated regular spermatogenesis of normal qualitative and quantitative appearance. The diameter of the seminiferous tubules was between 220 and 260 μm . The lamina propria appeared morphologically normal with a thickness of 5-7 μm .

Few data were available for the non-human primates concerning origin, history and age. In all cases the animals were large adult males of at least six years of age. The testis tissue from macaque I was obtained from an autopsy in the course of an unrelated investigation. The tissue from macaque II was obtained from the local state animal-home, where the animal had to be put down. The orang-outang was a large adult animal kept under modern conditions for many years in the local zoo (Hagenbecks Tierpark), where it still lives. Castration was necessary for veterinary reasons.

All tissue samples were similarly fixed in glutaraldehyde and prepared for the semithin section technique and electron microscopy (for ref. see Holstein and Roosen-Runge 1981). The quantitative studies were performed using photographs of serial sections (1 μm thick). All 25 μm ultrathin sections were prepared and subjected to electron microscopy to confirm the reliability of the light microscopical determinations. At least 100 clusters of elongate spermatids were analyzed from each sample.

In addition to the cluster analysis (es-Sc-ratio), and in order to obtain a broader base for comparison, the number of Sertoli cells in the tubule sections studied were determined as isolated elongate spermatids ("one-cell cluster"). All Sertoli cell nuclei in 80 - 100 μm long straight portions of tubules were counted. Finally, this figure was con-

Table 1. Elongate spermatid-Sertoli cell ratios and numerical Sertoli cell densities in four men, one ape and two monkeys

Species	Mean $\pm$ s.d. elongate spermatid- Sertoli cell ratio	Range	% isolated elongate spermatids ("one-cell cluster")	Numerical Sertoli cell density (per $10^6 \mu\text{m}^3$ tubule volume)
Human (23 years)	3.12 ± 1.53	2 - 13	59.9	38.3
Human (33 Years)	4.29 ± 2.92	2 - 13	56.7	41.0
Human (37 Years)	4.00 ± 2.54	2 - 10	58.6	35.8
Human (47 years)	4.00 ± 2.09	2 - 11	57.6	38.7
Macaque I (crab-eating)	12.36 ± 4.85	3 - 29	31.9	73.3
Macaque II (crab-eating)	6.36 ± 4.09	2 - 22	38.3	47.1
Orang-Outang	5.68 ± 2.26	3 - 18	26.1	63.1

verted to tubule volume units of $10^6 \mu\text{m}$ (seminiferous epithelium plus lumen, excluding lamina propria).

The results of this investigation (Table 1) show that the data for the four men are very similar. This is true for the medium es-Sc-ratios as well as for the range of cluster size. The values for Sertoli cell density lie within approximately one order of magnitude of one another, whereby slight inter-individual differences are apparent. Surprisingly, there is good concordance in the percentage of isolated spermatids ("one-cell clusters"). Possibly this represents a novel biological parameter which is characteristic for normal spermatogenetic activity in man.

The data derived from the non-human primates are more surprising. The very large discrepancy in the mean es-Sc-ratios in the two macaques is particularly dramatic. In macaque I the ratios are twice as

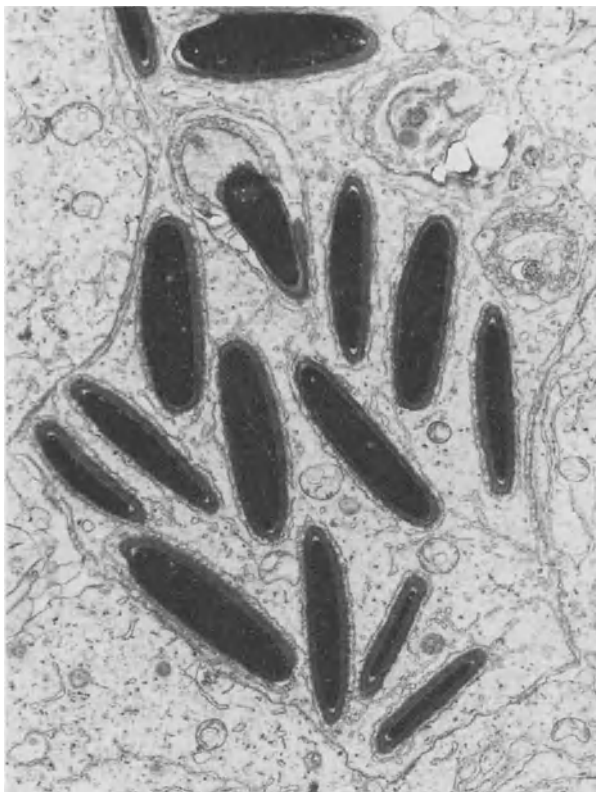


Fig. 4. Electron micrograph showing the heads of 15 elongate spermatids already embedded in the recesses of a single Sertoli cell in macaque I. By analysis of serial section it was revealed that the complete cluster consisted of 24 elongate spermatids

great as in macaque II, and are more like the values recorded for rodents. The large range between minimum and maximum cluster size is common to both macaques. The maximal cluster size of 29 observed in macaque I is, to our knowledge, the highest value ever recorded. The validity of this estimate was confirmed by electron microscopy of control sections (Fig. 4). The mean es-Sc-ratio found in macaque II is

similar to the value recorded by Russell and Peterson (1984) for the same species. The numerical Sertoli cell density in this animal is much less than in macaque I and corresponds more to human values. For the orang-outang the results presented elements of those found in both macaques.

The mean es-Sc-ratio was similar to that of macaque II, whereas the Sertoli cell density was more like that in macaque I. In all of the animals it was difficult to determine the significance of the isolated spermatids ("one-cell clusters"). It was frequently not possible, using either light or electron microscopy, to determine whether these represented a remnant of a previously released cluster, or whether the isolated spermatid was the sole survivor of an aborted cohort. Thus the data for "one-cell clusters" given in Table 1 are to be interpreted with caution.

Further comparisons are possible when the es-SC-ratios are plotted in the form of a frequency histogram (Figs. 5 and 6). The profiles are all very similar for the four human samples, with the two-cell cluster being most common. The frequency of more numerous cell clusters declines almost exponentially. In contrast, for macaque I the es-Sc-ratio displays virtually a normal distribution with a weak maximum at an es-Sc-ratio of 12. For macaque II the frequency distribution is more like that for the human samples, although the percentage frequency of the smaller clusters is lower.

For the orang-outang the normal distribution seen in macaque I is still readily apparent. However, there is a clear shift in the peak value towards smaller cluster size. One or two comments on the histological appearance of the monkey tissue samples are appropriate. The germinal epithelium of both the orang-outang and macaque I appeared very similar, and the Sertoli cells indicated no obvious morphological aberrations. In contrast, macaque II presented Sertoli cells containing large

Fig. 5. Percentage frequencies of the various cluster sizes (es-Sci-ratios) in the germinal epithelium of 4 men (age: 23, 33, 37 and 47 years). Figure appears on page 278

Fig. 6. Percentage frequencies of the various cluster sizes (es-Sc-ratios) in the germinal epithelium of two crab-eating macaques (macaque I and II) and an orang-outang. Figure appears on page 279

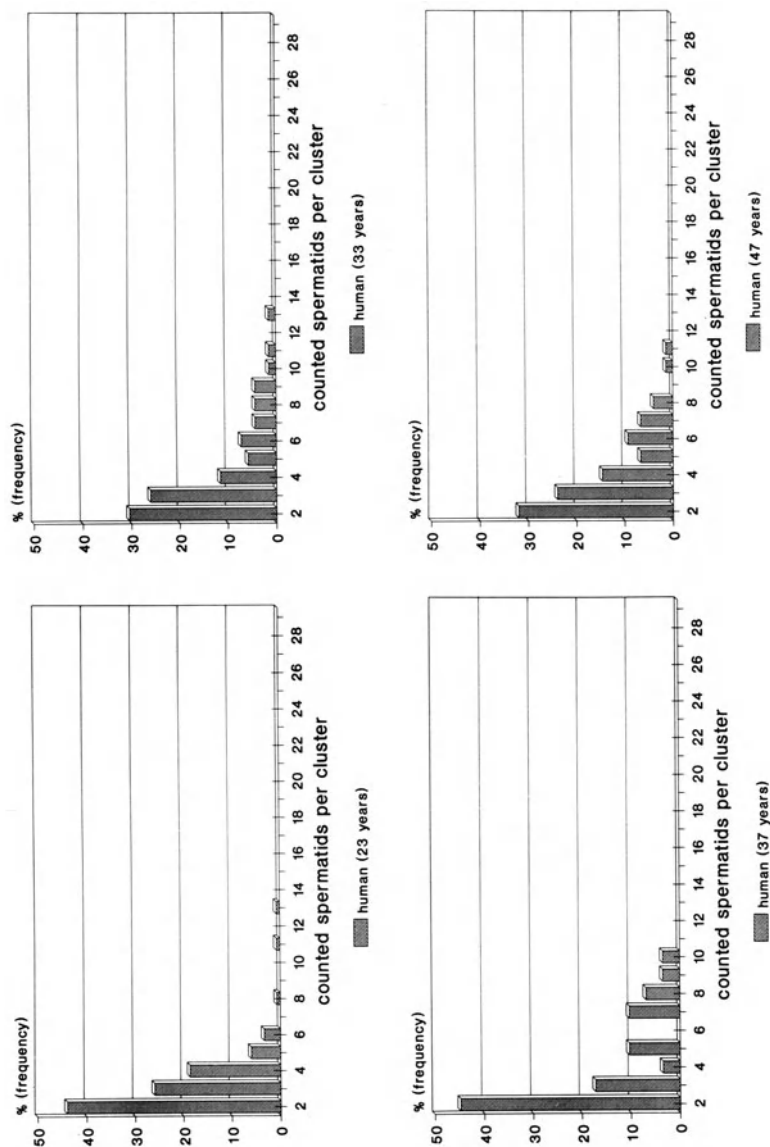


Fig. 5

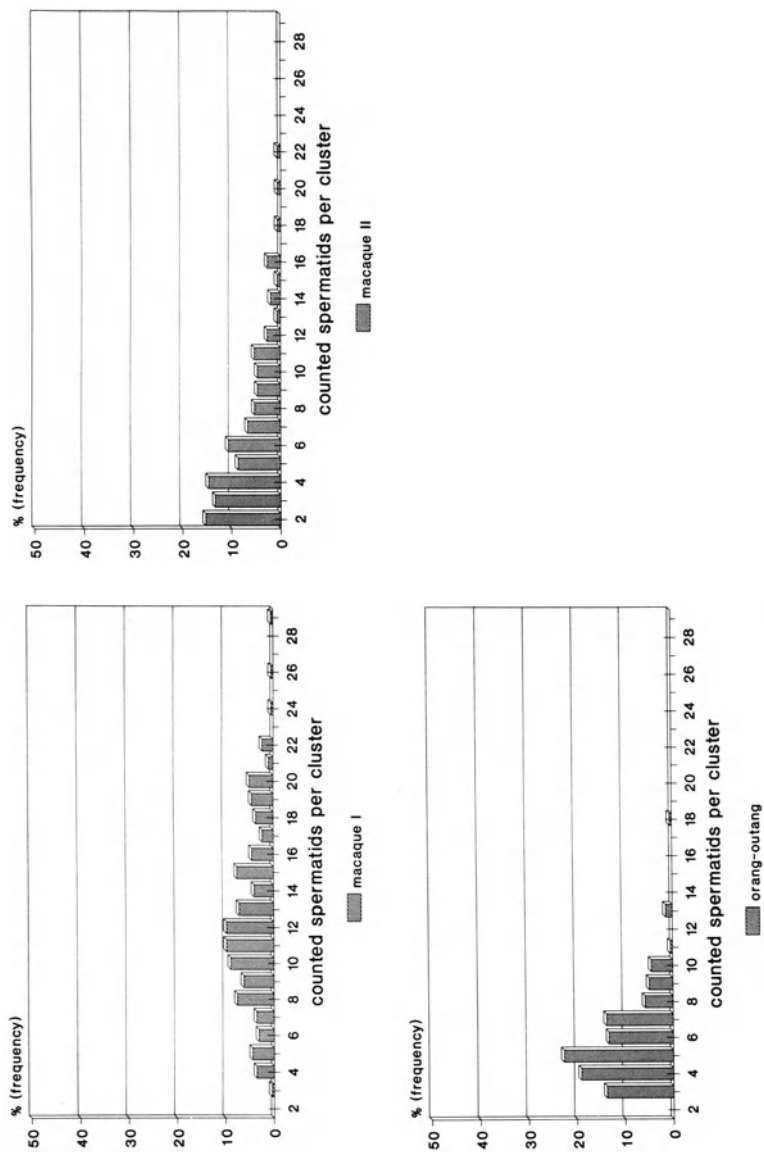
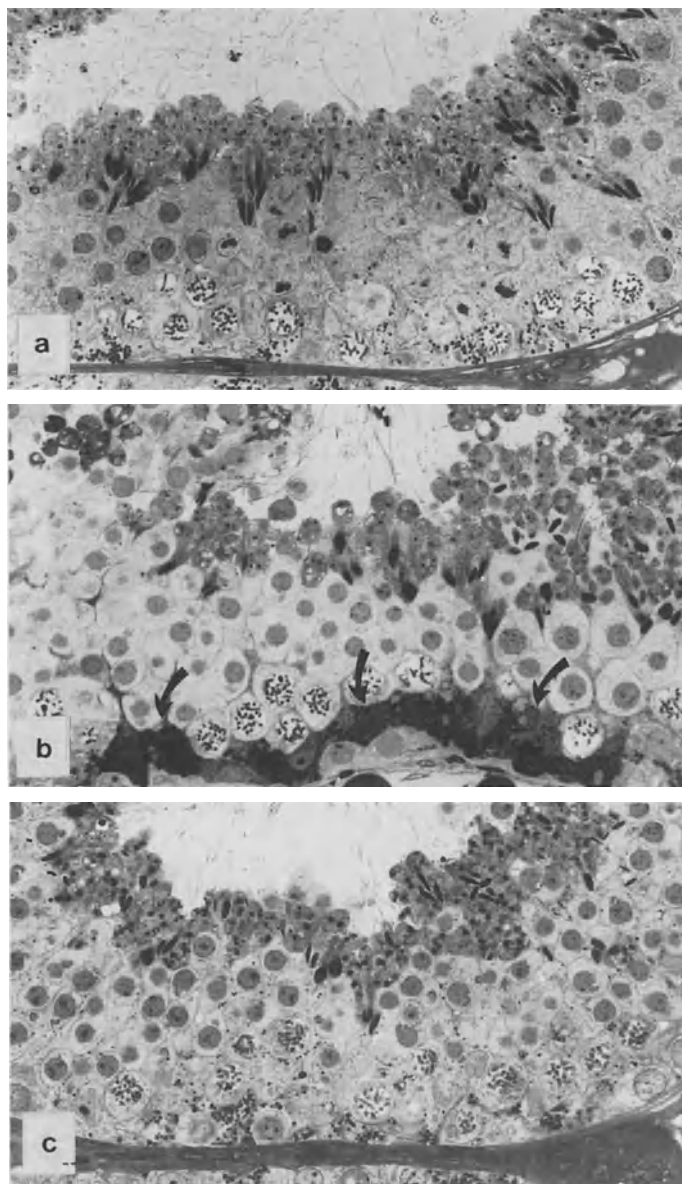


Fig. 6



numbers of lipid droplets and lysosomes (Fig. 7). Helical stage sequences could be readily observed in macaque II, whereas in macaque I and in the orang-outang they were clearly less frequent.

4 Concluding Remarks

Comparing the data obtained so far from the various species discussed here, there is an obvious decrease in spermatogenetic efficiency in the evolutionary branch leading to man. The reasons for this remain obscure. In addition to the quantitative and structural differences seen in the organization of the germinal epithelium, a kinetic modification of the cellular procession (e.g. mode of spermatogionogenesis) could play a role just as much as changes in other regulated parameters (e.g. elevation of physiological germ cell loss). How closely spermatogenetic efficiency and the organization of the germinal epithelium are linked can be seen from the study of the two macaques. In macaque I there was maximal spermatogenetic efficiency which led to the formation of larger single stages and a predominantly segmental order to the spermatogenetic wave. In contrast, macaque II indicated reduced spermatogenetic efficiency as well as Sertoli cell density, approaching values more typical of the human, with a corresponding change in the spatial organization of the germinal epithelium. These findings add weight to the hypothesis that the general organization of the spermatogenetic tissue in man and animals can be explained by a common morphogenetic principle (Schulze et al. 1987).

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Fig. 7 a-c. Light micrographs of the germinal epithelium of the three investigated animals. In comparison with macaque I (**a**) and the orang-outang (**c**) the Sertoli cells in macaque II are strikingly enriched with lipid droplets (arrows and lysosomes (**b**))

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Epididymal Proteins and Sperm Maturation

Trevor G. Cooper

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1 Introduction

It is appropriate that a Testis Workshop should have as an accessory appendage a session on the epididymis, for the mere production of sperm cells is insufficient for fertility and a period of extra-gonadal maturation and storage of spermatozoa is necessary. There have been recent advances in several areas of epididymal physiology and biochemistry and I shall address two of them. The main growth area, to which most of this review is devoted, is the identification of protein secretion products of the epididymal epithelium and their localization in the epididymis on epididymal spermatozoa and their putative rôles in fertilization. The epididymis is also responsible for modifying proteins already on the sperm when they enter the epididymis. Demonstrations of changes in the ability of sperm to respond to stimulation upon maturation have also provided clues as to what events occur to them in the epididymis.

2 Epididymal Sperm-Coating Proteins

The nature of epididymal epithelial protein secretions and their association with spermatozoa has been studied in several laboratory species. This review mainly concerns proteins that have been localized within epididymal tissue and on epididymal spermatozoa, especially with those that may be involved in influencing the fertilizing ability of spermatozoa.

2.1 In the Rat

The nature of the most important proteins secreted by the rat epididymis is given in Table 1. Various names, molecular sizes and isoelectric points have been given to what are now considered the same proteins, owing in part to the anomalous behaviour of glycoproteins in polyacrylamide gels. Identity in Table 1 is attributed on the basis of similar size, immunolocalization and, where known, amino acid or nucleotide sequences (see Cooper 1986, 1990; Shivaji et al. 1990). *In situ* hybridization has demonstrated the first appearance of mRNA of proteins B,C and SGP-1 in cells of the efferent ducts, and that of CRBP and SGP-2 in the initial segment. Higher signals were generated for SGP-1 in the efferent ducts, CRBP in the caput and SGP-2 in proximal corpus epididymidis (Garrett et al. 1991).

Less is known of other protein secretions, including a 22 kDa protein that is not unique to epididymal tissue, but present only in its secretions (Brooks 1985), a 26 kDa protein (Olson and Hinton 1985; Olson et al. 1987), Band III (Brooks 1983b), GP17 (Iusem et al. 1989), Protein G (Brooks, 1983a) and HIS-50, a 50 kDa protein secreted in the cauda epididymidis (Rifkin and Olson 1985).

The 22 kDa protein is probably the same as protein A' (Brooks 1981a,b), Protein I (Jones et al. 1980b), the 23 kDa protein of Jones and Brown (1982, 1987), the 20 kDa protein of Shabanowitz and Kilian (1987), complex 7 (Olson and Hinton 1985) and a component of the epididymal α -lactalbumin-like activity of Fraction III (Hamilton 1981). The α -lactalbumin activity claimed for epididymal proteins of different molecular weight (Jones and Brown 1982; Hamilton 1981; Byers et al. 1984a,b; Limpaseni and Chulavatnatol 1986) has not been

Table 1. The nature of proteins secreted by the rat epididymal epithelium and their possible roles in fertilization¹**Proteins B,C**

18-28 kDa, pI 4.9-6.0

(other names: proteins II,III; epididymal binding proteins (EPB) 1,2; sialo-glycoprotein; 18 kDa epididymal α -lactalbumin-like component)*homology:* with retinoic acid binding proteins**Proteins D,E**

27-37 kDa, pI 4.1-5.1

(other names: protein IV, sialoprotein (SP); specific epididymal protein (SEP); pre-albumin specific protein (PES); acidic epididymal glycoprotein (AEG); rat epididymal protein (REP); 32 kDa protein; major acidic sialoglycoprotein)*homology:* with lipid-binding proteins*functions:* promotion of sperm motility?, sperm-zona binding?, sperm-vitellus interaction?**Protein F**

66-70 kDa, pI 4.2-6.7

(other names: dimeric acidic glycoprotein (DAG); sulphated glycoprotein 2 (SGP-2); clusterin)*subunits:* Bands I,II 27-32, 35-41 kDa, pI 4.8-7.2, 3.7-6.5*(other names:* Bands 5,6; forms H,L)*homology:* lipid-binding proteins*functions:* immunosuppression?**Band 4**

60-70 kDa

(other names: sulphated glycoprotein 1 (SGP-1); prosaposin)*homology:* glycolipid/glycoprotein hydrolysis/transport**2D6 antigen**

23.2 kDa, pI 4.0-6.0

(other names: 26 kDa?)*function:* sperm-vitellus interaction?**Immobilin**

> 400 kDa

function: restriction of flagellar movement¹ Data from: Bayard et al. 1980; Brooks 1982, 1983a,b, 1987b; Brooks et al. 1986a,b; Brooks and Higgins 1980; Cameo and Blaquier 1976; Charest et al. 1988; Collard and Griswold 1987; Collard et al. 1988; Faye et al. 1980; Fournier-Delpech et al. 1985; Garberi et al. 1979, 1982; Goldin et al. 1989; Hamilton 1981; Jones and Brown 1987; Jones et al. 1980b, 1985; Lea and French 1981; Limpaseni and Chulavatnatol 1986; Mattmueller and Hinton 1991; Newcomer and Ong 1990; Ong and Chytil 1988; Ruiz-Bravo 1988; Sylvester et al. 1984, 1989, 1991; Turner and Reich 1987; Walker et al. 1990; Wong and Tsang 1982

confirmed (Brooks 1985; Hölpert and Cooper 1990a,b). The gene of the 18 kDa protein has been cloned (Walker et al. 1990) and the protein shows no similarity to rat milk α -lactalbumin (Moore et al. 1990).

2.2 In the Mouse

In the mouse two proteins of 18 kDa and 29 kDa show 86 % and 69 % homology with rat epididymal proteins B,C and D,E, respectively (Orgebin-Crist et al. 1990). Ghyselinck et al. (1989, 1990, 1991), Jimenez et al. (1990) and Faure et al. (1991) have identified a 24 kDa androgen-dependent epididymal specific protein with pI 7.2 and 8.2. Its gene has been cloned (1.4 kb) and computer databases have revealed homology with glutathione peroxidase. A different protein with similar mass (26 kDa) has also been identified in the epididymis (Murphy and Carroll, 1987). A 54 kDa sperm maturation antigen (SMA4) in the mouse epididymis (Vernon et al. 1987) is also known as the T21 antigen (Bunch and Saling 1991).

2.3 In the Hamster

Both poly- and mono-clonal antibodies against purified luminal fluid proteins (H1: Moore 1980) and mature spermatozoa (Smith et al. 1986; Hoos and Olson 1988) have identified epithelial secretion products in this species of 34 kDa (C5 antibody: Smith et al. 1986) and 23 kDa. The 23 kDa antibody cross reacts with rat sperm, where it is found on the sperm tail in association with a 26 kDa protein. This protein may be the same as that recognized by monoclonal antibody WS 64.23 against mature hamster spermatozoa (Moore et al. 1985), but from its localization within the epididymis, is unlikely to be EP3, a 23.8kDa secretion product of the hamster epididymis (González Echeverría et al. 1982).

2.4 In the Rabbit

Antibodies raised to cauda epididymal fluid from the rabbit epididymis contains sperm coating proteins designated R1, R2 and R3 with isoe-

lectric points of 3.43, 4.15 and 4.65, respectively (Moore 1980). An acrosome stabilizing factor (ASF) has been identified as a protein of Mr 259 - 370,000 and pI 5.3 - 7.0 consisting of 38 and 92 kDa subunits (Thomas et al. 1984, 1986). This protein is reported to be synthesized in the caput epididymidis *in vitro*, but not cauda or vas (Thomas et al. 1984) and is present in luminal fluid with increasing concentrations distally (Reynolds et al. 1989).

2.5 In the Pig

An androgen-dependent antagglutinin (M_r 250 kDa, consisting of four 63 kDa monomers) in porcine epididymal fluid prevents the *in vitro* agglutination of spermatozoa taken from the corpus and cauda in this species (Dacheux et al. 1983; Dacheux and Dacheux 1988b). Monoclonal and polyclonal antibodies raised against sperm integral membrane proteins demonstrate proteins that are increasingly associated with maturing epididymal spermatozoa and also present in epithelial cells. These proteins include a zona adhesion protein (AP_Z) of 55 kDa which has been identified as an epididymal secretion product.

2.6 In Man

Specific human epididymal proteins (R 0.31, R 0.43, R 0.68, R 1.01) have been given molecular weights and pI values in the range 38 kDa and 5.8, 21 kDa and 6.2, 69 kDa and 5.1 and 29 kDa and 6.8, respectively (Tézon et al. 1985a). Using recombinant DNA technology four genes coding for proteins specific for the human epididymis have been identified (Kirchhoff et al. 1990). Of these, HE4 codes for a small (700 bp, 10 kDa), cysteine-rich protein, pI 4.3, which has homology with extracellular protease inhibitors. *In situ* hybridization has demonstrated that its mRNA is expressed at low levels in the efferent ducts and caput and higher levels in corpus and cauda epididymidis (Kirchhoff et al. 1991).

3 Histochemical Localization of Sperm-Coating Proteins in Different Regions of the Epididymis

Immunohistochemical techniques localize proteins within different epithelial cell types in the cytoplasm or within particular cellular structures. Although it is not clear whether the co-localization of immunostain on spermatozoa within the epididymal lumen in tissue sections demonstrates a true association with the protein, or just a precipitation of the fluid protein on their surface, results obtained for various epididymal-specific proteins are summarized below.

3.1 In the Rat Epididymis

Proteins B,C

Proteins B,C (extracellular retinoic acid binding proteins) are present on both stereocilia and spermatozoa in the caput and corpus epididymidis, but a clear demonstration of its presence within the epithelium is lacking (Brooks 1987a). The localization of the cellular retinoic acid-binding protein is better known; this is definitely in the initial segment and proximal caput cells, but beyond the corpus epididymidis no staining of cell cytoplasm in the epididymis is observed. The secretory nature of this protein is revealed by the positive staining of both stereocilia and luminal spermatozoa (Porter et al. 1985). In sharp contrast to CRABP, cellular retinol binding protein (CRBP) in the epididymis is found only within the epithelial cells, not on spermatozoa in the lumen (Porter et al. 1983, 1985; Kato et al 1985).

Proteins D,E

Proteins D,E are not found in the initial segment but, depending on the antibodies used, are localized within epithelial cells in the caput and corpus, but not cauda, and on spermatozoa and stereocilia in all regions distal to the initial segment (Lea et al. 1978; Kohane et al. 1980a; Faye et al. 1980; Bayard et al. 1980; Brooks 1987a).

Sulphated Glycoproteins 1 and 2

Within the epididymis SGP-2 is found in epithelial cytoplasm, on stereocilia and on luminal spermatozoa in the caput and corpus epididymidis (Sylvester et al. 1984, 1991; Hermo et al. 1991). The relationship between the testicular and epididymal forms of SGP-2 (clusterin) and that in the fluid and on spermatozoa has been elucidated by a combination of immunohistochemical and physiological studies. Because the protein is localized within the apical parts of cells lining the rete and efferent ducts, but not on luminal spermatozoa in these regions, the removal of the testicular protein from the sperm by these cells was postulated. Within the epithelial cells the protein is indeed found within the endocytic apparatus of the principal cells. More distally in this segment, dense immunolabelling of epithelium is found together with labelled luminal sperm cells and this was considered to represent the adherence of the secreted epididymal protein to the spermatozoa. In this region of the duct, secretory vacuoles are filled with the antigen (Hermo et al. 1991). The form of SGP-2 on testicular and epididymal spermatozoa reflects that in the fluid in which they reside (Mattmueller and Hinton 1991; Sylvester et al. 1991). The regulation of this protein is interesting, being androgen-independent in the caput and not synthesised at all in the initial segment unless testicular fluid is withdrawn (Brooks 1983b), but it is premature to conclude that clusterin from the Sertoli cell acts as an inhibitor of the synthesis of the protein within the initial segment.

Immunohistochemistry for SGP-1 has only been applied to the caput epididymidis (Sylvester et al. 1989), but it is solely found within the epithelium and not on stereocilia or spermatozoa.

2D6 Antigen

This protein is not detectable immunologically in cells from the caput epididymidis and first appears in the proximal corpus regions, where antibodies label stereocilia and luminal spermatozoa (Jones et al. 1985). In the cauda it is not present within the epithelial cells but is still associated with stereocilia and spermatozoa.

Immobilin

Although this protein is associated with epithelial cells solely in the mid and distal caput epididymidis, stereocilia and spermatozoa are positively stained in all regions distally (Ruiz-Bravo 1988).

For the rat, the presence of a certain protein in the epithelial cells is either cotemporaneous with the its presence on sperm in that region or occurs on sperm a little more distally (*e.g.* 2D6 antigen, HIS-50).

The immunohistochemical reactivity in the epithelium discussed above related to principal cells. However, immunohistochemically positive material may also be found in epididymal clear cell cytoplasm. If this results from endocytosis of proteins from the lumen, the process is specific, since clear cells in the rat contain AEG (Lea et al. 1978) and immobilin (Ruiz-Bravo 1988) but not HIS-50 (Rifkin and Olson 1985), 2D6 antigen (Jones et al. 1985) or cellular retinoic acid-binding protein (CRABP: Porter et al. 1985). It is unclear if SGP-2 is present in clear cells; Sylvester et al. (1984) found anti-DAG in clear cells (termed halo cells in their paper) in immerse-fixed tissue with FITC-linked antibody but Hermo et al. (1991), using the same antibody linked to HRP, failed to detect SGP-2 in the clear cells of perfuse-fixed material.

3.2 In the Murine Epididymis

Glutathione peroxidase is restricted to cells in the caput epididymidis, but it binds to sperm heads in distal regions of the duct (Jimenez et al. 1990). From its more distal immunohistochemical localization (distal caput, corpus and cauda), a murine epididymal protein of 26 kDa (Murphy and Carroll 1987) is clearly not identical to the 24 kDa glutathione peroxidase.

The 54 kDa SMA4 sperm-coating protein (Vernon et al. 1987) is secreted by principal cells in the distal caput and corpus epididymidis (Vernon et al. 1982). An origin from "holocrine" (apical) cells has also been suggested on the basis of lectin (wheat germ agglutinin, WGA) staining of these cells, the protein and epididymal spermatozoa (Feuchter et al. 1987). However, the secretory function of apical cells has not been confirmed and no identification of the protein stained by lectin was made. Other WGA-positive sperm-coating proteins (GP-49 and

GP-83) have been identified in mouse epididymides as products of the corpus and cauda epithelium (Liu et al. 1991). A non tissue-specific antigen (stage specific embryonic antigen, SSEA-1) is secreted in an extremely discrete area in the mid-caput epididymidis, but is present on spermatozoa distal to this site (Fox et al. 1982).

3.3 In the Hamster Epididymis

The H1 antigen has located sperm epithelial cells of the caput and corpus but not cauda epididymidis (Moore 1980); but spermatozoa are labelled in all regions distal to its secretion. The 34 kDa protein (detected by the C5 monoclonal antibody) detects the antigen within epithelial cells of the corpus but not caput epididymidis (Smith et al. 1986). The 23 kDa antigen has been localized only in the proximal cauda epididymidis and is present on sperm tails in that region (Hoos and Olson 1988).

3.4 In the Rabbit Epididymis

Proteins R1, R2 and R3 are all localized within the distal caput and corpus epithelial cells and on stereocilia and luminal spermatozoa distal to these regions (Moore 1980). ASF is absent from both caput and proximal cauda cells, although present in the corpus region (Thomas et al. 1984). Uteroglobin (a progesterone-binding protein and possibly involved in immunosuppression of spermatozoa) is present in epithelial cells in the distal caput and proximal corpus epididymidis but not in cells in the cauda. On the stereocilia and spermatozoa it is found in all distal regions (El-Eltreby et al. 1983).

3.5 In the Porcine Epididymis

The antagglutinin is present within the epithelial cells from the initial segment to the distal corpus epididymidis and is found in association with spermatozoa in all regions distally (Dacheux and Dacheux 1988a;

Dacheux et al. 1983). The mono- and poly-clonal antibodies against integral sperm membrane proteins detect several sperm coating proteins that are secreted throughout the length of the tract (in epithelial cells, stereocilia and sperm in the mid-caput: Saxena et al. 1986b), but the intensity of staining is higher distally.

3.6 In the Human Epididymis

Immunofluorescence studies have revealed positive staining of epithelium in the distal caput and corpus epididymidis but of only stereocilia in the cauda (Tézon et al. 1985b). SSEA-1 has also been found to be present in the human epididymis (Fox et al. 1983).

In all species, spermatozoa come into contact sequentially with different proteins as they are moved through the epididymis. The particular sequence of contact with certain proteins may be just as important for protein function as the site of attachment of the protein.

4 The Cytochemical Location of Secreted Epididymal Proteins on Spermatozoa

As with the histochemical localization of proteins on spermatozoa, there is doubt about whether the results of cytochemical staining of epididymal proteins to spermatozoa released from the tract reflects the situation *in situ*. Localization can depend on whether cells have been fixed or permeabilized (Jones et al. 1985) and can alter with the concentration of first and second antibodies (Gaunt et al. 1983; Jones et al. 1985). It will also depend on how extensively the cells have been washed free of contaminating epididymal fluid. (In the epididymis, of course, the cells are “unwashed”, but see Section 3.) Complications may arise from the recognition of intracellular antigens, if the membranes have been damaged during washing (Jones et al. 1983) and, finally, the positioning of the proteins may change as sperm mature (Phillips et al. 1991). These reservations must be borne in mind when assessing the different parts of the sperm cell that seem to bind different proteins.

4.1 On Rat Spermatozoa

On spermatozoa, proteins B,C are found on both sperm heads and tails (Jones and Brown 1982). Although histochemical studies revealed that both sperm heads and tails are stained in tissue sections by antibodies to proteins D,E, cytochemical studies have not always confirmed this. Antibodies to purified proteins D,E are localized on the head and not tail (Brooks and Tiver 1983), whereas an antibody directed towards the luminal 32 kDa protein stains the tail but not the head (Brown et al. 1983; Cuasnicú et al. 1984c). Antibodies to the 2D6 antigen detect this protein on both the head and tail of spermatozoa (Gaunt 1983; Gaunt et al. 1983; Jones et al. 1985) whereas HIS-50 is solely found on the sperm head (Rifkin and Olson 1985).

4.2 On Murine Spermatozoa

Glutathione peroxidase (24 kDa) binds to sperm heads (Jimenez et al. 1990), whereas the 26 kDa protein is located on the sperm flagellum (Murphy and Carroll 1987) as is the 54 kDa SMA4 (Feuchter et al. 1981; Vernon et al. 1985; Bunch and Saling 1991).

4.3 On Hamster Spermatozoa

Protein H1 is found on the heads and tails of hamster spermatozoa (Moore 1980), whereas sperm cells are immunolabelled on the post-acrosomal region and mid-piece regions with C5 antibody (34 kDa protein: Smith et al. 1986). The 23 kDa antigen is localized on sperm tails alone in hamsters and rats (Hoos and Olson 1988).

4.4 On Rabbit Spermatozoa

Antibodies to protein R2 bind to rabbit sperm heads and tails, R1 only to heads and R3 only to tails (Moore 1981). Immunocytochemical labelling of spermatozoa with anti-ASF antibodies reveals it on sperm

heads and tails, but uteroglobin is only detectable on sperm tails (García et al 1988).

4.5 On Porcine Spermatozoa

The antagglutinin is found on both sperm heads and tails (Dacheux et al. 1983). Both the plasma membrane proteins 5.0 and 4.85 secreted by the pig epididymis are found on sperm heads and tails (Saxena et al. 1986a).

The domains and sub-domains of the sperm plasma membrane to which various secreted proteins adhere are most likely to be of importance to functions of specific sperm membranes associated with fertilization.

5 Importance of Secreted Epididymal Proteins for Fertilization

5.1 In the Rat

Effects of Secreted Epididymal Proteins on Fertilization

Evidence that proteins D,E are related to fertility has come from studies which have used antibodies against the protein. Cuasnicú et al. (1984c) incubated Anti-“DE” for 10 min with cauda spermatozoa and then used them in insemination studies. The percentage of eggs fertilized was reduced (6.6%) compared to controls of either sperm given no treatment (41.6%) or incubated with normal rabbit serum (NRS: 33.1%). In a further study, longer term incubation with the antibody was attempted by using caudal epididymal tubules cultured for 24h. These maintained the fertilizing capacity of the contained spermatozoa (49.3% vs non-cultured 56.6%), but when tubules were incubated in medium 199 containing anti-“DE”, spermatozoa subsequently removed from the tubules fertilized 16.6% of eggs compared to 52.6% from tubules cultured in medium alone or 52.2% in NRS. The results were interpreted as indicating access of the immunoglobulin to the epididymal lumen (see chapter by Cooper, this volume), although IgG was found on only 8.8% of sperm recovered from the incubated tubules.

The effect of active immunization against the most acidic epididymal glycoproteins of 18.5 and 32 kDa (most likely proteins B, C, D and E) was studied by Fournier-Delpech et al. (1985). Male rats of proven fertility were immunized with PES in Freund's adjuvant or Freund's adjuvant alone (1 injection per week for 4 wk) and after three weeks the rats were examined for serum antibodies and the motility and *in vitro* zona binding ability of sperm obtained from the cauda epididymidis. After incubation with eggs at 0C the percentage of eggs with 10 or more bound sperm was 0 for PES immunized animals, in contrast to 70 for the controls. During incubation at 34C, cauda sperm motility (% progressively motile) was 5% in 9 of the 10 PES immunized rats. In contrast, 40-50 % of sperm were progressively motile in 8/10 of the controls. After 2 weeks of treatment, 30% of the females that were mated with the treated males became pregnant and at 6 months no male rats were fertile. The percentage of female rats that gave birth after 1 week cohabitation with the treated males was, unfortunately, not examined in Freund's adjuvant-treated control immunized rats.

Effects of Secreted Epididymal Proteins on Sperm Motility

An effect of AEG (proteins D,E) on promoting the motility of "pre-motile" caput sperm (Pholpramool et al. 1983) *in vitro* was suggested by the authors themselves as being non-specific because bovine serum albumin was similarly effective. Unlike albumin, however, proteins D,E would at least normally make contact with spermatozoa in the epididymis and they are localized histochemically over the sperm flagellum. In contrast to a possible stimulatory action of AEG, the mucoprotein immobilin acts to restrict flagellar movement on account of its high viscosity (Usselman and Cone 1983; Usselman et al. 1985). Although synthesized in the caput epididymidis, the viscosity of fluid in this region is not so high and an interaction with lectin-like compounds has been suggested to induce the viscosity distally in the tract (Eksittikul and Chulavatnatol 1986).

Effects of Secreted Epididymal Proteins on Sperm-Zona Binding

There is some discrepancy in the literature about the fate of protein DE during fertilization. Kohane et al. (1980b) found that the binding of

anti-SEP (that identifies proteins C,D,E) was lost from sperm after 6h *in utero* or 4h *in vitro*. However, using anti-“DE”, an antigen localized on the plasma membrane over the acrosome was lost with membranes forming the acrosomal vesicles during the acrosome reaction *in vitro*, although some antigen also remained on the unvesiculated postacrosomal membranes (Cameo et al. 1983, 1986). This invited speculation that it was involved in further events of fertilization and, indeed, Orgebin-Crist and Fournier-Delpech (1982) demonstrated an effect of a PES (D,E and B,C?) on homologous zona binding by freshly collected testicular sperm, although no control proteins were evaluated. The localization of proteins D,E is on the plasma membrane over the acrosome and is consistent with such a function.

Effects of Secreted Epididymal Proteins on Vitellus Recognition

Of the other secreted proteins, the 2D6 antigen and 32 kDa (protein D,E) protein remained on sperm recovered from the uterus of mated rats and were even retained on the flagellum of fertilizing sperm (Gaunt 1983; Jones et al. 1985). Here the proteins extend onto the surface of the egg, with time spreading out from the site of entry of the sperm to cover most of the egg at 8h. While demonstrating well the intermixing of membranes of the egg and spermatozoon at fertilization, it is not known if these proteins are necessary for binding or fusion to the vitellus or contribute in any way to the development of the embryo. The 2D6 antigen is found on the post-acrosomal region of the sperm head, again consistent with a rôle in a post-zona fertilization event.

5.2 In the Mouse

Sperm maturation antigen 4 (SMA4) is present on mouse sperm taken from the uterus within 5.5 h of mating; it is not associated with sperm recovered later from the cumulus mass or zona pellucida. Such loss occurs *in vitro* but its relationship to capacitation is not established (Vernon et al. 1985).

5.3 In the Hamster

Effects of Secreted Epididymal Proteins on Sperm Motility

The hamster sperm antigen C5, present on mature (cauda) spermatozoa, but normally absent from less mature cells, is taken up by caput sperm after coculture with proximal corpus epididymal epithelial cells (Smith et al. 1986) and under such conditions the spermatozoa develop progressive motility (Moore et al. 1986). Another protein in epididymal luminal fluid (forward motility protein, FMP, see also Section 5.6, p. 302) has been shown to modify the circular swimming pattern of caput epididymal spermatozoa *in vitro* (Kann and Serres 1980). It can increase the number of eggs fertilized by caput sperm inseminated into the uterus, presumably by aiding transport through the utero-tubal junction (Kann and Raynaud 1982). The exact origin of this protein is not known.

The localization of the 23 kDa on the principal piece of mature spermatozoa antigen suggested to Hoos and Olson (1988) an involvement with the acquisition of motility. They argued that the 23 kDa protein may be the same as that recognized by monoclonal antibody WS 64.23 against mature hamster spermatozoa (Moore et al. 1985) which was localized only on the mid- and end-piece of the spermatozoon (Ellis et al. 1985). Antibodies to this 23 kDa antigen can reduce the *in vitro* fertilizing capacity of intact and zona-free eggs, but only as a consequence of its agglutinating ability (Ellis et al. 1985). Fertilization *in vivo* is reduced when antibodies against another epididymal luminal protein H1 antigen are instilled into the bursal cavity (Moore 1981).

Effects of Secreted Epididymal Proteins on Sperm-Zona Binding

EP3, a 23.8 kDa secretion product of the hamster epididymis (González Echeverría et al. 1982) is synthesized in the proximal corpus (Cuasnicú et al. 1984a). It is one of a mixture of epididymal proteins that can promote the binding of proximal corpus spermatozoa to the zona pellucida of hamster eggs *in vitro* (Cuasnicú et al. 1984b) and increase *in vitro* and *in vivo* fertilization by distal corpus spermatozoa (González Echeverría et al. 1984) but it has not yet been localized in the epithelium.

5.4 In the Rabbit

In the rabbit, antibodies to luminal fluid proteins R1, R2 and R3 all inhibit fertilization *in vivo* without inhibiting sperm motility (Moore 1981), suggesting a role for them in interactions between gametes. The function of acrosome stabilizing factor in protecting sperm head membranes and preventing the acrosome reaction is well documented in the rabbit (Wilson et al. 1987).

5.5 In the Pig

Effects of Secreted Epididymal Proteins on Sperm-Zona Binding

The function of the antagglutinin in fertilization has not been clarified, but epididymal proteins associated with the zona binding process have been demonstrated. The fact that caput spermatozoa, unlike corpus and cauda cells, could not bind to the zona pellucida (Peterson et al. 1984) prompted these workers to examine plasma membrane proteins in sperm cells of different maturity. Several proteins were present in plasma membranes from cauda, but not caput sperm, and those of 30, 45 and 70 kDa were associated with zona binding, as shown by their ability to block mature sperm-zona binding *in vitro*. Addition or exposure of these membrane proteins appeared upon epididymal transit, since proteins from cauda sperm could inhibit binding of mature cells to zonae, whereas those from the caput cells could not (Peterson et al. 1985), and antibodies to the cauda sperm proteins were not able to bind to the immature cells (Peterson et al. 1985; Peterson and Hunt 1989). Previous work had indicated that two plasma membrane proteins ("PM4.85" with 47 kDa and pI 4.7-5.2 and "PM5.0" with 44 kDa and pI 4.4-4.7; Russell et al. 1983) were present in greater amounts on cauda than caput sperm (Peterson et al. 1986). The 55 kDa "zona adhesion protein" (APz) is thought to be a minor component of the cell membrane (Peterson and Hunt 1989; Peterson et al. 1991).

5.6 In the Bull

Neither the localization in the epididymal epithelium, nor the domain of its association with luminal spermatozoa, is known for forward motility protein (FMP). No more has been heard of this protein, claimed to be a protein of bovine epididymal origin (Brandt et al. 1978), since much of its *in vitro* effects could be mimicked by anti-sticking agents (Stephens et al. 1981).

6 Pre-Existing Sperm Proteins

The addition of exogenous proteins to the surface of maturing spermatozoa is only one way in which the epididymis may modify maturing spermatozoa. Monoclonal antibodies raised against mature spermatozoa have not only detected some of these sperm-coating antigens, but also demonstrated antigens that are present on sperm as they enter the epididymis (see Eddy et al. 1985). Mature sperm antigens that are present in testicular spermatozoa include, in the mouse a 28 kDa antigen (1B3: Gaunt 1982) and an 200 kDa antigen (M42: Lakoski et al. 1988, 1989), in the rat a 40 kDa antigen (2B1: Gaunt et al. 1983), in the hamster a < 500 kDa antigen (Mabs 18.6 and 97.5: Moore et al. 1985; Ellis et al. 1985) and in the guinea pig an 70 kDa (PH-20: Phelps and Myles 1987).

In various species the localization or amount of immunolabelling of maturing sperm cells by these antibodies is altered in the epididymis: in the hamster, localization of 4 of 7 antigens is altered (Ellis et al. 1985), and in the pig 39 of 42 (Saxena et al. 1986b). These changing patterns of recognition may reflect the loss, masking, exposure or modification of pre-existing molecules in the immature cell membranes (see Jones 1989; Hall et al. 1991). How the epididymis is responsible for these modifications is only now beginning to be appreciated and is discussed below for proteins directly involved in fertilization.

6.1 Importance of Endogenous Sperm Proteins for Fertilization

In this section only sperm antigens with a putative rôle in fertilization will be addressed. In the mouse antibody M42 inhibits *in vitro* fertilization of zona-intact, but not zona-free eggs (Saling and Lakoski 1985), indicating interference with the process of zona binding. The antibody fails to stain the epididymal epithelial cells, indicating that this antigen is not an epididymal secretion product (Lakoski et al. 1988). In contrast, monoclonal M29 inhibits fertilization *in vitro* by zona-intact and zona-free eggs, suggesting an action of this antibody at the level of the vitellus (Saling et al. 1985). In the guinea pig, different monoclonal antibodies against mature spermatozoa also interfere with zona binding (PH-20: Primakoff et al. 1985) and sperm-egg fusion (PH-30: Primakoff et al. 1987).

Sperm enzymes may also be associated with sperm-egg recognition, *e.g.* a membrane-associated galactosyltransferase (GT) in the mouse (see Shur and Neely 1988) and proacrosin, an acrosomal proenzyme, present in testicular sperm in the boar (see chapter by Töpfer-Peterson this volume). Recently, changes to proacrosin have been shown to occur during maturation in the guinea pig epididymis (Anakwe et al. 1991), but exactly how the epididymal luminal environment modulates the activity of presumably intra-acrosomal enzymes remains to be clarified.

6.2 Changes in Endogenous Zona-Recognition Proteins on Maturation

M42 Antigen

The M42 antibody recognizes an antigen on the plasma membrane over the acrosome in sperm from all regions of the epididymis (Lakoski et al. 1988). However, it binds more to cauda and corpus cells than caput cells (240 and 185 %, respectively) and the antibody detects in Western blots a decrease in molecular size of the antigen: doublets of 195/210 or 220/240 exist in caput spermatozoa; 185/200 (235/260) in corpus spermatozoa and 185/200 (240/270) in cauda spermatozoa (Lakoski et al. 1988, 1989). As this maturational shift in molecular size did not occur in sperm retained for 2 weeks in the caput epididymidis

by ligation, an influence of epididymal secretions on the conversion was postulated. The nature of this influence has not been elucidated, although the antigen is sensitive to digestion by trypsin and collagenase (Lakoski et al. 1989). The relationship of the M42 antigen, facilitating the induction of the acrosome reaction, and P95 or sp56, putative tyrosine kinase-linked "zona receptors" within the sperm membrane (see Leyton and Saling 1989; Bleil and Wassarman 1990; Bunch and Saling 1991) is also not clear. Although it is present in caput spermatozoa, P95 is apparently uncoupled from the intracellular signalling systems present in mature cells.

PH-20 Antigen

In the guinea pig the antigen associated with zona binding (PH-20: Primakoff et al. 1985) is also present in testicular spermatozoa and is thus not an epididymal secretion product. Unlike the M42 antigen in the mouse, it changes its cytochemical localization in maturing sperm cells. In testicular caput and corpus epididymal sperm cells PH-20 is localized in both posterior and anterior head, as well as the tail. Sperm from the proximal cauda and more distally, however, bind most of the antibody over the posterior head (Phelps and Myles 1987). Whether this is due to changes in the sperm plasma membrane domain which recognizes the protein, or a change in the protein itself, and whether either of these changes is due to epididymal epithelial influences remains to be established. Like the 24 kDa integral membrane protein of rat spermatozoa (Moore et al. 1989), it is coupled to the membrane *via* phosphatidylinositol (Phelps et al. 1988). A shift in molecular size of PH-20 is also seen on maturation. Western blots of proteins extracted from maturing spermatozoa probed with the antibody reveal 68-70 kDa forms in the testis (range 63-70 kDa) but only 64 kDa (range 61-68 kDa) in cauda cells (Phelps and Myles 1987). This may also represent a shift to a form that can better interact with the zona pellucida in this species.

Galactosyltransferase

Significant evidence exists which supports the view that a sperm surface galactosyltransferase (GT) mediates sperm-zona binding in the mouse (Shur and Hall 1982a,b; DeGeyter et al. 1989). However, the

poor ability of caput sperm to bind to zonae, when studied under the same conditions as mature sperm cells (Saling 1982), is not related to the total enzyme activity per cell, which remains constant as sperm mature in the epididymis (Scully et al. 1987). A marked redistribution of the enzyme within the membrane is evident, however, when the sperm mature, with the enzyme signal changing from a diffuse to a discrete localization over the mature acrosome. The enzyme activity, when normalized per cell area, in immature spermatozoa is some 60% of those on cauda sperm (Scully et al. 1987). It has also been argued that inhibitors in epididymal fluid could reduce the inherent ability of caput spermatozoa to bind to the mouse zona, since caput sperm that are thoroughly washed (and centrifuged) can bind to zonae (McLaughlin and Shur, 1987). Despite the extreme fragility of rodent sperm to such treatments (Bunch and Saling 1991), these sperm are reported to be 82 % as effective as cauda sperm in binding to the zona pellucida (McLaughlin and Shur 1987). This implies that concomitant with the changes in "zona-receptors" that facilitate binding to the zona pellucida, is the addition of decapacitation factors that prevent their untimely exposure in the epididymis.

6.3 Changes in Endogenous Vitellus-Recognition Proteins on Maturation

The antibody PH-30 that inhibits guinea pig sperm-egg fusion is detected immunochemically for the first time in spermatozoa from the proximal cauda epididymis (Primakoff et al. 1987), but it is not known if this is an epithelial secretion product or pre-existing protein that becomes unmasked.

7 The Changing Response of Maturing Sperm Cells to Stimuli

Recent work has complemented the earlier identification of simple differences in "basal" activity of maturing sperm cells with functional differences in their responses to stimuli.

7.1 In the Ram

Williams et al. (1991) studied the maturation of ovine spermatozoa by examining the time course of the initiation of motility and induction of acrosome reaction by cells taken from different regions of the epididymis in unstimulated conditions *in vitro*. They confirmed the well known improvement in motility and ability to bind to, and penetrate, zona-free hamster eggs that accompanies maturation and were also able to show that spermatozoa from proximal regions of the tract were able to undergo acrosome reactions, but that they required more time compared to mature cells. The immature cells released less acrosin than mature cells and the calcium ionophore A23187 was only able to stimulate acrosome reactions in the more mature cells. The authors considered that these differences between mature and immature cells reflected changes in the sperm membranes that were previously shown to occur upon maturation in the epididymis in this species (Schlegel et al. 1986). In further studies, short-term incubation with dilauryl-phosphatidylcholine (PC12) promoted motility and acrosome reactions in caput and corpus spermatozoa, but the ability of these cells to fuse with zona-free hamster eggs was not improved (Graham et al. 1991). These results indicate that other maturational events occur to proximal cells before they are able to respond to external stimuli with a full fertilizing response.

7.2 In the Mouse

Unlike immature ovine spermatozoa, those from the mouse do respond with an acrosome reaction when challenged with the ionophore A23187 (Lakoski et al. 1988). However, more “physiological” stimuli (*i.e.* zona pellucidae) initiate acrosome reactions only in cauda and corpus cells: caput sperm cells are unresponsive (Lakoski et al. 1988). Since the M42 antibody prevents zona-induced acrosome reactions in mature cells, the maturational changes observed in molecular weight of the M42 antigen (dependent on epididymal epithelial activity) may be intimately connected with the development of the ability of maturing sperm cells to respond to such physiological stimuli.

7.3 In Other Species

The findings above are reminiscent of the findings of Rifkin and Olson (1985) that the cauda secretory protein HIS-50 is able to bind to rat corpus spermatozoa, but not caput sperm cells at scrotal temperature *in vitro*. As this interaction is electrostatic (the protein is not eluted with 400mM NaCl), the charge on caput sperm may be inadequate for binding to occur and the prior addition of glycoproteins may be necessary for the attachment of HIS-50. Another demonstration of sequential changes in sperm membranes on maturation is the failure of distal corpus spermatozoa from the hamster epididymis to fertilize eggs *in vitro* or *in vivo* after co-incubation with cultured proximal cauda epithelial cells, unless they have been held in the ligated corpus epididymal duct for 3 days (Moore and Hartman 1986; Moore HDM et al. 1987; Moore and Smith, 1988). Again, changes to the spermatozoon must occur during this time for them to respond to the epithelial cell secretions. The lipid or glycolipid-binding protein secretion products of the epididymal epithelium (see above) or of cholesterol binding proteins in luminal fluid in the ram (unpublished data cited in Graham et al. 1991) may mediate these changes.

8 Summary

As spermatozoa are moved through the epididymis they enter an ever changing environment which is a product of the various synthetic and secretory pathways of the epididymal epithelial cells lining the duct. Although the organic and inorganic components of the fluid, and the sequence in which they come into contact with maturing sperm cells, have been known for some time, the nature of the proteins and the specificity with which they bind to certain subdomains of the sperm cell membrane is only now becoming appreciated. Some of the proteins are likely to be transport proteins bringing to, or taking from these cells, ions and lipids that are presented to them *via* the epididymal epithelium from the circulation. The luminal environment is responsible, in as yet undefined ways, for modifying the size and location of proteins already present in testicular spermatozoa, that, in various species, have

roles in both zona-binding (M42, PH-20, GT) and vitellus (PH-30) recognition. The epithelium is also responsible for the elaboration and secretion of specific proteins that bind to various regions of the sperm cell as they pass through the organ. The order with which the sperm cells come into contact with these proteins may well be important for their resultant physiological effects on the sperm cell, with the secreted proteins only being able to bind, for example, after prior modification of the membrane charge or lipids. Some of these exogenous proteins are also involved in various aspects of fertilization: inducing motility (? DE), changing flagellar beating patterns (FMP?), necessitating a capacitation process (ASF), promoting zona-binding (APz, DE, EP3) or fusion with the vitellus (? DE, 2D6). All these findings provide evidence that, for nearly every step in the fertilization process, spermatozoa are influenced in some way by epididymal secretions.

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Physiology and Pathophysiology of Electrolyte Transport in the Epididymis

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1 Introduction

Although it was eighty years ago that early investigators of the functions of the epididymis noted certain differences in the characteristics of the spermatozoa obtained from different regions and proposed that

spermatozoa undergo a 'ripening' process during their epididymal passage (Tournade 1913), it was not until fifty-five years later that the principle of epididymal maturation of spermatozoa was refined with solid experimental supports (Bedford 1967; Orgebin-Crist 1967, 1969). It is now clear that important maturation changes take place in spermatozoa in the epididymis of most mammals including man and that these changes confer on the sperm the ability to fertilize eggs. Despite this, the mechanisms underlying sperm maturation are still obscure. To date, technological advances have made possible the study of epididymal functions at a cellular and molecular level and have brought light to our understanding of the underlying processes. Significant progress has been made in the area of androgen-regulated genes that control the biosynthesis and secretion of epididymal-specific macromolecules which are integral to the maturation of sperm plasma membrane. Interested readers are referred to the following excellent papers on this topic: Brooks 1987; Ghyselinck et al. 1990; Walker et al. 1990. The present chapter describes some studies made in our own laboratory on epididymal transport of electrolytes and fluid. Undoubtedly, this aspect of epididymal function is equally important for the maintenance of a favourable fluid environment on which sperm maturation and storage depend (Wong et al. 1982; Wong 1986). The introduction of cell culture techniques for the rat (Kierzenbaum et al. 1981, Beyers et al. 1986) and human (Cooper et al. 1990) epididymis facilitated study of the mechanism and regulation of electrolyte transport by the epididymis by the formation of an epithelial sheet on which transepithelial electrical (Cuthbert and Wong 1986; Wong 1988a, 1988b, 1988c), isotope flux (Fu et al. 1990) and microfluorescence (Wong and Huang 1990) measurements can be made on single cells as well as on cell monolayers. Furthermore, epididymal cell culture also permits access of patch-clamp pipettes to the luminal side of the epithelium so that the conductive properties of this membrane and its activation by intracellular messengers can be studied (Cook et al. 1990; Wong 1990c).

2 Electrolyte Transport by the Epididymis

2.1 Sodium Absorption

The epididymides of many mammalian species do not merely serve as a passive conduit for the sperm but play a dynamic role in influencing the environment in which the sperm exist. The epithelial cells lining the duct actively reabsorb sodium and water (Wong and Yeung 1977, 1978), and as a result, a major part of the testicular fluid returns to the blood by the time the testicular content reaches the cauda epididymidis (Levine and Marsh 1971; Turner et al. 1977). Water absorption is driven by active sodium absorption which is dependent on the presence of selective sodium channels in the apical membrane. Hence, sodium reabsorption is inhibitable by the Na^+ channel blocker, amiloride (Wong and Yeung; 1976, Wong YC et al. 1978) and is dependent on the $\text{Na}^+-\text{K}^+-\text{ATPase}$ in the basolateral membrane of the cell. There appear to be few endogenous substances that can regulate this sodium absorptive process in the epididymal epithelium though testosterone (Wong and Yeung 1977a) and mineralocorticoids (Au et al. 1978) are required to maintain a basal rate of absorption. Antifertility agents are also known to affect fluid reabsorption in the rat epididymis (Wong and Yeung 1977b; Wong et al. 1977).

2.2 Anion Secretion

In recent years, it has become apparent that exchange of electrolytes and water across the epithelium occur in both directions and that the epithelial cells can be driven to secrete chloride and bicarbonate (and hence fluid) when stimulated with β -adrenergic agonists (Wong and Chan 1987; Wong 1988a) and peptide hormones (Cuthbert and Wong 1968; Wong et al. 1989; Wong et al. 1990b; Wong et al. 1991a; Leung et al. 1992). In common with many other fluid secreting exocrine glands, secretion is mediated by a $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ symport, a Na^+-H^+ and a $\text{Cl}^--\text{HCO}_3^-$ exchanger located in the basolateral membrane of the cells (Wong 1988a, 1990). These membrane carriers utilize the energy derived from the Na^+ gradient (generated by basolaterally placed Na^+ -

K^+ -ATPase) to accumulate Cl^- and HCO_3^- inside the cell to above electrochemical equilibrium. When the cells are stimulated, there is a rise in cyclic adenosine 3',5' monophosphate (cAMP) which opens the Cl^- and HCO_3^- channels in the apical membrane allowing chloride and bicarbonate to exit from the cell and initiates secretion (Wong and Huang 1989). The K^+ channels in the basolateral membrane also play a major but probably permissive role by hyperpolarizing the membrane during secretion, hence maintaining a favourable electrochemical driving force for anion efflux (Wong 1989).

This secretion model has been derived from studies employing the short-circuit current, patch-clamp and microspectrofluorimetric techniques on cultured rat and human epididymal cells. Detailed account of these studies have been published and cannot be fully documented here. However, those which have direct bearings on the pathophysiology of obstructive azoospermia are given below:

1. Adrenergic agonists stimulate a rise in electrogenic anion secretion in cultured rat (Wong and Chan 1987; Wong 1988a) and human epididymal epithelia (Wong et al. 1991b) measured by the short-circuit current and the Ussing chamber technique. This increase in secretion was found to be inhibited by the addition of diphenylamine 2-carboxylate, a chloride channel blocker, to the apical bathing solution (Wong 1988b) or bumetanide, a blocker of the $Na^+-K^+-2Cl^-$ symport, to the basolateral side (Wong et al. 1990a). Addition of putative K^+ channel blockers to the basolateral side also inhibits the secretory response to secretory agonists indicating that the basolateral K^+ channels also play a role (Wong 1989).
2. Chloride channels with single channel conductance of about 8 pS has been identified in the apical membrane of cultured rat epididymal cells (Cook et al. 1990) and human fetal epididymal cells (Pollard et al. 1992). These channels exhibit slow kinetics and show linear I/V relationship resembling the 4-6 pS cAMP activated, non-rectifying chloride channels present in the pancreatic ductal cells (Gray et al. 1988; Gray et al. 1990). The regulation of the opening of the low conductance Cl^- channel is believed to be defective in cystic fibrosis (CF) (Berger et al. 1991).

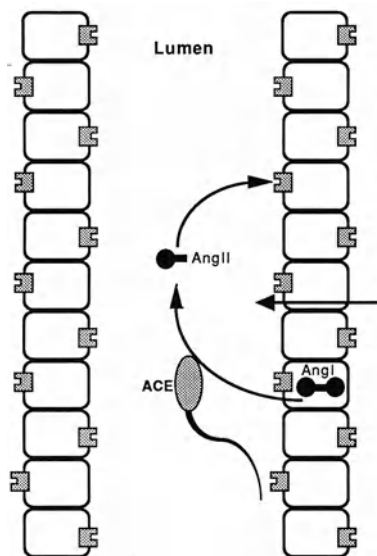


Fig. 1. Schematic diagram illustrating how intraluminal angiotensin II (AngII) controls anion (and fluid) secretion by the epididymis in the rat. Angiotensin I (AngI) formed by epididymal cells is secreted into lumen where it is converted by the sperm angiotensin-converting enzyme (ACE) into AngII. This octapeptide formed intraluminally interacts with the apical angiotensin receptors. This local control allows spermatozoa to regulate the fluid volume of their own environment (Wong and Uchendu 1990)

3. Chloride secretion is found to be stimulated by angiotensins (Wong et al. 1990b), bradykinin (Cuthbert and Wong 1986), endothelin (Wong et al. 1989; Wong et al. 1991c), calcitonin gene-related peptide (Leung et al. 1992). With the exception of the calcitonin gene-related peptide, the effects of these hormones are mediated through an increase in endogenous prostaglandin synthesis.
4. Secretion is subject to feedback control by spermatozoa acting from the luminal side of the epithelium. The likely substances that mediate this effect include sperm ATP acting on apical P₂-purinoceptors (Wong 1988c), and angiotensins formed *de novo* within the epididymal lumen by virtue of the high angiotensin converting enzyme

activity (ACE) inherent within the sperm (Wong and Uchendu 1990). It has been shown that rat epididymal cells in culture express renin and can form angiotensin I, which, in the presence of sperm ACE, can be converted to angiotensin II (Wong and Uchendu 1991). The endogenous angiotensin II formed *in vivo* then interacts with angiotensin receptors on the apical membrane of the epididymal cells and stimulate fluid secretion (Wong et al. 1990b). This mechanism constitutes a means by which spermatozoa can control the fluidity of their own environment (Fig. 1).

3 Adrenergic Stimulation of Anion Secretion

3.1 Receptor Subtypes Involved

Previous work has shown the stimulation of anion secretion by adrenergic agonists was largely mediated by the β -receptor, as propranolol markedly reduced the adrenaline-evoked increase in the short-circuit current (Wong and Chan 1988). However, more careful work employing specific agonists and antagonists as pharmacological tools have revealed that α -, β_1 - and β_2 - adrenoceptors are all involved. Adrenaline added to the basolateral side of the epithelium elicited a complex response which was seen to be made of at least 2 time-dependent components, an initial spike which was mediated by α - adrenoceptor activation followed by a sustained second phase mediated by the β -adrenoceptors (Fig. 2). The initial component became more prominent when a more potent α -adrenoceptor agonist, noradrenaline was used (Fig. 2). In all cases, the initial spike response was blocked by phentolamine while the second sustained phase was blocked by β -adrenergic blockers. Both atenolol and butoxamine, β_1 -selective and β_2 -selective antagonist, respectively, competitively inhibited the response of the sustained phase to adrenaline, indicating that the sustained response was in turn mediated by β_1 - and β_2 - adrenoceptors (Fig. 3). The significance of the involvement of multiple receptor subtypes in the adrenergic stimulation of secretion by the epididymal cells is unclear, although the major part of the response appears to be mediated by β -adrenoceptor activation.

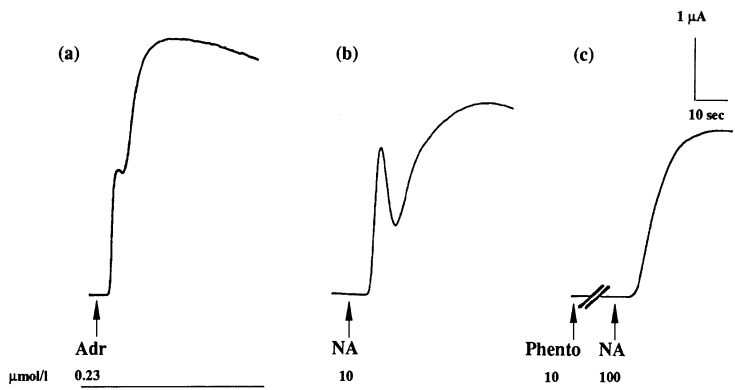


Fig. 2 a-c. Measurement of electrogenic anion secretion as short-circuit current (Isc) in three cultured rat epididymal epithelia (area 0.4 - 0.6 cm²). **a** Adrenaline (Adr, 0.23 mol/l), **b** noradrenaline (NA, 10 mol/l), and **c** phentolamine (Phento, 10 mol/l) followed by noradrenaline (NA, 100 mol/l), were added to the basolateral side of the epithelia. The arrows indicate the time at which the agents were added and the horizontal lines indicate zero SCC. Each record is representative of 10 to 15 separate experiments

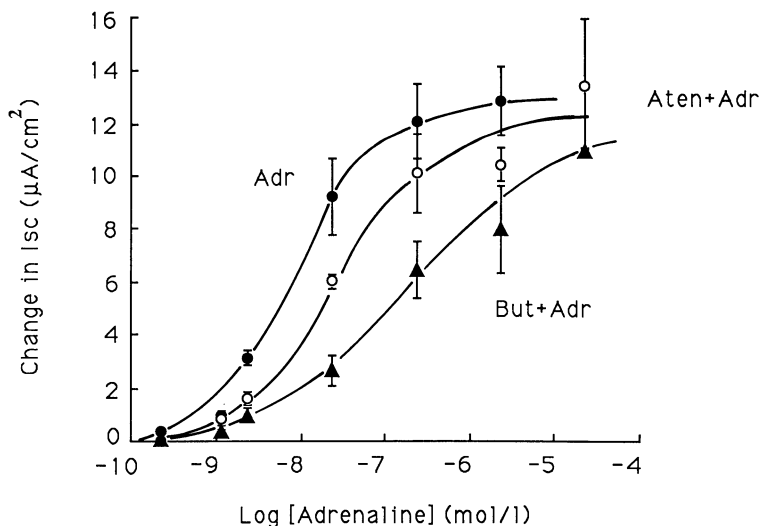


Fig. 3. Inhibition of adrenaline-induced secretion by β_1 - and β_2 -adrenoceptor antagonists. Concentration-response curve for adrenaline when adrenaline was added alone (●), or after pretreatment with atenolol, a β_1 -adrenoceptor antagonist (○), or with butoxamine, a β_2 -adrenoceptor (▲). Each point represents mean $\pm$ S.E.M. from 4 to 9 experiments. The response was measured as an increase in short-circuit current (Isc) evoked by adrenaline

3.2 Signal Transduction Mechanisms

In general, two signal transduction mechanisms mediate the secretory response of exocrine glands to autonomic nerve stimulation (Putney 1985; Donowitz and Welsh 1986). The stimulating effect of β -adrenergic agonists is transduced by cAMP-dependent regulatory pathway whereas the effects of α -adrenergic and cholinergic stimulation are mediated by a rise in intracellular calcium (Liedtke 1989). Epididymal

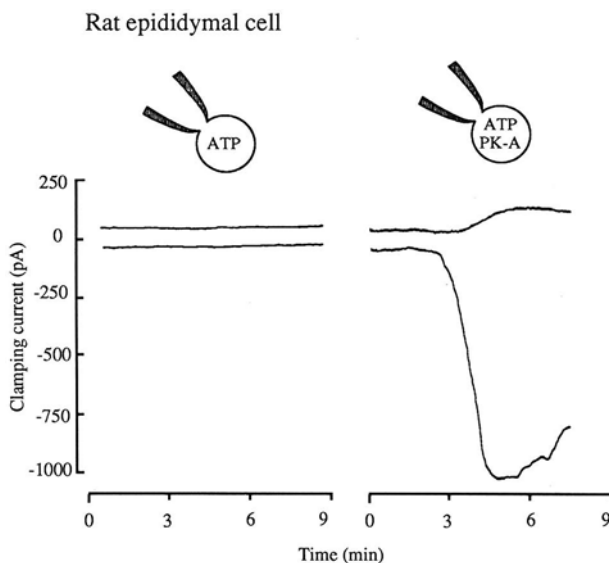


Fig. 4. Effect of cAMP-dependent protein-kinase (PK-A catalytic subunit) on whole-cell current in cultured rat epididymis. Pipette solution contained (in mmol/l): KCl (135), Na_2HPO_4 (1.2), MgCl_2 (1), HEPES (10) and glucose (11). The Ca^{2+} level was maintained at 120 nmol/l using EGTA- Ca^{2+} buffer. Bath solution contained (in mmol/l): NaCl (130), KCl (5), Na_2HPO_4 (1.2), MgCl_2 (1), CaCl_2 (1), HEPES (10) and glucose (11). The membrane potential was alternately clamped at 0 mV (for 1.5 s) and -80 mV (for 0.5 s) to study K^+ and Cl^- current respectively. Only the outline of the recordings is shown. *Left panel:* Inclusion of ATP (2 mmol/l) in the pipette had no effect on whole-cell current. *Right panel:* Inclusion of PK-A catalytic subunit (500 pmol/l) plus ATP (2 mmol/l) in the pipette evoked a Cl^- current 3 min after the formation of the whole-cell configuration

cells express mechanisms for both signal transduction pathways. The evidence for a role of cAMP in β -adrenergic stimulation is as follows: First, adrenaline evokes a short-circuit current response which is mimicked by exogenously applied cAMP or forskolin, a direct activator adenylate cyclase (Wong 1988a). Secondly, we demonstrated a 3-fold increase in cAMP concentration upon stimulation of the rat epididymal

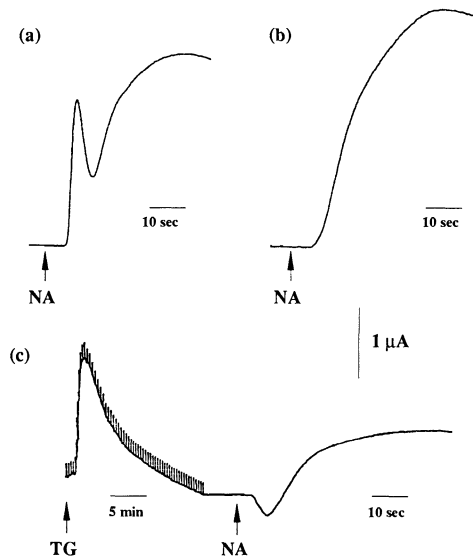


Fig. 5 a-c. Effect of chelating cytosolic Ca^{2+} with BAPTA-AM or depleting internal Ca^{2+} store with thapsigargin on the secretory response to noradrenaline. Short-circuit current (Isc) was measured in three separate rat epididymal monolayers (0.4 to 0.6 cm^2). **a** Control experiment in which noradrenaline was added to the basolateral side of the epithelium (NA, 10 mol/l). **b** Tissue was incubated with 100 mol/l BAPTA-AM for 30 min at 32°C before mounting in the Ussing Chambers for Isc measurement. Noradrenaline was added to the basolateral side of the epithelium (NA, 10 mol/l). **c** Tissue was treated with thapsigargin (TG, 1 mol/l) added to the apical side. This resulted in an increase in SCC due to release of Ca^{2+} from internal store. When the current has returned to the basal level, noradrenaline was added to the basolateral side (NA, 10 mol/l). The time scale has been expanded during response to noradrenaline. Note that BAPTA-AM (**b**) or thapsigargin (**c**) treatment abolished the initial spike in the responses to subsequent additions of noradrenaline

cells with adrenaline (Wong and Huang 1990). In parallel studies using the whole-cell tight-seal patch-clamp method, adrenaline induces a rise in whole-cell chloride current in single rat (Huang et al. 1990) and human (Wong et al. 1991b) epididymal cells. This effect can be mimicked by forskolin and blocked by propranolol (β -adrenergic antagonist) (Huang et al. 1991; Wong et al. 1991b). Likewise, agents which are known to interfere with the cAMP pathway were used and found that the adrenaline-induced rise in whole-cell chloride current was abolished when GDP β S, an inhibitor of G protein, or protein kinase A inhibitor was included in the patch pipette (cell cytosol) (Wong et al. 1991b). Finally, inclusion into the patch pipette (cell cytosol) of a phosphorylating 'cocktail' (C subunit of protein kinase A and ATP) evokes a whole-cell chloride current response in the rat and human epididymal cell indistinguishable from that of adrenaline (Fig. 4).

These pieces of evidence strongly support the contention that cAMP pathway is present in the epididymal cells and stimulation of this pathway by β -adrenergic agonists leads to secretion of anion and hence, osmotically, to water. Unlike the β -adrenoceptor agonists, the effect of α -adrenoceptor activation responsible for the initial spike is mediated by a rise in intracellular calcium. The evidence for this contention came from studies using BAPTA-AM and thapsigargin. The former compound is a membrane permeable calcium chelator (McCann et al. 1990) and the latter a microsomal Ca^{2+} uptake inhibitor (Thastrup et al. 1989) which has previously been shown to cause release of intracellular stored Ca^{2+} (Hanley et al. 1988). If the epididymal cells were first pretreated with these two agents to chelate cytosolic Ca^{2+} or to deplete internal Ca^{2+} store, subsequent addition of noradrenaline or adrenaline did not elicit the initial spike although the second sustained phase remained (Fig. 5). Results of these studies suggest that the α -adrenoceptor-evoked spike response is mediated by a release of Ca^{2+} from internal store and the sustained response is mediated by cAMP as second messenger. Microfluorimetric measurement on isolated rat epididymal cells has shown an increase in intracellular Ca^{2+} upon stimulation of the cell with noradrenaline (Wong et al. unpublished).

3.3 Second Messenger-Induced Membrane Conductance Changes

Not only are the α - and β -adrenergic effects mediated by different second messengers, the final effector events of different receptor-mediated agonists are expressed differently. The existing cell model of anion secretion by the epididymis and other exocrine glands in general features an apically located anion conductive pathway. Alteration of its activity by neurotransmitters and hormones is a principal control point determining the rate of chloride secretion (Welsh 1987). Under basal conditions, the conductance is low but can be activated by cAMP and Ca^{2+} upon stimulation of the cells with secretory agonists (Liedtke 1989). It has become increasingly apparent from studies in exocrine tissues, such as the Cl^- -secreting colonic epithelial cell line (Cliff and Frizzell 1990), that the conductances activated by cAMP and by Ca^{2+} have different properties with respect to the selective permeability to halides, time- and voltage-dependence of the second messenger-induced currents and sensitivity to anion channel blockers. For example, the cAMP-induced current showed no time- and voltage-dependent changes during voltage pulses. In contrast, the Ca^{2+} -activated current was outwardly rectified, activated during depolarizing voltage pulses, and inactivated during hyperpolarizing voltage pulses. We have shown that these features are also shared by the rat epididymal cells. Short-circuit current and whole-cell patch-clamp studies have revealed that the apical conductance evoked by cAMP was time- and voltage-independent with a halide permselectivity in the sequence $\text{Br}^- > \text{Cl}^- > \text{I}^-$, whereas the conductance evoked by Ca^{2+} was time- and voltage-dependent with the halide permselectivity sequence $\text{I}^- > \text{Br}^- > \text{Cl}^-$ (Wong et al. unpublished data). Evidence is accumulating that in cystic fibrosis, the cAMP-activated conductance is not functional (Reddy et al. 1991) (see below). The resulting decrease of anion and hence fluid secretion (and increase in absorption) in response to β -adrenergic stimulation has led to respiratory failures seen in cystic fibrosis (CF). It is interesting to note that the Ca^{2+} -activated anion conductance appears to be unaffected in cystic fibrosis (Boucher et al. 1989).

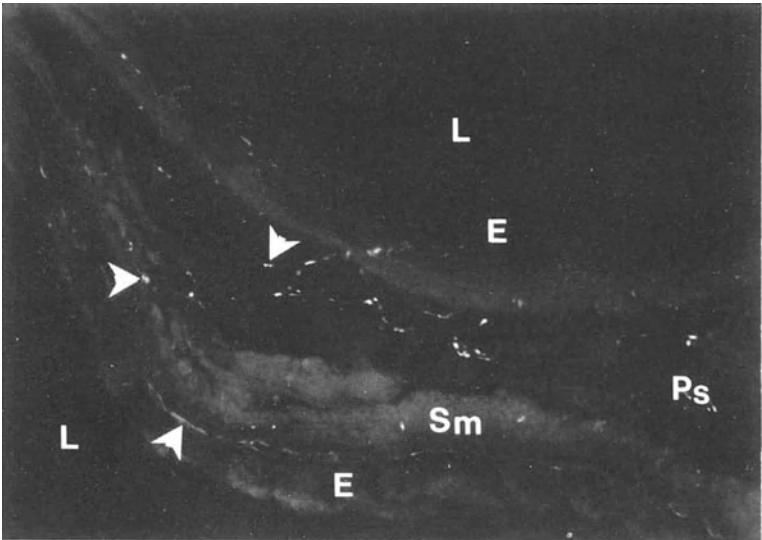
4 Adrenergic Innervation of the Epididymis

The adrenergic mechanisms described above may mediate the effect of sympathetic stimulation on electrolyte transport by the epididymis. Marked regional differences in the sympathetic innervation of the epididymis has been observed by earlier workers (Sjöstrand 1965; El-Badawi and Schenk 1967). In the proximal part of the epididymis the terminals of the sparsely distributed adrenergic neurons lie generally in close association with the blood vessels. However, in the cauda, rich networks terminate not only in the blood vessels and muscle layers but adrenergic nerve fibres have been seen to penetrate the basal membrane, forming an arborization network at the basal regions of the epithelial cells (Fig. 6). The interrelationships between the nerves and the epithelium are only poorly known and stimulation of electrolyte and water secretion may be one effect of increased sympathetic activity to the epididymis.

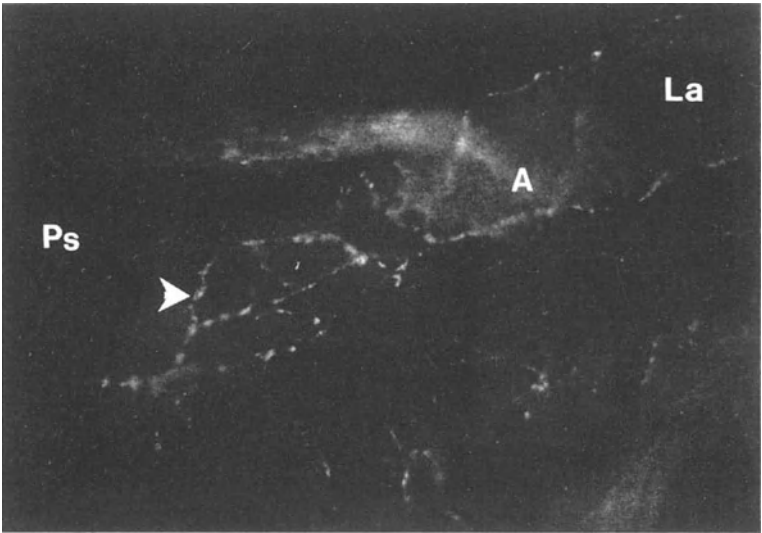
5 Electrolyte and Water Transport and Epididymal Fluid Environment

Both the absorptive (driven by active sodium transport) and secretory processes (driven by active anion secretion) described above influence water movement, outwards and inwards, respectively, across the epithelium. This is possible because the epididymal epithelium is water permeable. The presence in the epididymis of these transport processes with a predominant absorptive process plus a secretory process that

Fig. 6 a,b. Immunofluorescence staining of noradrenergic fibres in the cauda epididymidis of rats. The enzyme involved in the synthesis of noradrenaline from dopamine, namely, dopamine β hydroxylase (DBH) was used as a marker for the fibres (Eugene Tech International, Inc., New Jersey 07401, U.S.A., rabbit anti-rat DBH, 1:50 dilution). **a** Nerve fibres (*arrow heads*) were seen in the basal aspect of the epithelium (*E*), peritubular smooth muscle layer (*Sm*) as well as the peritubular interstitial space (*Ps*). No immunostaining was found in the lumen of the tubule (*L*) (x400). **b** Nerve fibres were also found in peritubular arteriole (*A*). No immunostaining was found in the lumen of the arteriole (*La*) (x400)



a



b

can be activated and regulated rapidly by autocoids and transmitters, provides the opportunity for fine tuning of the thickness of the fluid phase in the epididymal lumen where spermatozoa are bathed. Under normal conditions, absorption and secretion are matched and spermatozoa are bathed in a normal fluid environment. However, under pathological conditions, secretion may be decreased due to abnormal regulation by neurotransmitter or hormones leading to a disrupted fluid environment. This phenomenon is seen in the genetic diseases such as cystic fibrosis (CF), Young's syndrome, or in other chronic sinopulmonary diseases in which the patients have coexisting chronic infection of the nasal sinuses and the lung and obstructive azoospermia (see below). In all these cases, excessive secretion of mucoproteins by the epididymal cells without water secretion would reduce the fluidity of the lumen, depositing thick viscid mucous which blocks sperm passage (Wong 1990). Decreased fluid secretion results from failure of the cells to secrete anions and fluid in response to adrenergic nerve stimulation. An experimental correlate of this phenomenon was seen when male rats were treated with reserpine to disrupt adrenergic innervation of the epididymis (and other tissues). This treatment led to a decrease in fluid secretion by the epididymal tubule as evident from a rise in the thickness (viscosity) of the epididymal fluid with a concomitant increase in sperm and intraluminal protein concentrations (Wen and Wong 1988).

6 Molecular Basis of Abnormal Fluid Secretion

Much of our insight into the pathophysiology of obstructive azoospermia has been gained from CF research. Cystic fibrosis is the most fatal genetic disease in Caucasians. The disease characteristically affect three exocrine glands, namely sweat glands, pancreas and the airways, although other epithelial tissues such as the intestine and salivary glands are also involved to a lesser degree. In all these tissues, the primary disturbance is abnormal fluid secretion leading to obstruction of the ductal part of the gland with inspissated material.

It is now known that these manifestations of the disease have one common cause. The secretion of chloride (and hence fluid) by the epithelium in response to adrenergic agonists is impaired, although there

is evidence that sodium reabsorption is increased also (Cotton et al. 1987). Electrophysiology of cultured CF airway epithelial cells has localized the defect to an apical membrane that is impermeable to Cl (Widdicombe et al. 1985) and suggests a defect in Cl channel function in CF. Later, the patch-clamp technique was used to study single-channel currents in CF airway epithelial cells (Frizzell et al. 1986; Welsh and Liedtke 1986). It was found that in excised patches, apical membrane Cl⁻ channels were present, and their conductive and kinetic properties were normal. However, when channel activities were studied in the cell-attached mode, Cl⁻ channels did not open normally in response to agonists that stimulate cAMP production. There is no fault at the level of signal transduction because CF cells can generate cAMP in response to stimulation with secretomotor agonists. The defect must lie at a site distal to cyclic AMP generation. Experiments using subunit C of the cAMP-dependent protein kinase on excised membrane patches of the CF airway cells have further localized the defect to the regulatory protein of the apical Cl⁻ channels (Schoumacher et al. 1987). There appears to be a phosphorylation defect at a stage later than either cAMP generation or the activation of protein kinase A. The Cl⁻ channels which are responsible for CF have been identified to have small (a few pS) single channel conductance. They are nonrectifying and with anion permselectivity in the sequence Br > Cl > I. Chloride channels with these characteristics have also been found in the apical membrane of the rat epididymal cells (see section 2.2 above). They form the single-channel events that underly the whole-cell chloride current described earlier in the chapter.

7 Cystic Fibrosis Transmembrane Conductance Regulator

The discovery of the CF gene in 1989, together with the presumed structure of the protein product, has taken cystic fibrosis research one step forward. By means of a painstaking combination of chromosome walking and jumping and complementary DNA (cDNA) hybridization over half a million base pairs on the long arm of chromosome 7 band q 31 have been sequenced (Rommens et al. 1989; Riordan et al. 1989; Kerem et al. 1989). The putative protein coded for by the cDNA for

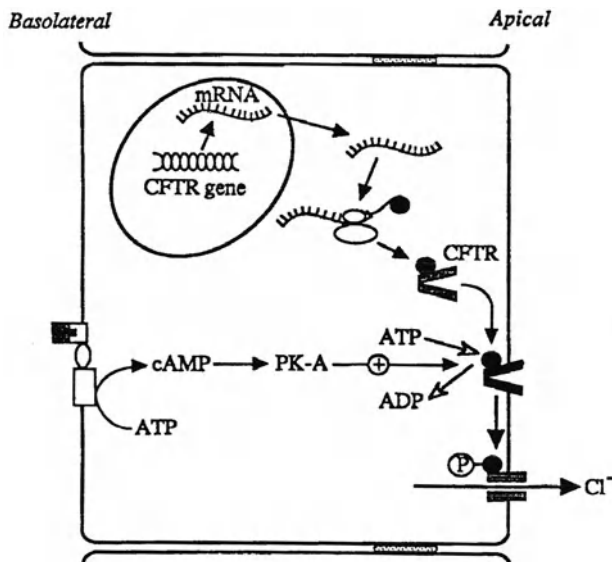


Fig. 7. Schematic diagram showing the role of cystic fibrosis transmembrane conductance regulator (CFTR) in chloride and fluid secretion by the epididymis. The CFTR gene encodes a protein CFTR which is associated with the apical (luminal) membrane of the epididymal cells. When the cells are stimulated the cAMP generated phosphorylates this protein (through PK-A) which confers a chloride conductance on the apical membrane of the cells. The result is an increase in chloride and bicarbonate secretion into the lumen followed by sodium and a net water secretion ensues

the cystic fibrosis locus consists of 1480 amino acids. The tri-nucleotide codon for the 508th amino acid from the N terminus (phenylalanine) was found to be deleted in 70% of patients with cystic fibrosis. On the basis of analogy and homology a tentative structure for the protein coded for by the gene has been deduced. The protein has been named the cystic fibrosis transmembrane conductance regulator (CFTR).

CFTR is a membrane-associated glycoprotein that can be phosphorylated *in vitro* by cAMP-dependent protein kinase. It is intimately related to the chloride channel of the CF epithelial cells (Fig. 7). How-

ever, it is not known whether CFTR is the Cl^- channel or a regulatory component linked to the Cl^- channel. What is known is that it is responsible for the increase in Cl^- conductance when phosphorylated (by the cAMP-dependent protein kinase) during stimulation of the cells with β -adrenergic agonists. In CF, mutation of the CF gene has resulted in mutant CFTR (F508) which cannot be phosphorylated by the cAMP-dependent protein kinase and hence the chloride channel cannot be activated during stimulation. Antibodies against human CFTR have been developed and preliminary results show that antibodies stained colonic epithelial cells, pancreatic duct and a number of CF epithelia but no staining was observed in non-epithelial tissues such as fibroblasts (Cohn et al. 1991, Puchelle et al. 1991). This would be expected if CFTR plays the role of chloride conductance in CF epithelia. The physiological role of CFTR has been confirmed by complementation studies. Introduction of plasmids containing cystic fibrosis transmembrane conductance regulator cDNA into cultured cystic fibrosis cells restores a chloride conductance response to cyclic AMP (Rich et al. 1990). More recently, Anderson et al. (1991) demonstrated that the cAMP-activated Cl^- conductance can be conferred on non-epithelial cells by expression of CF gene product.

8 The Role of CFTR in the Epididymis

Although no experiment has yet been performed on CF epididymal tissues, our electrophysiological measurements performed on normal rat and human epididymides have provided strong evidence that the epididymis is a CF tissue. The cellular mechanisms and neurohumoral control of secretion in the epididymis and the airways are very similar and this prompts us to speculate that the two tissues have similar properties with respect to their physiology and pathology. It has long been known that men with CF who have survived to adulthood are infertile because of obstructive azoospermia (di Sant'Agnese 1967, Seale et al. 1985). Sometimes the epididymis is absent and this morphological change may be secondary to defective secretion. Obstruction is caused by thick inspissated material blocking the lumen, a situation very similar to that seen in the airway and pancreatic ducts of CF pa-

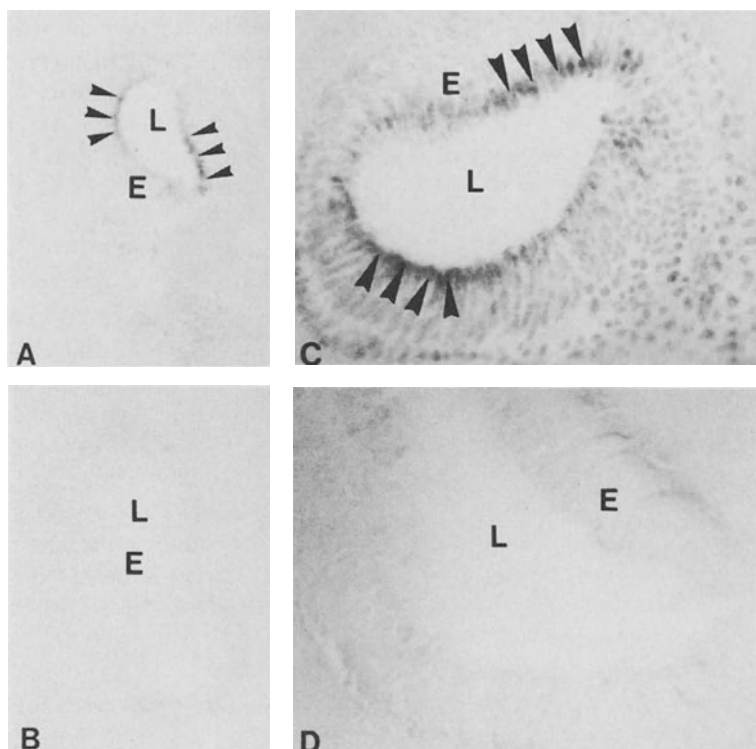


Fig. 8 A-D. Immunohistochemical staining of CFTR in human epididymis using mouse anti-human CFTR monoclonal antibody (Genzymes, Cambridge, MA, USA, Product code 1660-01, directed against R-domain of CFTR). **A** shows an epididymal tubule with its lumen (*L*) and epithelial lining (*E*). Immunoreactive CFTR (arrow heads) was seen at the luminal aspect of the epithelium (x50). **B** shows the buffer control of **A**. During the staining procedure, the phosphate buffer saline was added instead of the anti-CFTR antiserum. No immunostaining for CFTR was observed (x50). **C** shows an epididymal tubule at higher magnification with its lumen (*L*) and epithelial lining (*E*). Immunoreactive CFTR (arrow heads) was observed in the apical cytoplasm of the cells (x125). **D** shows the buffer control of **C**. No immunostaining was seen (x125)

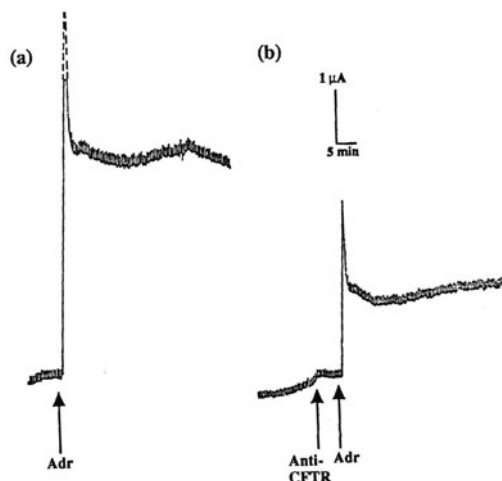


Fig. 9 a,b. The effect of the anti-human CFTR antibody (added to the apical side) on the short-circuit current (I_{sc}) response of a cultured human epididymal epithelium to basolateral application of adrenaline (**b**). The control response in another monolayer from the same batch of cells is shown in (**a**). The arrows indicate the time at which the agents were added. The two records were obtained from the same batch of cells after 52 days in culture

tients. One piece of direct evidence came from histochemical localization of CFTR in the epididymis using mouse anti-human CFTR monoclonal antibody (Genzymes, Cambridge, MA., USA, Product code 1660-01, directed against R-domain of CFTR). Staining was seen predominantly in the apical pole of the intact human epididymal epithelium (Fig. 8) as is expected if CFTR is linked to the cAMP-activated chloride channel in the apical membrane of the cell. Coleman and Harris (1991) have also provided evidence that the human fetal epididymal epithelial cells express the CFTR gene. Another observation made in our laboratory with a bearing on this issue is that the normal response of the cultured human epididymal epithelium to β -adrenergic agent is diminished if the apical membrane has been pretreated with an antibody directed against human CFTR (Fig. 9). This is a first direct piece of evidence for a functional role of CFTR in Cl^- transport in the human epididymis.

9 Obstructive Azoospermia and Gene Mutation

Of all the functional abnormalities present in cystic fibrosis, those affecting the lung are the most serious. Respiratory failure is the major cause of death of CF patients. Other exocrine glands like the pancreas, sweat gland and the intestine are also affected to varying degrees. It is now known that different forms of mutation in the CF gene may account for the different phenotypes (mild or severe disease, pancreas sufficiency or insufficiency). For example, F508 which constitutes 70% of the CF mutations is a 'severe' mutation, whereas 'mild' mutations are found in exons 1,9 and 12. Other halotypes are likely to be discovered soon. It is possible that different CFTR (with slightly different active sites) are involved in Cl^- secretion in different exocrine glands and single point mutation at one site may affect one exocrine gland more severely than other glands. It is also possible that different mutations may be found in different ethnic groups.

In recent years, the association of male infertility and chronic infections of the nasal sinuses and the lung (chronic sinopulmonary disease), in particular bronchiectasis, has drawn the attention of clinicians to the immotile cilia syndrome, CF and Young's syndrome. Among the three, CF and Young's syndrome have many features in common. In CF, recurrent pulmonary infection is associated with abnormal sweat tests, abnormal pancreatic function and male infertility (Kaplan et al. 1968). Young's syndrome is a combination of obstructive azoospermia and chronic sinopulmonary disease (Young 1970; Hendry et al. 1978; Handelsman et al. 1984). It has been reported that about 50 to 60% of patients with obstructive azoospermia had evidence of sinusitis, bronchitis or bronchiectasis (Hendry et al. 1978; Neville et al. 1983). Although CF is extremely rare in Orientals, Young's syndrome (sinopulmonary infections associated with acquired obstructive azoospermia) does exist in Orientals albeit showing a lower prevalence than exists in Caucasians (Wang et al. 1987). It is speculated that these abnormalities in respiratory and epididymal functions in the Orientals may be associated with defective Cl^- transport in the airway and epididymal epithelial cells as a result of mutations of the gene expressing CFTR. Unlike CF, these non- F508 mutants may manifest themselves as the 'milder' form of CF (without involvement of the sweat gland therefore not diag-

nosed as CF) whereas F508 CFTR is responsible for the more 'severe' form of CF phenotype which predominantly affects the Caucasians. To test this hypothesis to test, research is now in progress to study chloride secretion in the nasal and epididymal cells of men with combined chronic sinopulmonary infection and obstructive azoospermia and the responsiveness of the cells to adrenergic stimulation.

10 Conclusion

It is now clear that the maintenance of a normal fluid environment for sperm maturation and storage depends on the neurohumoral regulation of electrolyte and fluid transport in the epididymis. Stimulation of anion secretion and hence fluid secretion is mediated by complex cellular events, beginning with the interaction of neurotransmitters, hormones and autocooids with different membrane receptors located at the apical or basolateral membrane domains (Wong 1990). Activation of membrane receptors then triggers cascades of biochemical reactions leading to the formation of second messengers. Finally, these intracellular signals elicit permeability changes culminating in the secretion of electrolyte and fluid. Each step of this complex physiological event is vulnerable to pathological disturbances. It is believed that a thorough understanding of the underlying mechanisms of fluid secretion by the epididymis at the membrane and molecular level would offer an opportunity for therapeutic intervention with male infertility.

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Regulation of Mammalian Fertilization by Gamete Adhesion Molecules

Paul M. Wassarman

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1 Introduction

Summarized here are certain aspects of research performed in this laboratory during the past twelve years or so. The focus is on the current status of our attempts to identify and characterize those molecules associated with the surface of mouse eggs and sperm that regulate species-specific gamete adhesion before and after fusion of gametes. We have made considerable headway with the egg surface (zona pellucida) components and, consequently, this aspect constitutes the bulk of the presentation. However, some attention is paid to sperm surface

components involved in adhesion of sperm to eggs. It should be noted that there is evidence to suggest that conclusions drawn from our research on mice apply as well to many other mammals, including human beings.

Most of the research summarized here has been reviewed previously (Wassarman et al. 1985; Wassarman 1987a,b, 1988a,b,c, 1990, 1991a,b). Much more inclusive discussions of mammalian fertilization are available (e.g., Gwatkin 1977; Yanagimachi 1988; Wassarman 1991b) and should be consulted for a broader perspective of the field than that presented here.

2 Fertilization Pathway – Definition of Terms

The nomenclature we have adopted is in keeping with that used historically to describe molecules that support gamete adhesion during fertilization, for both vertebrate and invertebrate animal systems. This nomenclature also follows that used to describe molecules involved in the initial interactions between bacteriophage and bacteria and between viruses and animal cells (Luria et al. 1978; Crowell and Lonberg-Holm 1986). Such interactions may be considered somewhat analogous to those that occur between sperm and eggs. The egg surface component(s) to which sperm bind, we call *sperm receptors*. The sperm surface component(s) to which eggs bind, we call *egg-binding proteins*. Thus, species-specific adhesion between sperm and eggs is attributable to complexes formed between egg-binding proteins on sperm and complementary sperm receptors on eggs.

In mice, motile, capacitated, acrosome-intact sperm bind to the ovulated egg extracellular coat, or *zona pellucida* (Dietl 1989), by plasma membrane overlying their heads (Figs. 1 and 2). Thus, sperm receptors and egg-binding proteins are associated with egg zona pellucida and sperm plasma membrane, respectively. Following binding of sperm to eggs, sperm undergo the *acrosome reaction*, a form of cellular exocytosis required in order for sperm to penetrate the zona pellucida and fuse with egg plasma membrane (Kopf and Gerton 1991). The acrosome is a large, lysosome-like vesicle that overlies the sperm nucleus. The acrosome reaction involves fusion at many sites between plasma

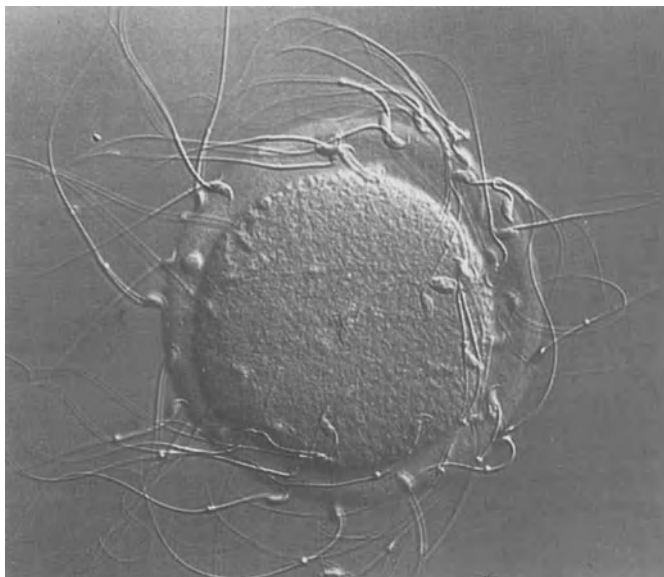


Fig. 1. Light micrograph of mouse sperm bound to the zona pellucida of an unfertilized mouse egg *in vitro*. The micrograph was taken using Nomarski differential interference contrast (DIC) microscopy

membrane overlying the anterior region of the sperm head and outer acrosomal membrane just beneath it. Hybrid membrane vesicles are formed and inner acrosomal membrane, with associated acrosomal contents, are exposed to the zona pellucida. Following fusion of gametes, fertilized eggs undergo the *cortical reaction*, a form of cellular exocytosis required for establishment of the *zona reaction* and “slow” or “secondary” block to polyspermy (Ducibella 1991). Cortical granules are small, lysosome-like vesicles that occupy a region of egg cytoplasm just beneath the plasma membrane. The cortical reaction involves fusion of cortical granule membrane with egg plasma membrane and release of cortical granule contents into the perivitelline space and, subsequently, into the zona pellucida. These contents induce the zona reaction some minutes after fertilization. The zona reaction includes the so-called “hardening” of the zona pellucida, as well as

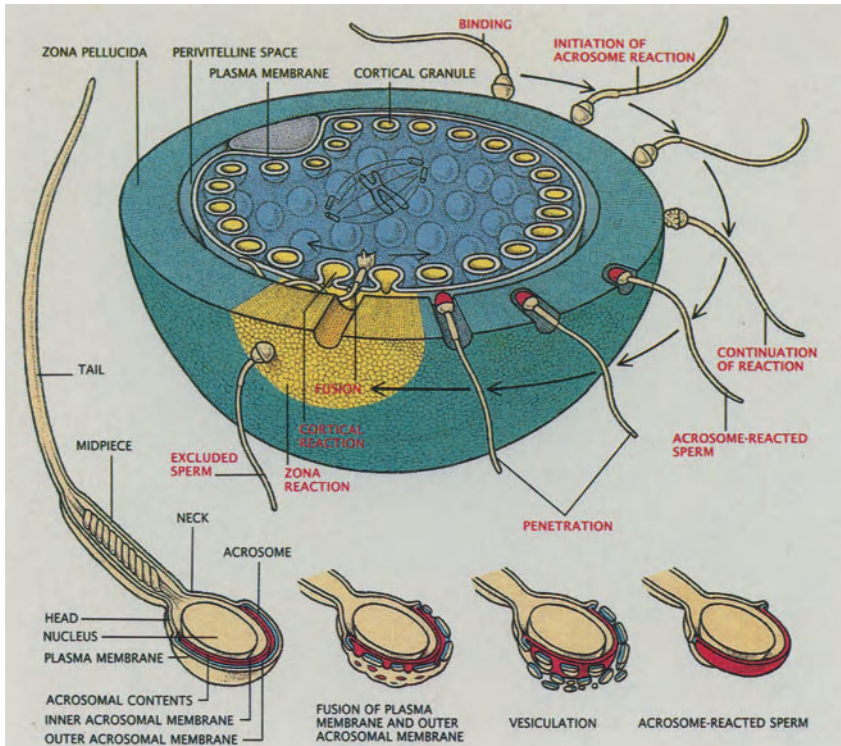


Fig. 2. Diagrammatic representation of the fertilization pathway (*top*) and the acrosome reaction (*bottom*) in mice. Depicted are: I. Binding of acrosome-intact sperm to the unfertilized egg zona pellucida (*aquamarine*). II. Initiation of the acrosome reaction (sperm exocytosis; fusion of sperm head plasma membrane (*beige*) and outer acrosomal membrane (*blue*) to form hybrid vesicles (*beige and blue*). III. Penetration of the egg zona pellucida by acrosome-reacted sperm with an exposed inner acrosomal membrane (*red*). IV. Fusion of acrosome-reacted sperm and egg plasma membrane (*white*) to produce a zygote. V. Initiation of the cortical reaction with cortical granule contents (*yellow*) deposited into the zona pellucida. VI. Initiation of the zona reaction. Steps V and VI are involved in the post-fertilization secondary block to polyspermy. This diagram is reprinted with the permission of *Scientific American* (taken from Wassarman, 1988b).

loss of its ability to bind free-swimming sperm. These changes in properties of the zona pellucida are considered to constitute a “slow” or “secondary” block to polyspermy.

3 Molecular Characteristics of mZP3 – The Mouse Sperm Receptor

The mouse sperm receptor is an 83,000 MW glycoprotein that we call mZP3 (Wassarman 1990). mZP3 and two other glycoproteins, mZP1 (200,000 MW) and mZP2 (120,000 MW), account for virtually all of the mass (3–4 ng) of the mouse egg zona pellucida (Bleil and Wassarman 1980a; Greve et al. 1982; Salzmann et al. 1983; Wassarman 1988a). mZP2 and mZP3 are present as heterodimers, every 140–150 Å or so, along the filaments that make up the extracellular coat (~7 µm thick) (Greve and Wassarman 1985; Wassarman 1988a; Wassarman and Mortillo 1991). The filaments, in turn, are interconnected by mZP1, a dimer of identical polypeptides held together by intermolecular disulfides (Wassarman 1988a). The three glycoproteins are held together in the zona pellucida by non-covalent bonds.

While mZP3 has a heterogeneous appearance following gel electrophoresis, it possesses a unique polypeptide consisting of 402 amino acids (44,000 MW) (Bleil and Wassarman 1980a; Kinloch et al. 1988; Ringuette et al. 1988). The polypeptide is particularly rich in proline, serine, and threonine residues, possesses very little α -helix, and is not significantly similar to any other entries in protein data bases. The heterogeneous appearance of mZP3 is attributable to its asparagine- (N-) linked and serine/threonine- (O-) linked oligosaccharides. mZP3 possesses 3 or 4 complex-type, N-linked oligosaccharides (the polypeptide has 6 potential N-linked glycosylation sites with the consensus sequence Asn-Xaa-Ser/Thr) and an undetermined number of O-linked oligosaccharides (Roller and Wassarman 1983; Salzmann et al. 1983; Wassarman 1988a). These oligosaccharides also account for the relatively acidic nature (pI~4.7) of mZP3.

mZP3 is encoded by a single-copy gene (chromosome 5 [Lunsford et al. 1990]) that contains eight exons (transcription unit, ~8.6 kb) and yields a 1.5 kb, polyadenylated, messenger-RNA (Kinloch et al. 1988;

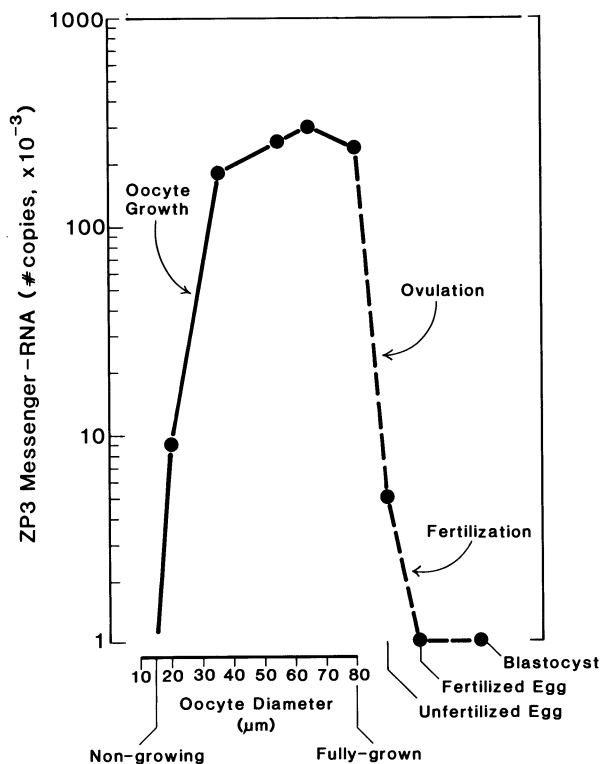


Fig. 3. Diagrammatic representation of the steady-state levels of mZP3 messenger-RNA in mouse oocytes, eggs, and preimplantation embryos, as determined by quantitative RNase protection assays (for details see Roller et al., 1989). The number of copies of mZP3 messenger-RNA ($\times 10^{-3}$) is plotted as a function of the stage of mouse development

Ringuette et al. 1988; Kinloch et al. 1990a; Kinloch and Wassarman 1989). The mZP3 gene is expressed only in growing oocytes, in no other cell type, and is regulated by *cis*-acting sequences in 5'-flanking region relatively close (within 470 bp) to the transcriptional start-site (Bleil and Wassarman 1980b; Philpott et al. 1987; Kinloch et al. 1988; Kinloch et al. 1990a; Kinloch and Wassarman 1989; Lira et al. 1990;

```

M MASSYFLFLCLLLCGGPELCNSQTLWLLPG 30
H .GL..Q.L.....AKQ.C..P..... 30

M GTPTPVGSSSPVKVECLEAELVVTVSRDLF 60
H .....GKLT.S.E..... 60

M GTGKLVQPGDLTLGSEGCQPRVSVDTDVVR 90
H .....I..E.....N.R.L...A.... 90

M FNAQLHECSSRVQMTKDALVYSTFLLHDPR 120
H .K.....N...V.E.....V...Q.. 120

M PVSGLSILRTNRVEVPIECRYPRQGNVSSH 150
H ..P.....AD..... 150

M PIQPTWVPFRATVSSEEKLAFLSLRLMEENW 180
H A.R.....ST.....V..... 180

M NTEKSAPTFHLGEVAHLQAEVQTGSHLPLQ 210
H ....LS.....Y.....L 210

M LFVDHCVATPSPLPDPNSSPHYFIVDFHGC 240
H ....R.....--.QTA...V..... 238

M LVDGLSESFSAFQVPRPRPETLQFTVDVFH 270
H ..... 268

M FANSSRNTLYITCHLKVAPANQIPDKLNKA 300
H .....I.....T...T..E.... 298

M CSFNKTSSQSWLPVEGDADICCCSHGNCSN 330
H ....RS.K..S.....EV.G...S.D.GS 328

M SSSSQFQIHGPRQWSKLVSRRRHVTDEAD 360
H ..R.RY.A..VS..P.SA..R...R.... 358

M VTVGPLIFLGKANDQTVEGWTASAQTSVAL 390
H .....S..A...AS.....L.. 388

M GLGLATVAFLTLAAIVLAVTRKCHSSSYLV 420
H .....A.....G...S..TP.HV. 418

M SLPQ 424
H ..S. 422

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Fig. 4. Comparison of primary structures of mouse ZP3 (mZP3; M) and hamster ZP3 (hZP3; H) polypeptides using the single letter amino acid code. For hZP3, only those positions where the amino acid sequence differs from mZP3 are shown. A two amino acid deletion in hZP3 is indicated by dashes at positions 223 and 224 of mZP3. The predicted potential signal sequences are italicized (residues 1-22), and locations of cysteine residues (C) and potential N-linked glycosylation sites (N) are underlined (for details see Kinloch et al., 1990b)

Schickler et al. 1992). mZP3 messenger-RNA accumulates to extremely high steady-state levels during oocyte growth (~300,000 copies/oocyte) and falls to undetectable levels (<1,000 copies/oocyte) in the fertilized egg (Fig. 3) (Kinloch et al. 1990a; Roller et al. 1989). The nascent mZP3 polypeptide consists of 424 amino acids, with the N-terminal 22 amino acids constituting a potential signal-sequence that is removed from the polypeptide prior to secretion of mature mZP3 by growing oocytes (Kinloch et al. 1988; Ringuette et al. 1988; Kinloch et al. 1990a; Kinloch and Wassarman 1989). Molecular probes directed against the mZP3 gene recognize the homologous gene in a variety of mammals, from hamsters to human beings, suggesting considerable conservation of the gene during evolution (Ringuette et al. 1986; Kinloch et al. 1990b; Chamberlin and Dean 1990; Kinloch and Wassarman, unpublished). For example, a comparison of mZP3 and hamster ZP3 (hZP3) polypeptide primary structures (402 and 400 amino acids, respectively) indicates that they are 81% identical, with the positions of all 13 cysteine residues conserved (Kinloch et al. 1990b; Moller et al. 1990) (Fig. 4).

4 Biological Characteristics of mZP3

mZP3 fulfills many of the predictions that can be made about the behavior of a *bona fide* sperm receptor *in vitro* (Bleil 1991; Wassarman 1990). For example:

1. Purified egg mZP3, at nanomolar concentrations, inhibits binding of sperm to ovulated eggs (and, consequently, inhibits fertilization) (Bleil and Wassarman 1980c; Wassarman 1987a,b; Wassarman 1990). Presumably, at low concentrations, exogenous mZP3 interacts with egg-binding proteins on sperm and, thereby, prevents the sperm from binding to eggs.
2. Purified egg mZP3 only binds to plasma membrane surrounding heads of acrosome-intact sperm (Bleil and Wassarman 1986; Wassarman 1987a,b; Wassarman 1990; Mortillo and Wassarman 1991). This is consistent with the fact that acrosome-intact, but not acrosome-reacted sperm can bind to ovulated eggs.

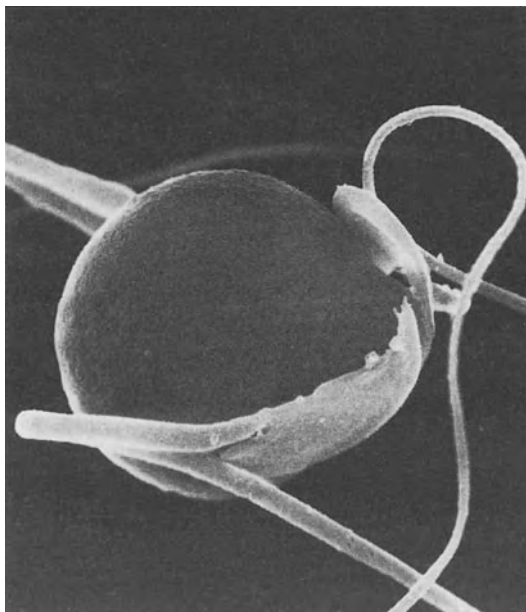


Fig. 5. Scanning electron micrograph of mouse sperm bound to a silica bead possessing covalently-linked mZP3 (for details see Vazquez et al., 1989)

3. Sperm bind to glass beads to which egg mZP3 is covalently attached (Fig. 5), as well as to embryonal carcinoma (EC) cells transfected with the mZP3 gene and secreting mZP3 (Fig. 6) (Vazquez and Wassarman 1989; Wassarman 1990; Kinloch et al. 1991).
4. Purified egg mZP3 is capable of inducing sperm to undergo the acrosome reaction (Bleil and Wassarman 1983; Florman et al. 1984; Wassarman 1987a,b; Wassarman 1990). mZP3 apparently activates the signal transduction pathway normally associated with cellular exocytosis (Kopf and Gerton 1991).
5. Purified *embryo* mZP3 (~83,000 MW), although indistinguishable from egg mZP3 following gel electrophoresis, does not exhibit the biological properties of a sperm receptor, as described in (i-iv) (Bleil and Wassarman 1980c; Wassarman et al. 1985; Wassarman 1987a,b; Wassarman 1990).

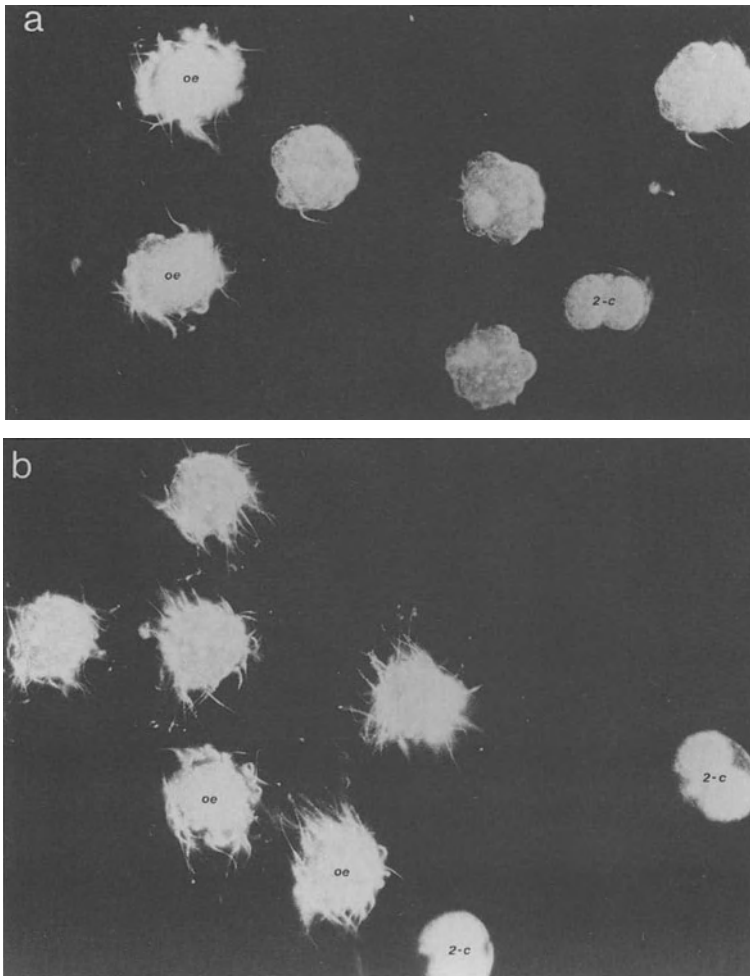


Fig. 6 a,b. Analysis of binding of mouse sperm to transfected and untransfected EC-cell aggregates *in vitro*. **a** Photomicrograph of a sample containing four untransfected EC-cell aggregates, two ovulated eggs (*oe*), and one two-cell embryo (*2-c*). Note that sperm are bound to ovulated eggs, but not to EC-cell aggregates or the embryo. **b** Photomicrograph of a sample containing four transfected EC-cell aggregates, two ovulated eggs (*oe*), and two two-cell embryos (*2-c*). Note that sperm are bound to ovulated eggs and EC-cell aggregates, but not to two-cell embryos (for details see Kinloch et al., 1991). Bar, 30 μ m

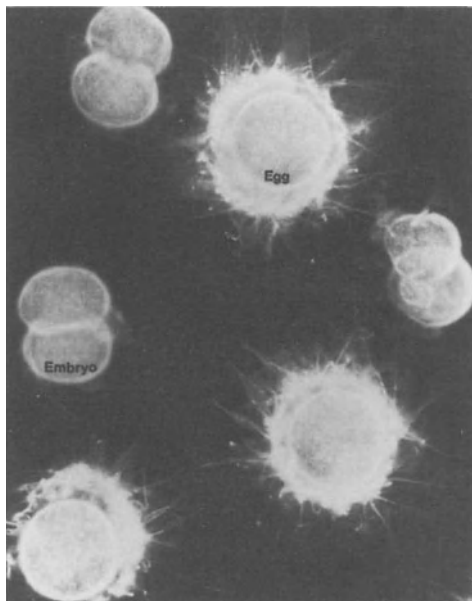


Fig. 7. Photomicrograph of mouse sperm bound to unfertilized mouse eggs in the presence of two-cell embryos *in vitro*. Sperm bind to the zona pellucida of unfertilized mouse eggs, but not to the zona pellucida of two-cell embryos due to inactivation of sperm receptors following fertilization. The micrograph was taken using dark-field optics

Thus, while egg mZP3 is active as a sperm receptor (and acrosome reaction-inducer), embryo mZP3 is inactive. This final point is especially relevant, since sperm can bind to the unfertilized, but not to the fertilized egg zona pellucida (one aspect of the *zona reaction* and “slow” block to polyspermy) (Fig. 7) (Gwatkin 1977; Bleil and Wassarman 1980c; Wassarman 1987a,b; Wassarman 1990).

These and other observations strongly suggest that mZP3 is the mouse egg *primary* receptor for sperm. Apparently, each sperm binds to the egg zona pellucida by as many as tens-of-thousands of mZP3 molecules (Bleil and Wassarman 1986; Wassarman 1990; Mortillo and Wassarman 1991). Once bound, sperm undergo the acrosome reaction and, as

a result, lose the plasma membrane (and egg-binding proteins) that adheres to the zona pellucida. The inner acrosomal membrane, with associated acrosomal components, is now exposed to the zona pellucida.

5 Maintenance of Acrosome-Reacted Sperm Binding

To remain bound to eggs, acrosome-reacted sperm bind by their inner acrosomal membrane (exposed as a result of the acrosome reaction) to mZP2 (Bleil et al. 1988). Purified egg mZP2 binds to inner acrosomal membrane of acrosome-reacted sperm, but not to plasma membrane of acrosome-intact sperm (Bleil et al. 1988; Mortillo and Wassarman 1991). Thus, mZP2 acts as a *secondary* receptor for sperm. The interaction between sperm and mZP2 may be mediated by a sperm protease, perhaps acrosin (Urch 1991), since it can be prevented by serine-protease inhibitors (Bleil et al. 1988). Bound, acrosome-reacted sperm are able to penetrate the zona pellucida and fuse with egg plasma membrane (i.e., fertilize the egg). In this context, it remains a curiosity that free-swimming, acrosome-reacted sperm are unable to bind to the zona pellucida of unfertilized eggs.

6 Molecular Basis of mZP3 Function

Gamete adhesion in mice is an example of *carbohydrate-mediated* cellular adhesion (Wassarman 1989, 1990, 1991a). Sperm bind to a specific class of O-linked oligosaccharides (~3,900 MW) that are covalently linked to mZP3 (Florman et al. 1984; Florman and Wassarman 1985; Wassarman 1990). When these oligosaccharides are removed from egg mZP3 (alkaline-borohydride) and purified (HPLC sizing and ionic exchange), they retain the properties of a sperm receptor (but, do not act as an acrosome reaction-inducer). On the other hand, the mZP3 polypeptide with its N-linked oligosaccharides intact, does not behave as a sperm receptor. Thus, mZP3 O-linked oligosaccharides are essential for sperm receptor function.

O-Linked oligosaccharides responsible for the sperm receptor function of mZP3 are located on the carboxy-terminal half of the polypep-

tide (Rosiere and Wassarman 1990; Rosiere and Wassarman, unpublished). This region of the mZP3 polypeptide is characteristically resistant to proteases (e.g., papain and V8 protease), perhaps, due to a relatively high degree of glycosylation (especially N-linked glycosylation).

While the primary structure of the sperm receptor O-linked oligosaccharides has yet to be determined, it is clear that certain sugar residues are essential for binding of sperm. One of these is a galactose residue at the non-reducing terminus of the oligosaccharide, that is in α -linkage with the penultimate sugar residue (Bleil and Wassarman 1988; Wassarman 1989, 1991a). Either removal of the galactose (by digestion with α -galactosidase) or conversion of its C-6 hydroxyl to an aldehyde (by oxidation with galactose oxidase) inactivates the oligosaccharide (or purified egg mZP3). There is circumstantial evidence that a fucose residue may also be essential (Bleil and Wassarman 1988) and that the O-linked oligosaccharide may possess repeating N-acetylglucosamine units (Shalgi et al. 1990).

7 The Zona Reaction

Following fertilization (or artificial activation), mZP3 and mZP2 are inactivated as primary and secondary sperm receptors, respectively. Apparently, mZP3 is modified by a cortical granule glycosidase(s) to a form of the glycoprotein called mZP3_f (Wassarman 1989, 1991a; Bleil and Wassarman, unpublished), whereas, mZP2 is modified by a cortical granule protease (Bleil et al. 1981; Moller and Wassarman 1989) to a form of the glycoprotein called mZP2_f. The latter apparently also accounts for the so-called "hardening" of the zona pellucida. Collectively, conversion of mZP2 and mZP3 to mZP2_f and mZP3_f, respectively, constitutes the so-called zona reaction (Fig. 7).

8 Mouse Egg-Binding Proteins

Several different sperm proteins have been proposed as candidates for the role of egg-binding protein in mice. The list of different sperm proteins is expanded further when other mammals are included (Wassar-

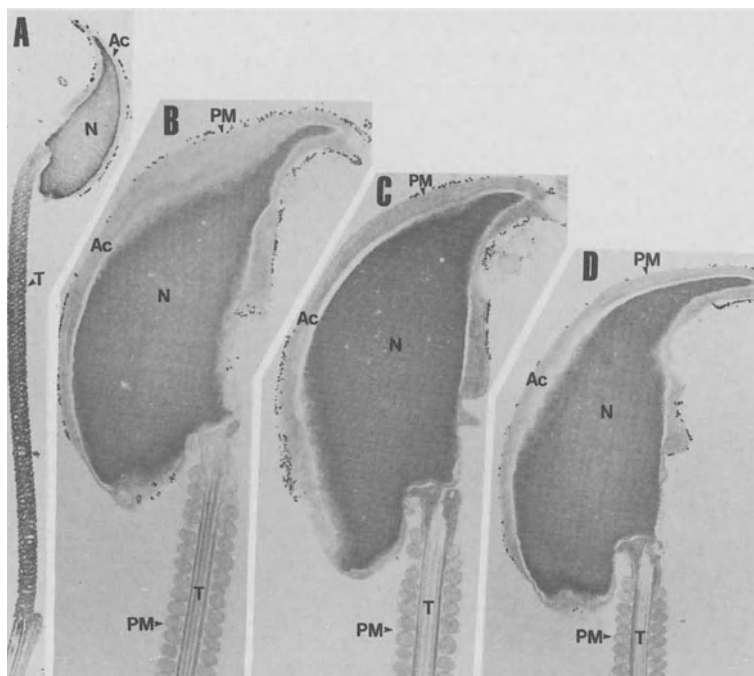


Fig. 8 A-D. Transmission electron micrographs of mZP3 gold-probe bound to acrosome-intact mouse sperm. Shown are representative examples of the distribution of mZP3 gold-probes associated with acrosome-intact sperm (**A-D**). Note the presence of gold particles over virtually all regions of plasma membrane encompassing the sperm head. Note the absence of gold particles over the sperm tail. Fifty nm gold particles were used in **A** and 30 nm gold particles were used in **B-D** (for details see Mortillo and Wassarman, 1991). N, nucleus. Ac, acrosome. PM, plasma membrane. T, tail

man 1991a,b). Thus, the nature of the egg-binding protein remains problematic, although most studies suggest that the candidates specifically recognize carbohydrate moieties of zona pellucida glycoprotein(s).

Light and electron microscopic evidence suggests that the egg-binding protein(s) that recognizes mZP3 is located in plasma membrane overlying the mouse sperm head (Fig. 8) (Bleil and Wassarman 1986;

Mortillo and Wassarman 1991). Such evidence indicates that the *primary* egg-binding protein(s) is not an abundant sperm protein (10^4 - 10^5 molecules/cell) and that it is not localized to a particular region of the acrosome-intact sperm head. Most of this egg-binding protein is lost as a result of the acrosome reaction (formation of hybrid membrane vesicles), with only a small amount remaining at the posterior end of the sperm head (in the region that fuses with egg plasma membrane).

Light and electron microscopic evidence suggests that the egg-binding protein(s) that recognizes mZP2 is located in membrane surrounding the acrosome (Bleil and Wassarman 1986; Mortillo and Wassarman 1991). Such evidence indicates that the *secondary* egg-binding protein(s) is associated with both inner and outer acrosomal membrane. In this context, acrosin, a potential secondary egg-binding protein (Bleil et al. 1988; Urch 1991), has been localized to both inner and outer acrosomal membrane prior to the acrosome reaction (Urch 1991).

9 sp56, A Putative Mouse Egg-Binding Protein

Our attempts to identify a mouse sperm protein that binds specifically to mZP3 on Western blots have proved unsuccessful, although we do not have a satisfactory answer for this lack of success. However, using purified egg mZP3 as an affinity probe and radiolabeled (^{125}I) Denny - Jaffee reagent (Jaffee et al. 1980; Denny and Blobel 1984) as a heterobifunctional, photoactivatable crosslinker, a 56,000 MW sperm protein has been identified and proposed to be a primary mouse egg-binding protein (Bleil and Wassarman 1990; Wassarman 1991a). Although other sperm proteins were radiolabeled by the affinity probe, as much as 90% of the radiolabel was associated with sp56. sp56 is located in plasma membrane overlying acrosome-intact sperm heads (Fig. 9), is lost following the acrosome reaction, and binds tightly to mZP3- and galactose-affinity columns. It is possible that sp56 exists as a multimer in sperm plasma membrane and that it undergoes redistribution in the membrane during the course of the mZP3-induced acrosome reaction. There is ultrastructural evidence that massive lateral displacement of transmembrane sperm glycoproteins overlying the acrosome is, indeed,

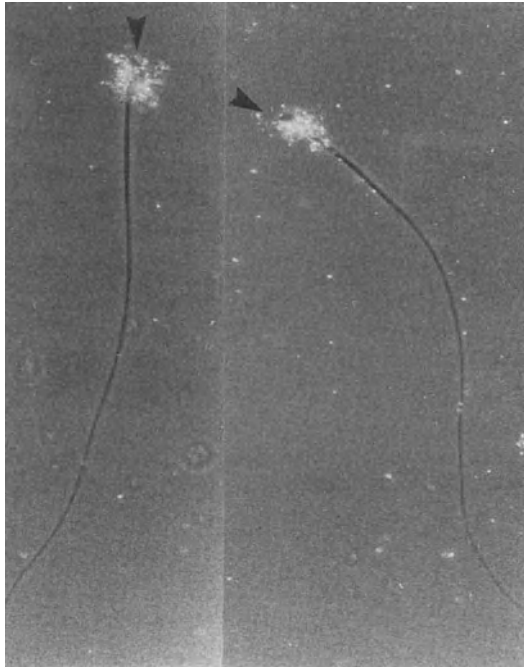


Fig. 9. Whole-mount autoradiographic visualization of ^{125}I -Denny-Jaffee reagent covalently bound to acrosome-intact sperm. Shown are two typical examples of acrosome-intact sperm with silver grains overlying their heads (*arrowheads*). Acrosome-reacted sperm had only background levels of silver grains associated with their heads (for details see Bleil and Wassarman, 1990)

an early step in the acrosome reaction (Aguas and Pinto da Silva 1989). It remains to be determined whether or not sp56 corresponds to any of the proteins with similar molecular weights previously proposed to be the primary egg-binding protein, such as proacrosin (Jones et al. 1988), PH-20 antigen (Lathrop et al. 1990), galactose-binding protein (Abdullah and Kierszenbaum 1989), and galactosyl transferase (Macek and Shur 1988).

10 Final Comments

The preceding summary highlights research carried out in my laboratory on several gamete adhesion molecules that participate during fertilization in mice. Overall, our results suggest that two zona pellucida glycoproteins, mZP3 and mZP2, serve as primary and secondary sperm receptors, respectively, during fertilization in mice. These glycoproteins are synthesized and secreted by growing oocytes and assembled, together with mZP1, into an extracellular matrix. The complementary primary and secondary egg-binding proteins are associated with plasma membrane surrounding acrosome-intact sperm heads and inner acrosomal membrane of acrosome-reacted sperm, respectively. The interaction between mZP3 and its complementary egg-binding protein, possibly a sperm plasma membrane protein called sp56, is carbohydrate-mediated. It is possible that proacrosin (or acrosin), or some other sperm serine-protease, is the secondary egg-binding protein that interacts with mZP2. Both mZP3 and mZP2 are modified by cortical granule enzymes (glycosidase(s) and protease(s), respectively) shortly after fertilization, thereby destroying their sperm receptor functions. Thus, egg and sperm surface adhesion molecules regulate the process of fertilization in mice. It is likely that homologous egg and sperm surface molecules play the same role in other mammals, including human beings.

Considerable progress has been made; however, there is still much to be learned about the molecular basis of gamete adhesion during fertilization in mammals. While some of the principal players have been identified, others remain elusive, or at least controversial. The primary and secondary egg-binding proteins on sperm fall into the latter category. However, there is reason to believe that the next few years will see important pieces added to the “fertilization puzzle”. For example, we should be able to decipher the “oligosaccharide code” apparently responsible for species-specific gamete adhesion and the enzymology responsible for the “slow” block to polyspermy. Clarification of these and other aspects of mammalian fertilization will certainly be of both academic and practical interest. Hopefully, the knowledge gained will be used in ways that enrich our lives.

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Sperm Interaction with the Zona Pellucida: Role of a Tyrosine Kinase Receptor for ZP3 in Fertilization

Donna Bunch, Lisette Leyton, and Patricia Saling

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1 Introduction

At the time of fertilization, mammalian eggs are enveloped by an egg-specific extracellular matrix, the zona pellucida (zp). For fertilization to occur, sperm must recognize and bind to the zp. ZP-binding triggers exocytosis in the acrosomal region of the sperm head (the acrosome re-

action, AR), which is mandatory for sperm passage through the zp and access to the egg plasma membrane where gamete fusion occurs. Thus, the related events of sperm-zp binding and zp-triggered ARs are key regulators of fertilization. Although sperm-zp interaction carries species specificity, the fundamental mechanisms of communication between gametes at this level appear conserved among many animals (Saling 1989; Wassarman 1988).

To understand the process of gamete interaction in detail, it is necessary to define the proteins involved. Regarding the zp, its composition has been well-characterized in a number of mammals. Studies in the mouse have provided the fullest insight into the functions of the zp's 3 major glycoproteins, ZP1, ZP2, and ZP3 (Bleil and Wassarman 1980a,b; 1983; 1986; Bleil et al. 1981; Florman and Wassarman 1985; Florman et al. 1984; Wassarman and Mortillo 1991). These proteins have been purified, and the genes encoding both ZP2 and ZP3 have been identified (Liang et al. 1990; Ringuette et al. 1986, 1988). Studies of sperm interaction with zp suggest that receptors on the sperm plasma membrane interact with ZP3 [termed primary binding], whereas components of the acrosomal matrix interact with ZP2 following the AR [termed secondary binding] (Bleil and Wassarman 1980a, 1986; Bleil et al. 1988; Jones et al. 1988). In addition, evidence indicates that ZP3 serves not only as the ligand for primary sperm binding, but is also responsible for triggering the AR (Bleil and Wassarman 1983).

While molecules of the zp that participate in fertilization have been defined, much less information is available about the complementary sperm proteins that function in gamete recognition and binding. In our laboratory, a 95 kD receptor protein for ZP3, termed p95, has been identified in mouse sperm (Leyton and Saling 1989a). A description of some of the evidence that supports this conclusion is provided below. A highly significant feature of this receptor for ZP3 is that it is a protein tyrosine kinase (PTK), with characteristics similar to other PTK receptors (eg, EGF-R, PDGF-R and insulin-R [Hunter 1987; Ullrich and Schlessinger 1990; Yarden and Ullrich 1988]), including ligand-induced receptor aggregation, autophosphorylation on tyrosine residues, and ligand-stimulated kinase activity. PTK genes and their products are highly conserved across eukaryotic cells, and PTKs are

thought to be critical regulators of many cellular responses (Hunter 1987; Ullrich and Schlessinger 1990; Yarden and Ullrich 1986). Given the anticipated conservation of a PTK receptor like p95, we have examined sperm from 3 other species and have identified a protein of similar M_r that also contains phosphotyrosine (PY) in human, bull, and cat sperm.

1.1 Identification of ZP3-Binding Proteins

To look for sperm proteins that bind ZP3, we employed a Western blot assay (Leyton and Saling 1989a). For any investigation of ZP3-binding proteins, it is necessary to optimize the conditions for sperm preparation so as to preserve the plasma membrane overlying the acrosome. Mouse sperm exposed briefly to Ca^{+2} using non-capacitating conditions (Tris, NaCl, and $CaCl_2$ for 30 min) demonstrate minimal levels of spontaneous ARs. Such preparations also display maximal zp-binding activity regardless of capacitation status (Lee and Storey 1985; Saling and Storey 1979). Sperm prepared using these conditions were dissolved in SDS and the proteins separated by SDS-PAGE. Western blots of these gels were probed directly with ^{125}I -whole zp, ^{125}I -ZP2 or ^{125}I -ZP3. When blots containing separated sperm proteins were probed directly with ^{125}I -whole zp or ^{125}I -ZP3, two sperm proteins, with M_r 95 kD and 42 kD, are detected prominently; faint reactivity may also be observed with low M_r components running near the dye front of the gel. ^{125}I -ZP2 does not bind to either the 95 kD or 42 kD proteins, nor to any other sperm protein, when the cells are prepared in this manner. We have not yet established the relationship between the 95 kD and the 42 kD proteins.

1.2 Identification of Substrates for Protein Tyrosine Kinase (PTK)

In mouse sperm, a physiological inducer of acrosomal exocytosis is ZP3. To examine whether a signalling pathway known to play a critical role in somatic cell function might also be involved in this stimulated exocytotic process of gametes, we examined tyrosine kinase ac-

tivity in mouse sperm (Leyton and Saling 1989a). Anti-PY antibodies, indicating the presence of PY residues (Glenney et al. 1988), react on immunoblots with three mouse sperm proteins of M_r 52, 75, and 95 kD. Indirect immunofluorescence of fixed cells using the same immunoprobe localizes reactivity to the acrosomal region of the sperm head. The 52 and 75 kD phosphoproteins are detected only in capacitated sperm, whereas the 95 kD protein is detected in both fresh and capacitated sperm. High levels of PY reactivity are also seen in detergent (RIPA; Kamps and Sefton 1988) extracts of mouse sperm; such reactivity depends on the presence of Na_3VO_4 during extraction to inactivate endogenous phosphatase. For the 95 kD protein, the level of anti-PY antibody immuno-reactivity is enhanced approximately two-fold by capacitation and four-fold by sperm interaction with solubilized zp proteins. These findings suggest that a 95 kD protein located in the acrosomal region of the sperm head is phosphorylated on tyrosine residues in response to zp binding.

1.3 Evidence that Aggregation of Sperm Receptors for ZP3 Triggers the AR

To try to understand the bifunctional role of ZP3 (binding and AR inducing activities), we examined the hypothesis that ZP3 functions to aggregate sperm receptor molecules. By analogy with findings in a variety of other extracellular signal transducing systems, including receptors for growth factors and insulin, this aggregation event could initiate the cascade resulting in the AR. To test this hypothesis, we generated monospecific polyclonal antibodies against ZP2 and against ZP3, and examined the effects of these probes on capacitated sperm incubated in the absence or presence of various zp protein preparations. For some experiments, we used proteolytic fragments of ZP3, a preparation known to retain specific binding, but not AR-inducing, activity (Florman and Wassarman 1985; Florman et al. 1984).

Capacitated sperm were exposed to zp glycopeptides followed by specific polyclonal antibodies against either ZP2 or ZP3. The sperm's acrosomal status was monitored by the CTC assay (Saling and Storey 1979; Ward and Storey 1984). After capacitation, nearly 80% of the

sperm display intact acrosomes. Treatment of such a sperm population with solubilized whole zp induces the AR in the majority of the sperm. In contrast, treatment with zp glycopeptides fails to induce the AR-pattern even though the glycopeptides bind to the sperm. However, exposure of the sperm to zp glycopeptides followed by anti-ZP3 IgG induces the AR to the same extent as exposure to whole zp. This induction of the AR is specific for anti-ZP3 IgG, and no induction is seen if the zp glycopeptides are omitted. Furthermore, zp glycopeptide-treated sperm do not undergo ARs in the presence of univalent anti-ZP3 Fab fragments. But, if cells from this same preparation are incubated subsequently with goat anti-rabbit IgG, ARs are observed in the majority of the population. These findings are consistent with the hypothesis that aggregation of sperm molecules recognized by ZP3 glycopeptides triggers the events leading to acrosomal exocytosis.

2 A Working Hypothesis for Ligand-Induced Acrosomal Exocytosis

Our own results, and many others from the literature, have enabled us to formulate a working hypothesis for the relationship between ZP3 binding and AR induction; key elements in this model are analogous to findings in a variety of other systems for ligand dependent cellular responsiveness (Cunningham et al. 1991; Ullrich and Schlessinger 1990). We suggest that acrosomal exocytosis is triggered by the aggregation of receptors for ZP3 (ZP3-Rs) in the sperm's plasma membrane. We have identified a 95 kD protein as a putative ZP3-R. We have also found that, in response to zp binding, the PY level of a 95 kD sperm protein is increased. The simplest interpretation of these findings, supported by indirect evidence (Leyton and Saling 1989a), is that ZP3 binding and tyrosine kinase substrate activities reside in the same 95 kD protein. [For convenience of discussion, we term this protein p95.] In other systems, such as the insulin receptor and several growth factor receptors, the receptor is itself a protein tyrosine kinase (PTK) which is stimulated by ligand binding and which autophosphorylates and phosphorylates other substrates. Based on results from these systems, we

hypothesize that p95 is a ZP3 receptor and behaves as others members of the PTK receptor family.

With these concepts in mind, we have related sperm-zp binding to AR triggering, as follows: (i) for primary binding between zp and acrosome-intact sperm, ZP3 serves as ligand and p95 as receptor; (ii) bound multivalent ZP3 facilitates the oligomerization of ZP3-R; (iii) oligomerization and/or ligand binding stimulates the autophosphorylation of p95 on tyrosine residues, initiating the intracellular cascade that results finally in acrosomal exocytosis.

In this working hypothesis, we suggest that oligomers formed in the sperm's plasma membrane constitute the functional units responsible for AR triggering. We specify p95 as one of the components of this signalling aggregate, but other proteins (that need not bind ZP3 directly) may also participate. Aggregate formation could occur spontaneously in capacitated sperm, due to the increase in plasma membrane fluidity that accompanies capacitation and may explain the occurrence of spontaneous ARs. ZP3 may be viewed as a specific accelerator of oligomer formation by cross-linking at least some of its constituents. If this interpretation is correct, PTK activity would be expected to increase with capacitation, and to increase further following exposure to ZP3. Our experimental findings are consistent with this interpretation.

3 Evidence that p95 is a Tyrosine Kinase Receptor for ZP3

3.1 Preparation of Bioactive Sperm Membranes

To be considered for receptor function, it is imperative to demonstrate the location of p95 in the sperm plasma membrane. Toward this end, we have generated a plasma membrane fraction from mouse sperm that retains zp binding activity (Bunch and Saling 1991). Briefly, washed cauda epididymal mouse sperm are demembranated by vortexing. After a low-speed spin to remove demembranated cells, the crude membrane supernatant may be separated into 3 bands and a pellet on a discontinuous sucrose gradient. Ultrastructural analysis of this material indicates Bands 2 and 3 are highly membrane-rich, although mitochondrial contamination can also be seen in Band 3.

Two related types of experiments, (i) vectorial labelling of sperm membranes with ^{125}I using Iodobeads, followed by demembration, and (ii) indirect immunofluorescence using anti-mouse sperm mAbs directed against regional domains of the sperm cell, allowed determination of plasma membrane fractionation as a function of vortexing. We found that 90% of the recovered plasma membrane fractionates in Band 2, and that the principal membrane region released arises from the plasma membrane of the anterior sperm head. To measure the bioactivity of the recovered membranes, we developed a competitive binding assay, and found that crude membranes contain zp binding activity. This activity can be accounted for fully in Band 2 membranes, whereas Band 3 displays insignificant activity.

3.2 Bioactive Sperm Membranes Contain Ligand-Responsive Tyrosine Kinase Activity

If our model for zp-binding and AR triggering is correct, we would expect that membranes containing zp-binding activity will display intrinsic kinase activity that can be stimulated by exposure to its ligand, zp proteins. In addition we would also expect that the bioactive preparation will contain tyrosine-phosphorylated p95. When crude membranes and membrane fractions are assayed for kinase activity in the presence of [^{32}P]-ATP, electrophoresis followed by autoradiography reveals the presence of > 25 phosphoproteins in both the crude membranes and in Band 2 (Fig. 1, lanes A and B, respectively). It is notable that in both of these preparations, a phosphoprotein with M_r 95,000 is detected. Much of the observed ^{32}P incorporation depends specifically upon PTK activity (either directly or as an activator of other Ser/Thr kinases) since Tyrphostin RG-50864, a specific inhibitor of tyrosine kinases (Gazit et al. 1989; Lyall et al. 1989), decreases the extent of phosphorylation in these membranes by > 80% (data not shown). The profile in Band 3 presents a sharp contrast in which few proteins are phosphorylated (Fig. 1, lane C). In the latter preparation, the major phosphoproteins migrate with M_r 35,000 and 60,000; no phosphoprotein in the 95 kD range is detected in Band 3 preparations. Control experiments show that when parallel samples of these membrane prep-

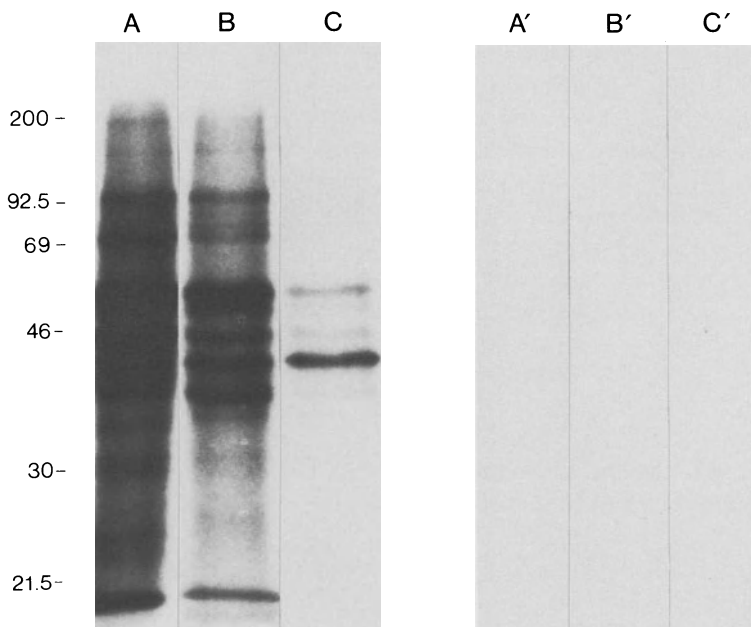


Fig. 1. Kinase assays in a cell-free system. Crude membranes (A,A') and plasma membrane fractions (B,B': Band 2 ; C,C': Band 3) were subjected to kinase assay for 30 min at 37°C in the presence of [^{32} P]-ATP in the absence (A-C) or presence (A'-C') of excess unlabelled ATP. Proteins were electrophoresed under disulfide reducing conditions. Each lane contains proteins recovered from 10^7 sperm. Molecular weight markers ($\times 10^{-3}$) are indicated to the left of lane A. The autoradiogram indicates that a large number of phosphoproteins were present in the crude membranes (lane A). The majority of these phosphorylated proteins, including a 95 kD protein, fractionated with Band 2 (lane B), a plasma membrane enriched preparation that contains zp receptor activity. No incorporation of ^{32} P was detected in the controls (lanes A'-C')

arations are incubated in the presence of excess unlabelled ATP (Fig. 1, A'-C'), ^{32} P is not incorporated into the sperm proteins. Thus, in addition to expressing zp-binding activity, crude membranes and membranes in Band 2 also appear to contain an associated kinase(s) that is active in a cell free system. Furthermore, immunoblot analysis of these

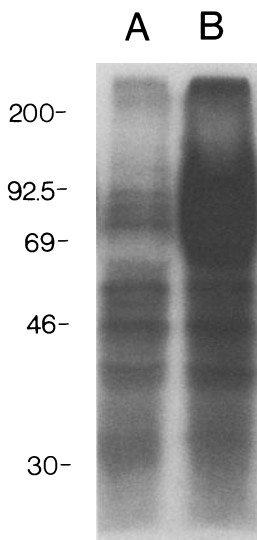


Fig. 2. Kinase assay in the presence of solubilized zp proteins. Kinase assays were conducted using crude membranes in the absence (lane A) or presence of solubilized zp proteins at a concentration of 90 $\mu\text{g/ml}$ (lane B). Control samples were treated with 130 $\mu\text{g/ml}$ BSA instead of zp. Molecular weight markers ($\times 10^{-3}$) are indicated to the left of lane A. The autoradiogram shows that zp proteins enhanced total kinase activity present in the crude membranes, particularly in the range of 80-120 kD (lane B). The methods for recovery and use of zp proteins may be found in Leyton et al. 1989

same membrane fractions using anti-PY Ab indicates that tyrosine phosphorylated p95 is highly enriched in Band 2, accounting for ~75-80% of the total activity in whole sperm (LeGuen et al. 1992).

When sperm membranes are incubated in the presence of solubilized zp proteins, we observe an increase in overall kinase activity (Figure 2, lane B). Crude membranes were treated for 10 min with 90 $\mu\text{g/ml}$ zp proteins and kinase assays were conducted subsequently. Control samples were treated with 130 $\mu\text{g/ml}$ BSA instead of zp proteins. Phosphorylation of proteins in the region 80 to 120 kD is particularly enhanced following exposure to zp proteins. No kinase activ-

ity is detected in control samples containing either solubilized zp proteins alone or a sample identical to that in lane B, but incubated with an excess of unlabelled ATP (data not shown).

3.3 The Role of p95 in Fertilization

Our earlier results suggest that the sperm's ZP3 receptor shares several characteristics with known PTK receptors, such as location in the plasma membrane, activation by ligand-induced receptor aggregation (Leyton and Saling 1989b), and increased PY content in response to ligand binding (Leyton and Saling 1989a). Since stimulation of the PTK activity associated with this type of receptor appears essential for signalling action, the use of specific inhibitors of PTK activity would provide information on the mechanism whereby the cell responds to a particular ligand.

If PTK activation is an initial signal leading to the AR, then inhibition of PTK activity should have a profound effect on this event. To determine whether PTK inhibition would affect the ability of sperm to undergo zp-triggered ARs, the PTK inhibitor genistein (Akiyama et al. 1987) was employed. Sperm were capacitated *in vitro* in the presence of genistein and then incubated with solubilized zp proteins. Following an additional 30 min incubation, the status of the acrosome was assayed using chlortetracycline. We find that, in the presence of genistein, the sperm's acrosomal status is indistinguishable from that of control sperm without zp proteins, i.e., ARs are not triggered despite the presence of zp in the inhibitor-treated samples.

Together with our earlier findings, these data suggest two important conclusions: (i) p95 is a key regulatory sperm molecule for the occurrence of fertilization in mice, and (ii) a receptor-effector system similar to those in somatic cells which are responsive to various growth factors and hormones may also exist in mammalian sperm.

4 Testable Predictions of Our Model

Our data suggest that p95 is similar in functional respects to other PTK receptors. This receptor family also shares structural similarity with an extracellular domain specialized for ligand binding, a single-pass transmembrane domain, and an intracellular domain containing an intrinsic tyrosine kinase, capable of auto- and substrate-phosphorylation (Ullrich and Schlessinger 1990). Our data are consistent with this same structure for p95. Thus, it should be possible to generate specific probes for these different domains, so that different aspects of receptor function can be analyzed independently. In this regard, we have already employed anti-PY antibodies to study various characteristics of the intracellular catalytic domain of p95, as discussed above.

Of particular interest with respect to analysis of our working hypothesis is the generation of probes that interact with the extracellular domain of p95. Such reagents could be utilized as ZP3 substitutes, permitting careful scrutiny of several predictions of the model presented earlier. As described above, work from our lab and many other labs indicates that the consequence of ZP3 binding to sperm can be assessed in at least three different ways: i) Sperm-zp binding is competitively blocked by soluble ZP3, presumably by occupancy of the ligand binding site on the sperm receptor (Bleil and Wassarman 1980a); ii) Acrosomal exocytosis is triggered by ZP3, presumably by aggregation of ZP3 receptors in the sperm plasma membrane (Leyton and Saling 1989b); and iii) Protein tyrosine kinase is activated by ZP3, presumably by stimulation of the PTK intrinsic to p95 located in the sperm plasma membrane (Leyton and Saling 1989a).

Generation of an antibody directed specifically against the extracellular domain of p95 would permit a thorough analysis of the role of p95 in these steps of the fertilization process. Moreover, the ability to use bivalent *versus* univalent forms of such a reagent would add considerably to our understanding of these interactions at the molecular level.

If our model is valid, we expect that an anti-p95 antibody will act as a ligand (ZP3) substitute and block sperm-zp binding. We would expect this antibody to exert a similar effect regardless of whether its bivalent or univalent form is employed, paralleling the similar effect of

both intact ZP3 glycoprotein and pronase-digested ZP3 glycopeptides on sperm-zp binding (Florman et al. 1984). If the aggregation of p95 molecules in the sperm plasma membrane is an initial part of the trigger for the AR cascade, then we would also predict that an anti-p95 antibody would induce acrosomal exocytosis in sperm in the absence of ZP3. In this case, however, marked differences should be apparent depending upon the antibody's valency: while activity equivalent to that observed with the natural ligand is expected with bivalent antibody, no AR triggering is expected with Fab fragments since these will lack the ability to cross-link ZP3 receptors. Finally, we can examine the effect of anti-p95 antibody binding on PTK activity. For other PTK receptors, ligand induced receptor aggregation is the initiating event that triggers intrinsic PTK activation of the oligomerized receptors (Heffetz and Zick 1986; Hempstead et al. 1983; Yarden and Schlessinger 1987). In the case of p95 as the ZP3 receptor, then, we expect that anti-p95 bivalent antibody application to sperm will result in full stimulation of intrinsic PTK activity, whereas anti-p95 Fabs may exert a partial, if any, effect on PTK activity. We have recently developed an anti-p95 monoclonal antibody, termed LL95, that appears to be directed against the extracellular domain of the receptor, and are currently using this reagent to examine the soundness of our model.

5 Summary

Successful interaction between sperm and egg is an obvious essential aspect of fertility. In recent years, technological advances have accelerated our pace of understanding the molecular mechanisms that are involved in such interaction. Studies utilizing mouse gametes, both *in vitro* and *in vivo*, have contributed the most complete conceptual framework for understanding gamete interaction at the molecular level.

Our studies on sperm-zp interaction have identified p95 as a ZP3 receptor and as a member of the highly conserved family of PTK receptor proteins. Independently, remarkable conservation among ZP3 from several different mammals, at both the nucleic acid and amino acid levels, has been reported; for instance, 67% of the deduced amino acid sequence of human ZP3 is identical to that of mouse ZP3 (Cham-

berlain and Dean 1990). Thus the available evidence suggests that both receptor and ligand involved in mammalian gamete interaction belong to conserved protein families. These lines of reasoning prompt the prediction that other mammalian sperm including human contain a receptor for ZP3 that is homologous to murine p95. If this is the case, then studies such as ours in model systems are likely to provide the information required to develop novel and specific methods for the regulation of fertility.

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Characterization of Inhibitory and Stimulatory Effects of Zona Pellucida Glycoproteins on the Proacrosin/Acrosin System

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1 Acrosin and its Role in Gamete Interaction

Proacrosin, the zymogen form of the serine protease acrosin, is located in the acrosome of mammalian spermatozoa. During the process of gamete interaction, acrosin is believed to assist sperm penetration of the surrounding egg shell, the zona pellucida. This penetration is supported by a proteolytic attack of acrosin against the zona glycoproteins.

After the acrosomal reaction has taken place the pH of the surrounding media changes from acidic to neutral. This leads to the activation of the zymogen proacrosin. We were able to mimic this process

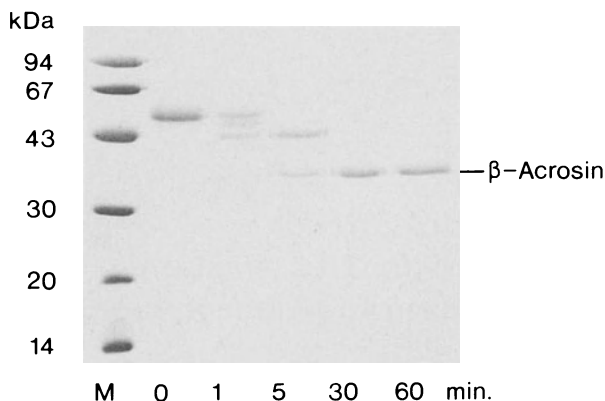


Fig. 1. Purified proacrosin was activated in 300 mMTris/HCl pH 8.0 at 37° C. At the time intervals given aliquots (3 mg/lane) were taken from the activation mixture and immediately subjected to SDS-PAGE (12.5 %) under reducing conditions and stained with Coomassie Blue

in vitro using purified boar proacrosin. A pH-change to neutral induces proacrosin activation and conversion which finally leads to the fairly stable b-acrosin. During this process several distinct and well characterized acrosin forms appear (Fig. 1). A processing mechanism which is probably induced by activated proacrosin is responsible for activation starting from the C-terminal end (Baba et al. 1989a). Conversion stops *in vitro* with the generation of b-acrosin with a molecular weight of 35 kDa.

Proacrosin is associated with the inner acrosomal membrane at the equatorial segment or within the acrosomal matrix (Tesarik et al. 1988; Richardson et al. 1991). Immediately after the acrosomal reaction, proacrosin establishes contact with the glycoproteins of the zona pellucida. There is striking evidence that proacrosin/acrosin functions as a "secondary ligand" (Jones 1990). It is believed that this binding prevents the detachment of the spermatozoa from the zona pellucida. This hypothesis is in agreement with other observations made by Bleil et al. (1988) where a sperm protein with trypsin-like activity binding to the ZP2-fraction was found to serve as a secondary sperm receptor in mice.

Given these facts, it is obviously of interest to study the activation of proacrosin in the presence of zona glycoproteins. The experiments described in this paper were carried out to elucidate the interaction of zona glycoproteins (ZP) with the proacrosin/acrosin system.

2 Carbohydrates as Zona-Substitute

In our experiments we used not only zona glycoproteins but also fucoidan, a sulfate-rich polysaccharide. The validity of using fucoidan as a ZP-substitute is based on several established precedents and criteria:

1. Fucoidan blocks fertilization in a variety of mammalian species (hamster, human, boar, rat and mouse) and is especially effective in inhibiting binding of acrosome-reacted guinea-pig spermatozoa to homologous eggs (Huang et al. 1982; Ahuja 1982).
2. Fucoidan has been used to identify adhesion molecules on different cell types, most notably binding from sea urchin spermatozoa (Glabe et al. 1982).
3. Similar proteins are recognized by fucoidan and ZP glycoproteins when they are used to probe Western blots containing detergent- or acid-extracted proteins from guinea pig spermatozoa (Jones and Lancaster 1988).

2.1 Acrosin and its Binding to Carbohydrates

We successfully detected proacrosin and all acrosin forms generated during the activation process using both biotinylated zona glycoproteins and biotinylated fucoidan (Fig. 2). This result indicates that sulfated oligosaccharides such as fucoidan and the sulfated proteoglycans from the zona pellucida recognize similar acrosin forms.

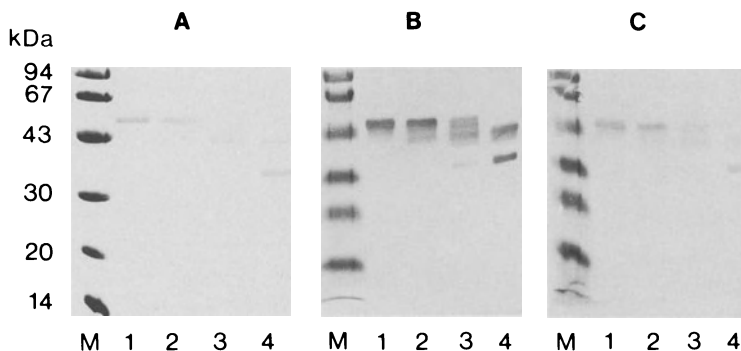


Fig. 2 A-C. Purified proacrosin was activated in 300 mM Tris/HCl pH 8.0 at 37° C. At frequent intervals (0 min: lane 1; 5 min: lane 2; 10 min: lane 3; 30 min: lane 4) 1 mg aliquots were taken and subjected to SDS-PAGE (12.5 %) under reducing conditions. Coomassie-Blue-Staining (A), Western-blot using biotinylated fucoidan (B) and biotinylated ZP (C)

2.2 Activation and Autodegradation as Proacrosin/Acrosin by Carbohydrates and Zona Pellucida Proteins

Glycosaminoglycans such as heparin, chondroitinsulfate and fucoidan (Parrish et al. 1979) as well as the glycoproteins of the zona pellucida (Töpfer-Petersen and Cechová 1990) stimulate proacrosin activation. These observations may be explained in two ways. Either glycosaminoglycans and ZP glycoproteins stimulate activity as a result of binding to the generated acrosin forms, or the binding of these saccharides to proacrosin enhances the molecular conversion of proacrosin into acrosin. This second mechanism of glycosaminoglycan stimulation of proacrosin conversion was postulated by Parrish in 1979. However, apart from the above mentioned activity measurements, no protein-chemical analyses have been performed so far.

Proacrosin was incubated in a neutral activation buffer containing different concentrations of ZP glycoproteins or fucoidan. At frequent time intervals aliquots were subjected to SDS-polyacrylamide gel electrophoresis. In parallel another aliquot was used for measuring the resulting amidolytic activity using a synthetic chromogenic substrate (Fig. 3 and 4).

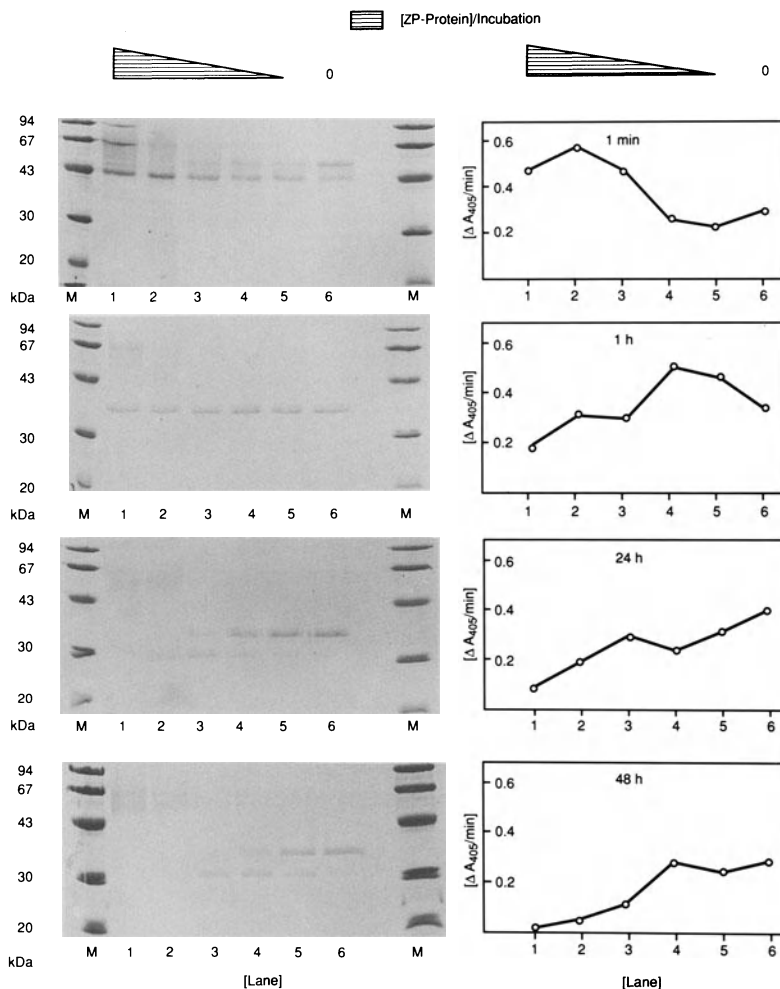


Fig. 3. Purified proacrosin (0.2 mg/ml) was activated in 300 mM Tris/HCl pH 8.0 at 37° C with different amounts of ZP-protein. At time intervals given aliquots (2 mg proacrosin/lane) were removed from the reaction mixture. One aliquot was immediately treated at 100°C for 5 min with 5 ml of SDS sample buffer and the reduced proteins were subjected to SDS-PAGE. The other aliquot was used for activity measurement using an amidolytic assay. Molar (pro)acrosin/ZP-protein ratios: Lane 1-5: 0.3, 0.6, 3.0, 6.0, 30.0/ 1 respectively. Lane 6: control without additives (Eberspächer et al. 1991)

In comparison to a control experiment without ZP glycoproteins, an accelerated conversion and activation of proacrosin was observed after one minute of incubation in the presence of ZP glycoproteins (Fig. 1). The more ZP glycoproteins were used the less converted acrosin could be detected after prolonged incubation times (Fig. 3, lane 1 and 2). This was also expressed in the decreased activity measured in parallel. This observation indicates that an autocatalytic degradation process takes place in the presence of ZP glycoproteins. To rule out the possibility that this surprising effect was due to a protease contamination in the zona preparation, a control experiment was performed. Using a very sensitive protease detection system (Boehringer, Mannheim, Germany), no significant amount of unspecific protease activity could be detected. During this autodegradation process an intermediate acrosin form of 30 kDa appeared (Fig. 3, lane 3 and 4). Sequence analysis demonstrated that this intermediate form has an N-terminal sequence identical to that of the heavy chain of mature b-acrosin, indicating that the processing of proacrosin into b-acrosin from the C-terminal end (Baba et al. 1989b) takes place.

The substitution of ZP glycoproteins for fucoidan in this experiment produced almost the same results (Fig. 4).

Autodegradation of acrosin was also observed *in vitro* when we used other negatively charged molecules like heparin, chondroitinsulfate and even DNA (Eberspächer et al. 1991). All these compounds carry clusters of negative charges. Whether this rather unspecific property is responsible for the observed effect awaits further elucidation.

Although normally active b-acrosin was detected after a prolonged incubation time in the presence of fucoidan (Fig. 4, lanes 2 and 3), almost no amidolytic activity was measured. This apparent inhibition clearly has no influence on the autodegradation mechanism (Fig. 4, lanes 1 to 4). A less pronounced inhibition could also be observed in the presence of ZP glycoproteins (Fig. 3, 1 hour time-point). Applying Michaelis Menten kinetics, different types of inhibition for zona glycoproteins or fucoidan were observed (Fig. 5).

The enzyme kinetics with b-acrosin in the presence of different concentrations of ZP glycoproteins revealed a competitive type of inhibition with an inhibition constant K_I of 10^{-7} M. In contrast to this,

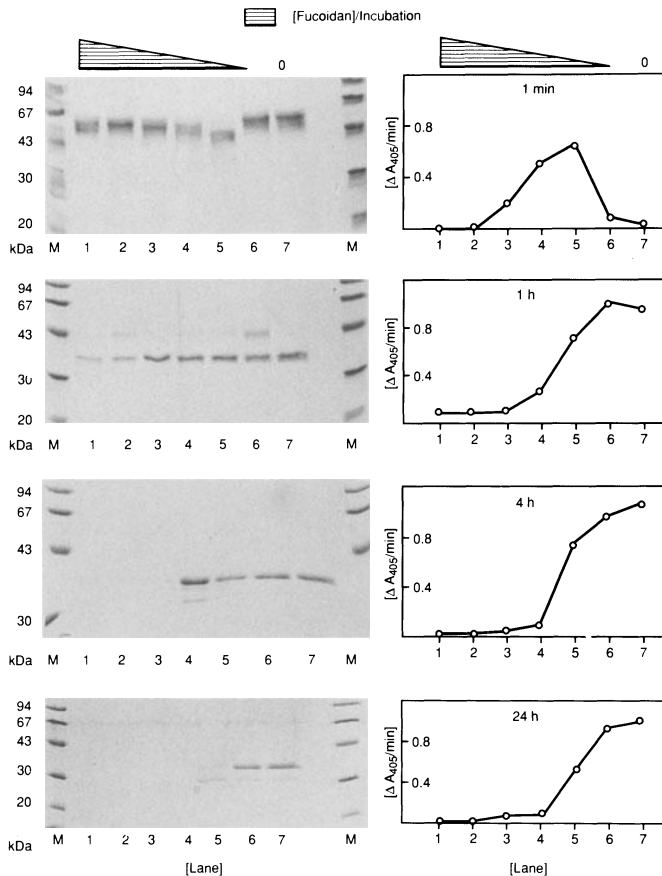


Fig. 4. Purified proacrosin (0.2 mg/ml) was activated in 300 mM Tris/HCl pH 8.0 at 37° C with different amounts of fucoidan. At time intervals given aliquots (2 mg proacrosin/lane) were removed from the reaction mixture. One aliquot was immediately treated at 100° C for 5 min with 5 ml of SDS sample buffer and the reduced proteins were subjected to SDS-PAGE. The other aliquot (20 ml) was used for activity measurement using an amidolytic assay (Eberspächer et al. 1991)

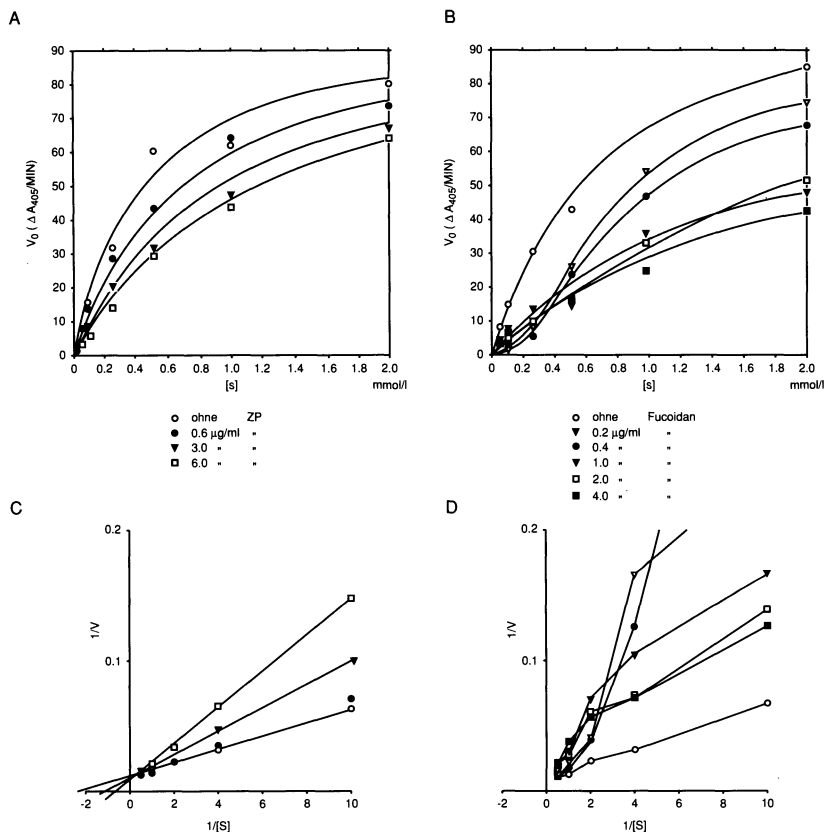


Fig. 5 A-D. Inhibition of b-acrosin by ZP-glycoproteins (**A** and **B**) or fucoidan (**C** and **D**). Michaelis-Menten-plot (**A** and **C**); Lineweaver-Burk-plot (**B** and **D**)

kinetics observed with fucoidan do not fit any common model of inhibition. These observations imply different types of interaction between b-acrosin with fucoidan or ZP glycoproteins.

3 Binding of Proacrosin Peptides to Fucoidin and/or Zona Proteins

This assumption is further supported by experiments analyzing the binding of fucoidan and ZP glycoproteins to proacrosin peptide fragments (Fig. 6).

The peptides resulting from a tryptic digest of proacrosin were separated by HPLC (Fig. 6a). ELISA-plates were covered with the peptides obtained and analyzed for binding to either biotinylated fucoidan or ZP glycoproteins using a streptavidin-peroxidase conjugate (Fig. 6b and c). Although biotinylated ZP glycoproteins show a very strong affinity towards proacrosin and all generated acrosin forms (Fig. 2), no significant binding towards the tryptic proacrosin fragments could be demonstrated (Fig. 6c). In contrast to ZP glycoproteins, fucoidan recognizes distinct proacrosin peptides (Fig. 6b). These results indicate that binding of ZP glycoproteins is dependent on more intact acrosin structures than fucoidan binding is. An unspecific binding of the negatively charged fucoidan towards positively charged regions in the proacrosin molecule would be another possible explanation.

4 Interaction of Zona Pellucida and Acrosin – A Hypothesis

It is in combining these results that we can define a hypothesis for the biological function of the interaction demonstrated between ZP glycoproteins and proacrosin/acrosin (Fig. 7).

Spermatozoa penetrate the zona pellucida by boring small tunnels, but finally only one sperm fuses with the egg. Activation of proacrosin occurs in the presence of ZP glycoproteins after the acrosomal reaction has taken place. Sperm transit is supported by a proteolytic attack directed against the zona by the generated active acrosin forms. Proacrosin activation and conversion into active acrosins is enhanced in the presence of zona pellucida. Due to the loss of its hydrophobic C-terminal end, generated b-acrosin might exhibit less binding affinity towards the acrosomal matrix. Therefore b-acrosin loses contact with the spermatozoa and remains deposited at the sidewall of the zona pellucida tunnels (Stambaugh and Smith 1976). Now autodegradation of

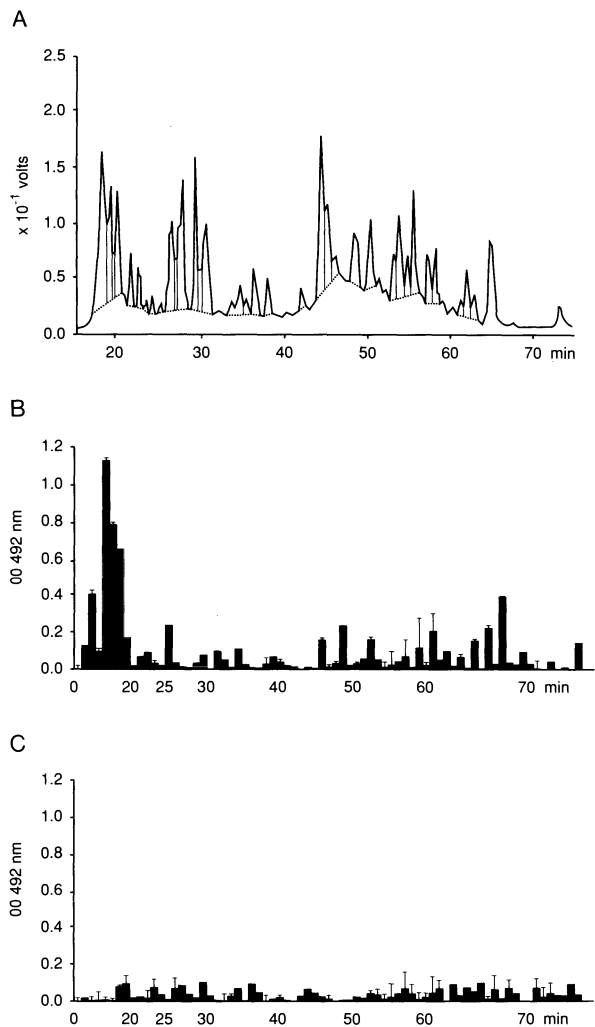


Fig. 6 A-C. A HPLC-run after tryptic digestion of proacrosin. ELISA-like assay using biotinylated fucoidan (**B**) and ZP (**C**) to detect the outcoming peptides of the HPLC run above

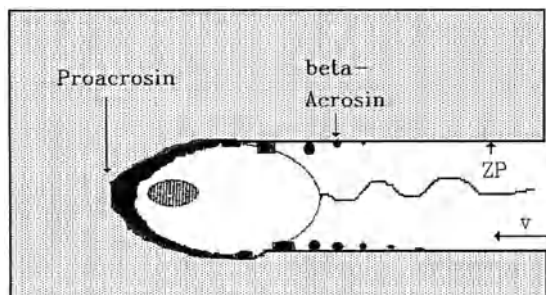


Fig. 7. Schematic representation of a spermatozoa penetrating the zona pellucida

acrosin takes place, because the molar ratio between ZP glycoproteins versus acrosin has changed in a direction where autolysis of acrosin is favoured.

5 Conclusions

Accelerated proacrosin activation and conversion occurs in the presence of zona glycoproteins, supporting the transit of sperm through the zona pellucida.

Induced autoproteolysis of acrosin by glycoproteins of the zona pellucida might be a kind of protection mechanism for the egg during and after the fertilization process. This mechanism is believed to prevent complete removal of the zona pellucida, the presence of which is absolutely necessary for the survival of the early embryo until implantation.

Using fucoidan as a zona pellucida analogue in experiments is questionable because of different binding characteristics of fucoidan and zona glycoproteins towards acrosin peptides and different types of acrosin inhibition.

Accelerated activation and conversion of proacrosin is dependent on the interaction of proacrosin with negatively charged surfaces.

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Carbohydrate-Binding Proteins Involved in Gamete Interaction in the Pig

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1 Gamete Interaction: General Concepts and Background

An essential event in the complex, multistep process of mammalian species-specific fertilization is gamete recognition through specific complementary molecules located on the external surfaces of the spermatozoon and the oocyte (Lillie 1913; Yanagimachi 1988). Increasing evidence points out that sperm-egg recognitions and binding actually involve carbohydrate-protein interaction (Ahuja 1982; Huang et al. 1982; Macek and Shur 1988; Jones and Williams 1990; Miller and Ax 1990). Remarkably, this interaction has been conserved in the process of fertilization throughout the whole evolutionary scale (Dale 1991). Thus, inhibition experiments with a variety of polysaccharides show that mannose and fucose contain egg surface components of the marine

brown alga *Fucus*, which interact with complementary carbohydrate-binding structures on the sperm cell surface (Bolwell et al. 1979). Similarly, Hoshi (1984) has presented evidence suggesting that sperm-egg binding in ascidians may be mediated by the enzyme α -fucosidase located on the sperm head. Moreover, the freshwater bivalve *Unio* spermatozoa bind to its homologous egg at a part of the oocyte surface that binds lectins, suggesting that the sperm receptors are also fucosyl-containing glycoproteins (Focarelli et al. 1988). In the sea urchin, the sperm specific protein, bindin, a 24 kDA major protein (Gao et al. 1986) associated with the inner acrosomal membrane and exposed during the acrosome reaction, mediates the adhesion to the egg's vitelline layer by a lectin-like activity (Vacquier and Moy 1977; Glabe et al. 1982). Bindin has a strong affinity for sulfated, fucose-containing carbohydrate structures on proteoglycans of the egg surface (De Angelis and Gabel 1987). Sulfate esters of the vitelline coat and arginine residues of the sperm protein appear to be critical for the binding (De Angelis and Gable 1988; 1990).

Several sugars have been also shown to inhibit the sperm-zona pellucida interaction in mammals. In particular, fucoidan (an algal heteropolysaccharide composed of sulfated L-fucose) and dextran sulfate are potent inhibitors of the binding of spermatozoa from a wide variety of species to their homologous oocyte's external coat, ranging from mammals to lower organisms (Ahuja et al. 1982; Huang et al. 1982; Jones and Williams 1990). Other sulfated polysaccharides (i.e. chondroitin sulfate A and C and heparin) are unable to block this interaction (Peterson et al. 1984). Since similar molecules are recognized by fucoidan and zona pellucida glycoproteins in Western blot analysis, it is believed that the interaction of fucoidan with spermatozoa mimics that of its natural substrate (i.e. the zona pellucida).

Species-specific recognition in fertilization may have evolved by co-evolution of complementary molecules on the limiting surface of both gametes. Understanding the molecular basis for species-specific fertilization thus requires the identification and characterization of those molecules involved in gamete recognition and binding. In mammals, sperm-binding molecules reside in the zona pellucida of the female gamete, which is a glycoprotein matrix encompassing the egg (Wassarman 1990). In the mouse, a paradigm species for fertilization

studies, the sperm receptor activity is attributable to certain oligosaccharide chains O-linked to the polypeptide backbone of the zona pellucida glycoprotein 3 (ZP3) (Bleil and Wassarman 1988; Bleil 1991). This sperm receptor has been cloned and sequenced in a number of mammalian species (Kinloch et al. 1988; Ringuette et al. 1988; Chamberlain and Dean 1990; Kinloch et al. 1990) and its genomic organization, oocyte-specific gene expression, and developmental regulation have been thoroughly studied (Ringuette et al. 1986; Philpott et al. 1987; Chamberlain and Dean 1989).

A variety of putative high- and low-molecular mass sperm zona pellucida-binding proteins have been identified in a number of mammalian species by a) incubating Western blots of sperm proteins with labeled zonal pellucida proteins and/or fucoidan; b) with the help of specific polyclonal or monoclonal antisera, which inhibit sperm-zona binding; or c) by using crosslinking reagents (O'Rand et al. 1985; Töpfer-Petersen and Henschen 1987; Brown and Jones 1987; O'Rand et al. 1988; Leyton and Saling 1989a; Jonáková et al. 1991; Hanqing et al. 1991; Bleil 1991). Sperm zona-binding proteins (ZBPs) have been divided into four categories based upon their molecular mass (O'Rand 1988). Among the high molecular mass ZBPs, a 95 kDa mouse protein that also binds anti-phosphotyrosine antibodies, has been reported by Leyton and Saling (1989a). Also in the mouse, Bleil and Wassarman (1989) have identified, by cross-linking experiments, a 56 kDa sperm surface protein which has many of the properties expected for a sperm's zona pellucida recognition protein.

The intra-acrosomal enzyme (pro)acrosin (50-65 kDa) and the extracellular membrane-bound enzyme β -1,4-galactosyltransferase (Gal-Tase, 45-55 kDa) are examples of well-characterized sperm receptor binding proteins (Jones 1991; Urch and Patel 1991; Shur 1986; Shur 1988; Macek and Shur 1988; Macek et al. 1991). Another enzyme, α -mannosidase, has also been reported to play a role in sperm-zona interaction in the rat (Tulsiani et al. 1989).

In addition to enzymes, mammalian spermatozoa of every species investigated so far also possess a group of low molecular mass (14-20 kDa) sperm-associated zona pellucida recognition proteins (O'Rand et al. 1985; O'Rand 1988; Jones and Williams 1990; Jonáková et al. 1991; Hanqing et al. 1991). The rabbit RSA proteins (O'Rand et al.

1988), the 5 kDa mouse proteinase inhibitor binding component (Poirier et al. 1986; Robinson et al. 1987; Benau and Storey 1987), and the boar spermadhesin protein family described recently (Jonáková et al. 199; Sanz et al. 1991, 1992a, 1992b, 1992c; see below) all belong to this group.

The relationship between similar zona pellucida-binding proteins identified in different species, as well as the relative contribution of the members of the different groups of ZPB molecules to the process of species-specific sperm-egg recognition and binding have not been evaluated. Here we will briefly summarize the current information available on spermatozoa-associated zona pellucida-binding proteins in the pig.

2 Complementary Receptors for Egg-Binding on Boar Spermatozoa

In intact boar spermatozoa, Töpfer-Petersen et al. (1985) localized fucose-binding proteins, by means of electron microscopy using fucosylperoxidase-collodial gold, at the plasma membrane covering the rostral tip of the sperm head (Fig. 1a). In acrosome-reacted spermatozoa, however, ultrastructural data demonstrated that other fucose-binding sites were localized inside the acrosome and were exposed during the course of the acrosome reaction (Friess et al. 1987a,b) (Fig. 1b). The zona-binding pattern showed a striking similarity with the fucose-binding pattern. Moreover, the sperm-zona interaction was completely inhibited by different fucosyl conjugates (Töpfer-Petersen et al. 1990a), suggesting that both ligands, fucose and zona glycoproteins, may bind to the same receptor(s). To examine the nature of these putative sperm-zona binding molecule(s) at the molecular level, fucose-binding proteins were visualized, after SDS-polyacrylamide gel electrophoretic separation of boar sperm extracts and electrotransfer to nitrocellulose sheets, using fucosylperoxidase as ligand. Enzymatic amplification showed two bands with apparent molecular masses of 53 Da and 17 kDa (Töpfer-Petersen et al. 1985). The 53 kDa porcine sperm protein has been purified to apparent homogeneity (Töpfer-Petersen and Henschen 1987) and was identified as the two-chain serine protease

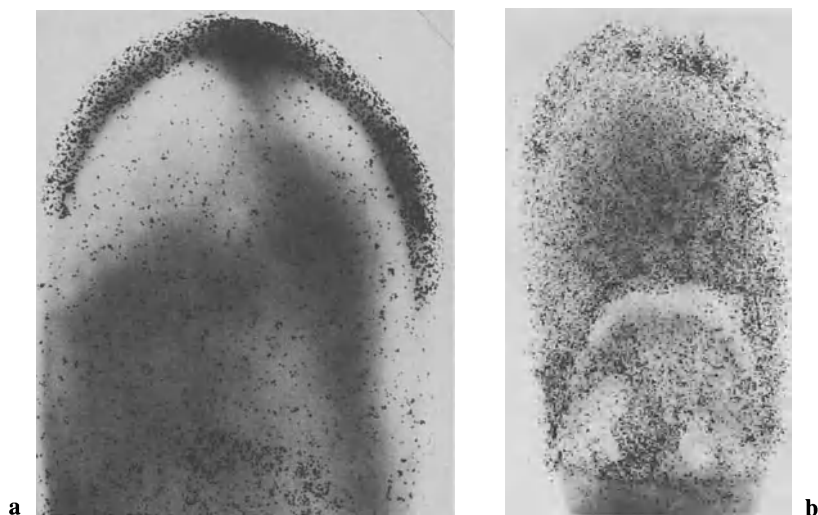


Fig. 1 a,b. Topography of fucose-binding proteins of boar sperm demonstrated by transmission electron microscopy using fucose-horse radish peroxidase-gold (fuc-HRP-gold) as a label. **a** Total specimen preparation of intact spermatozoon. Strong binding of fuc-HRP-gold on the head is limited to the plasma membrane overlying the apical part of the acrosome. (Reprinted with permission from Töpfer-Petersen et al. (1985)). **b** Exposure of fucose-binding activity after induction of the acrosome reaction by addition of the calcium-ionophore A23 187. The entire acrosomal area is heavily labeled. (Reprinted with permission from Friess et al. (1987))

acrosin (Töpfer-Petersen and Henschen 1987, 1988; Brown and Jones 1987; Jones and Brown 1987). The 17 kDA band has been recently shown to contain several proteins, which belong to the novel protein family called spermadhesin (Jonáková et al. 1991; Sanz et al. 1991, 1992a, 1992b, 1992c).

2.1 Spermadhesins: Primary Sperm Receptors Involved in Gamete Interaction

In mammalian species in which acrosome-intact spermatozoa bind to the zona pellucida, like the pig, the complementary sperm zona pellucida-, carbohydrate-binding primary counter-receptors are mainly associated with the anterior head membrane overlying the acrosomal cap (Peterson et. al. 1981; Töpfer-Petersen et al. 1985; Mortillo and Wasarman 1991). Recently, we have characterized several low molecular mass zona pellucida-binding proteins isolated from boar sperm extracts (Jonáková et al. 1991; Sanz et al. 1991, 1992a, 1992b, 1992c). These proteins, denoted AQN-1, AQN-3, AWN-1 and AWN-2, have their biological origin in secretions of the male accessory glands, and become sperm-associated upon ejaculation.

The following lines of evidence indicated that these sperm proteins fulfill the criteria expected for primary receptors which involve a gamete interaction through a carbohydrate-recognition mechanism. Thus, a) the interaction of biotinylated zona pellucida glycoproteins with blots containing the isolated boar proteins was inhibited by previous incubation with fucoidan (Fig. 2a); b) topographical localization of AQN-1 and AWN-1 on boar sperm, studied by indirect immunofluorescence, showed that the proteins are predominantly on the surface membrane overlying the acrosomal cap region of the sperm head (Fig. 2b); and c) *in vitro*, the isolated proteins effectively blocked the capa-

Fig. 2a-d. **a** Lane a, binding of biotinylated zona poellucida (2.5 µg/ml) to isolated AWN-1 eletrophoresed on a SDS-polyacrylamide (10-25 %) gel and electroblotted onto mitrocellulose sheet. Lane b, the same experiment as in a) carried out after incubation of the immobilized protein with 100 µg/ml fucoidan. In the left, molecular mass markers: Lysozyme (14kDa); soybean trypsin inhibitor (21 kDa); carbonic anhydrase (30 kDa); ovalbumin (43 kDa); bovine serum albumin (67 kDa); phosphorylase b (94 kDa). **b** Topographical localization of AWN-1 on the surface of capacitated boar spermatozoa using chicken monospecific polyclonal antibodies. **c** and **d** Inhibition of the sperm-oocyte binding by isolated AWN-1. Washed zona pellucida-encased oocytes were incubated with buffer (**c**) or with 0.2 mg/ml isoleted AWN-1 (**d**), washed and then mixed with capacitated spermatozoa. Pictures were taken with a fluorescence microscope (magnification: 100 x objective). The same results shown in **a-d** were obtained with AQN-1, AQN-3 and AWN-2

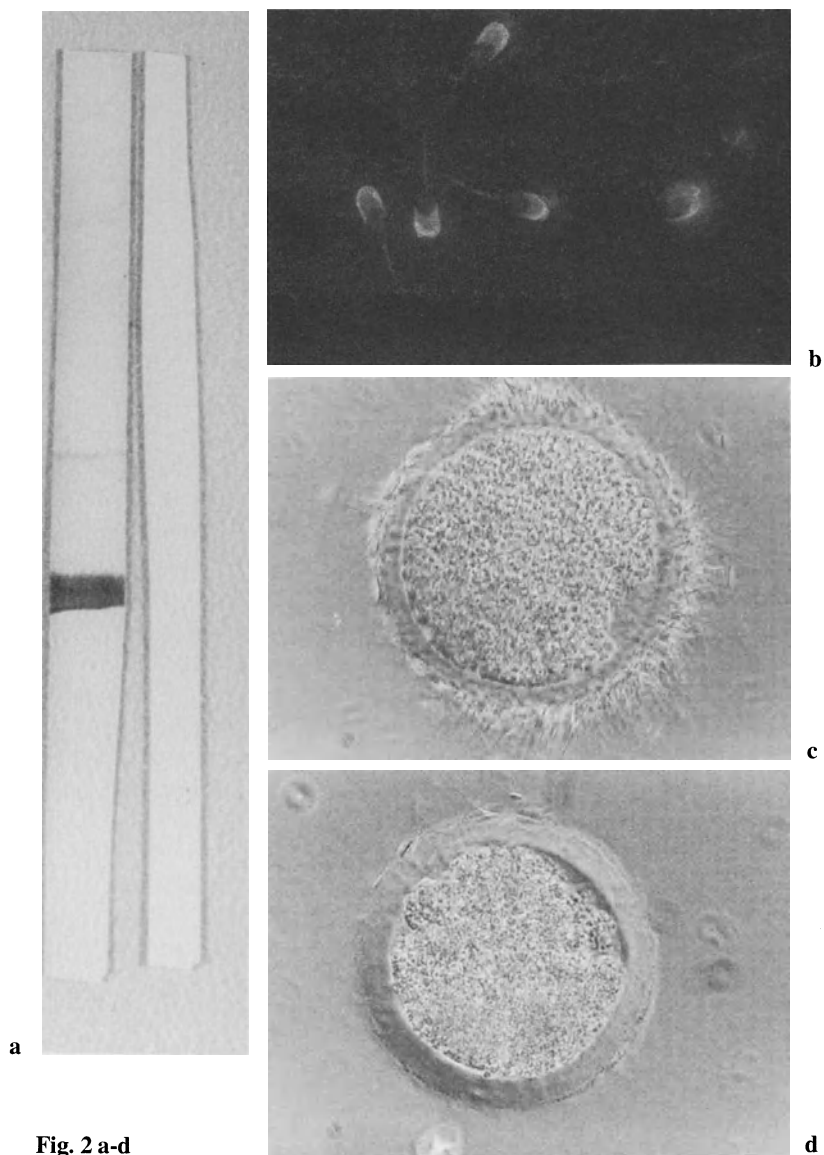


Fig. 2 a-d

citated sperm-oocyte interaction in a dose-dependent manner when freshly isolated zona-encased oocytes were preincubated with different concentrations of the proteins (Fig. 2c-d).

The complete covalent structure of AQN-1, AQN-3, AWN-1, and AWN-2 have been reported (Sanz et al. 1991, 1992a, 1992b, 1992c) (Fig. 3). They are 111 to 133 amino acid residues long, contain two disulphide bridges between Cys⁹-Cys³⁰ and Cys⁵³-Cys⁷⁴, and are not glycosylated. AWN-2 contains an acetylated N-terminus (Sanz et al. 1992a), but an otherwise identical polypeptide chain as AWN-1 (Sanz et al. 1992) and, therefore, may be considered as isoforms of a single chemical unit, AWN. Comparison of the amino acid sequences of spermadhesins (Fig. 3) clearly showed that AQN-1, AQN-3, and AWN are evolutionally related. Their primary structures are 40 to 60 % identical, but failed to show any significant similarity to any protein structure deposited in the Protein Sequence Data Banks. Therefore, these boar sperm-associated proteins may belong to a novel protein family involved in fertilization. We have coined the term "spermadhesin" to denote this family collectively.

Although spermadhesins AQN-1, AQN-3, and AWN all bind solubilized zona pellucida glycoprotein(s), their ligand-binding specificity (including macromolecules and ions) as well as the protein ligand-binding domain(s) remain to be characterized. Interestingly, the interaction of spermadhesins with zona pellucida for fucoidan is not inhibited by disulphide bridge reduction, indicating that a linear sequence within the protein structure may be responsible for their carbohydrate-binding ability. From X-ray crystal structure analysis of macromolecular complexes of carbohydrate-binding proteins with their saccharide ligand, we have learned that the carbohydrate-binding site mainly contains polar planar side chains and hydroxyl-containing amino acids, which are extensively used in the intricate network of hy-

Fig. 3. Alignment of the primary structures of boar spermadhesins. *, the amino acid sequence of AQN-2 is identical with that of AWN-1 except that the former contains an N-terminal acetyl group. Identical residues are in boxes. Two disulphide bridges between nearest-neighbour cysteine residues are conserved in all boar spermadhesins. Residue 95 of AQN-3 has been tentatively assigned as N-methylhistidine (Sanz et al. 1991)

AWN-1: *A W N R R S R S C G G V L R D P P G K I
 AQN-1: A Q N K G P H K C G G V L R D L S G R I
 AQN-3: A Q N K G S D D C G G F L K N Y S G W I

21 30 40
 AWN-1: F N S D G P Q K D C V W T I K V K P H F
 AQN-1: S T Y E G P K T D C I W T I L A K P G S
 AQN-3: S Y Y K A L T T N C V W T I E M K P G H

41 50 60
 AWN-1: H V V L A I P P L N L S C G K E Y V E L
 AQN-1: R V F V A I P Y L N L A C G K E Y V E V
 AQN-3: K I I L Q I L P L N L T C G K E Y L E V

61 70 80
 AWN-1: L D G P P G S E I I G K I C G G I S L V
 AQN-1: Q D G L P G A G N Y G K L C S G I G L T
 AQN-3: R D Q R A G P D N F L K V C G G T G F V

91 90 100
 AWN-1: F R S S S N I A T I K R L R T S G H R A
 AQN-1: Y Q S S S N A L S I K Y S R T A G H S A
 AQN-3: Y Q S S X N V A T V K Y S R D S H H P A

101 110 120
 AWN-1: S P F H I Y Y Y A D P E G P L P F P Y F
 AQN-1: S S F D I Y Y Y G D S
 AQN-3: S S F N V Y F Y G I P Q G A K A

121 130
 AWN-1: E R Q T I I A T E K N I P

drogen bonds formed in the protein-sugar complex (Quiocho 1988). Analysis of the primary structure of spermadhesins reveals that the polypeptide stretch between residues 69 and 111 in all of them fulfills the requirements listed in Fig. 3. The possible involvement of this part of spermadhesins in carbohydrate binding requires further investigation. Ultimately, a three-dimensional structure will be needed to understand this point. Another unresolved question is whether different spermadhesins recognize the same or closely related carbohydrate moieties on the zona pellucida.

Low molecular mass sperm proteins (15-18 kDa), which closely resemble the boar spermadhesins, have been identified in every mammalian species investigated so far (O'Rand et al. 1985). The paradigm molecules in this group are the rabbit sperm autoantigens (RSA) (O'Rand et al. 1988). Structural work on RSA peptides identified a 10-residue sequence (PGGGTLPPSG), which defines an autoantigenic epitope and which shows limited sequence similarity to certain segments of the sea urchin protein bindin (O'Rand and Widgren 1989). This particular amino acid stretch has no similar sequence within the boar spermadhesin structures (Fig. 3). Further structural characterization of RSA proteins is needed to definitively assess the relationship between the RSA proteins, the boar spermadhesins, and bindin. Relevant to this point, we had previously noticed a weak similarity between the N-terminal amino acid sequences of the boar spermadhesins and bindin (Jonáková et al. 1991). Completion of the primary structure of spermadhesins revealed that the sequence similarity between the boar and the sea urchin proteins was restricted to their N-terminal parts, however. Whether this analogy is a result of divergent or convergent evolution, as well as its significance, if any, requires further study.

In the boar, Hanqing and coworkers (1991) have described similar zona pellucida-binding proteins, which show identical cell compartmentalization and biological origin as spermadhesins. Although no structural data of these boar proteins are available, we believe that they may belong to the spermadhesin family. The availability of the primary structure of the boar spermadhesins may help in the identification of homologous proteins in other species, and may lead to a better understanding of the molecular basis of gamete interaction.

In addition to their zona pellucida-binding ability, spermadhesins AQN-1 and AWN also bind to a Kunitz-type soybean trypsin inhibitor and to a seminal plasma acrosin inhibitor of the Kazal-type (Sanz et al. 1992d). The binding of one of those serine proteinase inhibitors does not hinder the simultaneous binding of solubilized zona pellucida glycoproteins to AQN-1. We argue that both ligands may interact in a non-exclusive manner with different spermadhesin domains. However, interaction of the AWN-inhibitor complex with solubilized zona pellucida glycoproteins displaces the bound inhibitor. In this case, whether both ligands bind to the same or to structurally related binding site(s) remains to be established.

Interestingly, both a Kazal-type and a Kunitz-type proteinase inhibitor (which possess a similar proteinase-binding loop but are otherwise structurally unrelated) are able to bind to the same spermadhesin. It could be hypothesized that both inhibitors may utilize the protease-binding site for interaction with its sperm acceptor molecules. No detectable enzymatic activity is associated with AQN-1 or AWN. Thus, although spermadhesins may share some features with trypsin-like enzymes, their inhibitor-binding site is different from the active site of serine proteinase.

In the mouse, a 15 kDa inhibitor acceptor protein, located on the anterior portion of ejaculated spermatozoa, which possesses zona pellucida binding site(s) has been described (Poirier et al. 1986; Robinson et al. 1987; Benau and Storey 1987). This 15 kDa protein may be a mouse counterpart of the boar spermadhesins, though no structural data of the mouse protein have been reported.

Seminal plasma inhibitors, once bound to sperm surface acceptor molecules, are thought to play a pivotal role in sperm capacitation (Schill et al. 1975; Zanefeld 1976). The current hypothesis is that these inhibitors may stabilize or protect sperm surface membrane specific sites for sperm-egg interaction (Rogers and Bentwood 1982). Therefore, our results may imply that some spermadhesins, besides their role as sperm-zona pellucida adhesion molecules, may be also involved in sperm capacitation.

Spermadhesins AQN-1, AQN-2, AQN-3, and AWN are also heparin-binding proteins (Sanz et al. 1992). *In vitro*, heparin and zona pellucida binding to a given spermadhesin are mutually exclusive. The

fact that AQN-2, which is structurally similar to other spermadhesins, binds heparin but does not bind zona pellucida (Jonáková et al. 1991), strongly suggests that both ligands may interact with different spermadhesin domains. Furthermore, near its N-terminus AWN contains the amino acid sequence ³NRRSRS⁸, which resembles the consensus sequence XBBXB (B, basic amino acid) identified in the heparin-binding regions of many proteins (Barkalow and Schwarzbauer 1991). In our laboratories, the initial experiments on proteolytically dissecting zona pellucida binding site within the structure of AWN revealed that its N-terminal domain, comprising residues 1-15 and the putative heparin-binding region, is not essential for zona pellucida binding.

Analysis of the primary structure of AQN-1 reveals that it contains the amino acid sequence ¹⁷SGRIST²² within its first disulphide loop. This polypeptide is very similar to the sequence stretches ⁹⁹RGRISCT¹⁰⁵ and ¹⁸⁹SGRITCT¹⁹⁵ involved in the binding of heparin to the first heparin-binding domain of fibronectin (Kornblihtt et al. 1985). Jaikaria et al. (1991) have proposed a model for the interaction of fibronectin with heparin in which a disulphide-bonded structure would expose five spatially close loops that contain at their tips potential heparin interaction sites with the essential arginine residues (see Fig. 5 in Jaikaria et al. 1991). The regions of fibronectin 99-105 and 198-195 correspond in this model to loops II and IV, respectively. Interestingly, the amino acid sequence 17-22 of AQN-1 resides within a disulphide-bonded loop between Cys⁹ and Cys³⁰ (Sanz et al. 1992b). However, similar sequences are not present within the structures of the other heparin-binding spermadhesins. This raises two questions, whether different spermadhesins utilize the same or different protein regions for interaction with glycosaminoglycans, and whether heparin elicits different responses upon interaction with different spermadhesins. These points are currently under investigation in our laboratories.

Heparin-binding proteins on spermatozoa are known to act as extrinsic regulatory capacitation factors which interact with oviductal heparin-like glycosaminoglycans and thereby potentiate the zona pellucida-induced acrosome reaction (Florman and First 1988a, 1988b; Chandonnet et al. 1990; Miller et al. 1990; Florman and Babcock 1991). Interestingly, heparin and zona pellucida seem to induce the

Table 1. Ligand-binding capability of isolated boar spermadhesins

Ligand	AQN-1	AQN-2	AQN-3	AWN
Zona pellucida	+	-	+	+
Fucoidan	+	-	+	+
Proteinase inhibitors	+	-	-	+
Heparin	+	+	+	+

acrosome reaction through activation of the same intracellular pathway upon interaction with their respective, or with a common, sperm surface receptor(s) (for a recent review, see Florman and Babcock 1991). Our data show that some boar spermadhesins are common receptors for both ligands, and reinforce our conclusion that they may be involved in two important aspects of fertilization: sperm capacitation and sperm-egg recognition and binding events.

Data from different laboratories suggest that the zona pellucida glycoprotein 3 (ZP3) triggers the acrosome reaction by aggregation of ZP3 binding sites on the sperm head (Leyton and Saling 1988b; Macek et al. 1991). Similarly, Aarons et al. (1991) reported that immunoaggregation of a proteinase inhibitor SVI of seminal plasma origin, which binds to a site on a 15 kDa protein on the sperm head known to participate in zona binding, is sufficient to induce the acrosome reaction. Since spermadhesins combine within the same molecule zona pellucida-, heparin-, and/or serine proteinase inhibitor-binding activity (Table 1), it is tempting to speculate that clustering of spermadhesin molecules (i.e. AQN-1 and/or AWN) on the sperm surface, induced by the repeating structure of glycosaminoglycans or the zona pellucida network, could be part of the signal transmission which triggers the acrosome reaction. This working hypothesis deserves further detailed investigation.

2.2 Acrosin May Act as a Secondary Zona Pellucida Binding Receptor

Srivastava et al. (1965) provided the first direct evidence that an acrosomal protease had the ability to degrade the zona pellucida network of

rabbit eggs. This observation became the foundation for the hypothesis, nowadays fully accepted, that sperm hydrolases might play an important role in fertilization by facilitating sperm passage through the egg envelopes (reviewed by Urch 1991). Recently, several studies have indicated that, in addition to its proteolytic activity, (pro)acrosin also possesses zona pellucida binding capability (Töpfer-Petersen and Henschen 1987, 1988; Brown and Jones 1987; Jones and Brown 1987). However, acrosin is an intra-acrosomal enzyme and, thus, it can only play a role in sperm binding as a secondary receptor once the acrosome reaction has taken place.

Boar acrosin (Fig. 4) combines several functional properties within a single molecule: the catalytic triad of the serine proteinases, a membrane-associating domain, and the carbohydrate-binding site(s) by which the molecule binds to the zona pellucida. A feature of acrosin, not observed in other serine proteinases, is a 125-residue C-terminal extension containing an unusual clustering of prolines, including a stretch of 23 consecutive proline residues (Baba et al. 1989). Also unusual for a serine proteinase, acrosin autoactivates by modification of both the N-terminal and the C-terminal ends. Autocatalytic cleavage of the Arg²³-Val²⁴ peptide bond leads to the formation of the disulphide-bonded two-chain enzyme (Fock-Nüzel et al. 1984; Cechova et al. 1988; Töpfer-Petersen et al. 1990b), while liberation of certain polypeptide segments from the C-terminus of the heavy chain, including the proline-rich segment (Baba et al. 1989), correlates with the appearance of the lower molecular mass β -form (38 kDa) (Zelezna and Cechova 1982), which seems to be the most stable form of the enzyme. The high proline content of the C-terminal conversion peptide could account for the pronounced affinity of acrosin to membranes (Zelezna et al. 1989). However, this hypothesis awaits experimental confirmation.

The carbohydrate-binding ability of acrosin is independent of its proteolytic activity (Töpfer-Petersen and Henschen 1988). The zona pellucida binding activity has been mapped to the heavy chain (Töpfer-Petersen et al. 1990a), and further narrowed to an N-terminal 15 kDa peptide comprising its first 100-110 residues (Töpfer-Petersen et al. 1990c). On the other hand, studies on the interaction of zona pellucida glycoproteins, sulfated carbohydrates, and synthetic polymers with boar procacrosin indicate that it may involve (poly)sulfate groups on zona

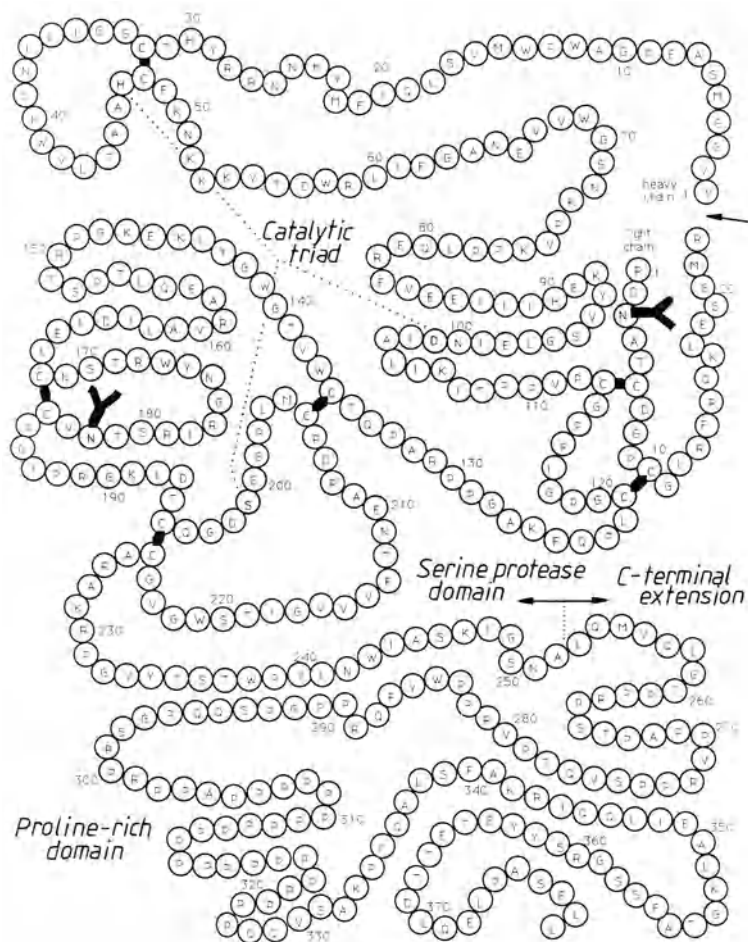


Fig. 4. Schematic drawing of full-length 53 kDa boar acrosin showing its major molecular features. The sequence has been taken from Baba et al. (1989). Amino acid residues are in the standard one-letter code. The arrow points to the autoproteolytic cleavage site between Arg²³-Val²⁴, which produces the two-chain enzyme. N-glycosylation sites (Y) and disulphide bridges are taken from Töpfer-Petersen et al. (1990a)

glycoproteins that bind with high affinity ($K_d = 1.2\text{--}5.0 \times 10^{-8}$ M) to complementary site(s) on proacrosin, and that the spatial orientation of the sulfate groups may play a role in the determination of binding selectivity to certain basic residues on the protein surface (Jones 1991; Urch and Patel 1991).

Interestingly, both the glycan binding affinity and specificity are similar to that reported for the interaction of the sea urchin sperm protein bindin with egg jelly polysulfated fucans (Glabe et al. 1982). Boar acrosin also resembles the sea urchin sperm protein bindin in that both are found in the acrosome and both adhere to the inner acrosomal membrane (Jones et al. 1988). However, their amino acid sequences (Gao et al. 1986; Baba et al. 1989) do not show any similarity, and bindin does not possess any known enzymatic activity. Thus, as pointed out by Urch and Patel (1991), the similarity of cellular location and biological function may reflect convergent evolution.

Finally, Bleil and Wassarman (1986) and Mortillo and Wassarman (1991) have reported that the mouse secondary sperm receptor, zona pellucida glycoprotein 2 (ZP2) (Wassarman 1988), binds exclusively to the inner acrosomal membrane of acrosome-reacted spermatozoa, and have hypothesized that acrosin might be its ligand. Whether acrosin actually binds to ZP2 and, if so, whether this interaction is attributable to the enzyme's substrate-binding domain or to its lectin-like activity, are areas for further study.

3 An Emerging Model for Sperm-Egg Interaction in the Pig

O'Rand and coworkers (1986) have described a model by which alternating cycles of binding to, digestion of zona proteins, and release from the zona pellucida, together with the forward motility of the spermatozoon, would be required to achieve sperm penetration through the oocyte's extracellular coat. Our data showing that spermadhesins may act as dual receptor molecules in sperm capacitation and sperm-egg interaction events provide a new insight to this general model. Thus, it is conceivable that acrosome-intact spermatozoa bind egg's zona pellucida carbohydrate moieties, probably on the boar counterpart of mouse ZP3, through primary carbohydrate-binding receptors, i.e. spermad-

hesins, located on the plasma membrane over the acrosomal cap. This interaction may lead to these acrosome reaction. In addition, a certain population of spermatozoa, approaching the oocyte through the matrix of the cumulus, may have already begun the acrosome reaction, in part as a consequence of interaction of glycosaminoglycans with spermadhesins. Full-length acrosin may then function as a secondary binding link between the acrosome-reacted spermatozoon and the oocyte's zona pellucida via its membrane-associating domain and the carbohydrate-binding sites. Acrosin may then locally digest the zona pellucida network, and auto-processing may release it from the (C-terminal) membrane-bound domain, allowing the free spermatozoon to move deeper through the zona pellucida.

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The Epididymis as a Site of Contraceptive Attack

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1 Introduction

Drastic measures are required to curtail the ever burgeoning world population. Fortunately, the male partner is becoming more aware of family planning (WHO 1980) and the problem seems to be less one of a willingness to contribute than a lack of a suitable method (but see Nieschlag, this volume). As an alternative to the testicular approach, the epididymis has often been mooted as an attractive site for attack by a contraceptive designed for use by men (Hinton 1980; Ford and Waites 1986) for three main reasons: (a) the onset of infertility (and rapid return to fertility after withdrawal of treatment) should be far quicker than any agent attacking the testicular production of spermatozoa; (b) damage to the genetic material, a possible sequela of action on dividing germ cells, may be obviated when attack is on maturing cells and (c) the natural function of the epididymis (that of mixing its secretions with spermatozoa) would be exploited. Concerning point (a), there may not be much difference in onset of action between an agent acting on spermiation or acting at the level of the caput epididymidis. Considering point (b), recent evidence has shown that administration of cyclophosphamide can cause embryonic malformation when given to male rats in which the ligated efferent ducts prevent spermatozoa from being attacked by the drug in the testis (Robaire, personal communication). The prime advantage of an epididymal approach, therefore, would be that the drug of choice would be delivered to, and thoroughly mixed with, spermatozoa, thus preventing the normal sperm-secretion interactions or creating a hostile environment for spermatozoa.

2 Postulate

A variety of possibilities exist for contraceptives acting reversibly in the epididymis (see Fig. 1). The basic tenet is that the epididymis influences spermatozoa in three ways (a) by a process of maturation that requires an optimum environment and the necessary time for the spermatozoa to be affected, (b) by a variable period of storage of potentially fertilizing sperm cells held in a quiescent state and (c) by regulat-

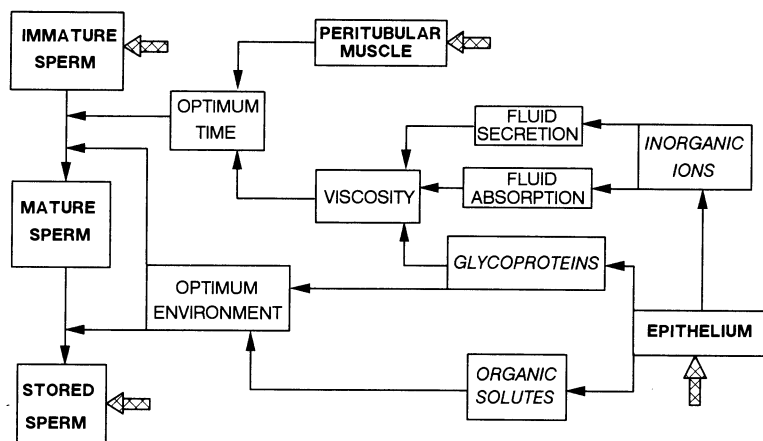


Fig. 1. Processes susceptible to attack by antifertility agents acting in the epididymis. Putative contraceptive agents could attack (a) the epithelium, which secretes inorganic and organic compounds that modulate the environment of maturing and stored sperm cells, (b) the peritubular muscles that control the transit of spermatozoa and thus the time course of interaction with epithelial secretions or (c) the sperm cells themselves

ing the transit of spermatozoa through the organ. Disruption of any process should prevent the normal production of fertile spermatozoa.

The time taken for the majority of spermatozoa to achieve fertilizing potential varies from 1 to 6 days (Orgebin-Crist and Olson 1984) and it has been argued that decreasing this time would lead to the ejaculation of immature spermatozoa incapable of fertilizing eggs. The means to reduce such transit time could involve either the peritubular musculature, increasing the distal transport of luminal spermatozoa by physical means, or epithelial secretion or absorption, such that a less viscous luminal fluid offering less resistance to distal flow would result. Action on the epithelium could result from an increased fluid secretion, driven by apical Cl^- and HCO_3^- ions (Wong 1988) or decreased fluid absorption, driven by Na^+ in the cauda (Wong and Yeung 1978) or Cl^- in the caput (Wong et al. 1978). An alternative to modifying ion transport by the epithelium would be to reduce the secretion of viscous glycoproteins. Modifying the secreted glycoproteins and

other organic compounds present in high concentration in the epididymis (carnitine, GPC, inositol) may also affect the environment (osmolarity, ionic strength, pH) in a way detrimental to the maturation or storage of spermatozoa. However, there is little evidence as yet that any of these secretions are essential for either sperm maturation or storage.

The scheme above, however, does permit a rationale for the design of a contraceptive acting on the epididymis and it is the purpose of this review to summarize the work that has been done in this area. There are several reviews of contraceptives acting in the male, most of which act on the testis (Jeffcoate and Sandler 1982; Zatuchni et al. 1986) but only those aspects pertinent to the epididymis will be cited here. It should be borne in mind that some compounds appear to act on both the testis and the epididymis. Putative contraceptive drugs could interact with the peritubular muscles, the epithelial cells or the contained spermatozoa (Fig. 1) and each of these compartments will be examined in turn.

3 Male Contraception via Action on the Epididymal Neuromusculature

3.1 Background

A consideration of the normal muscular activity of the epididymal tubule and its effect on luminal contents is required to appreciate the effect of antifertility agents acting at this site. In the rat the innervation of the peritubular muscle in the epididymis increases distally where both cholinergic and adrenergic fibres have been demonstrated by cholinesterase activity and noradrenalin localisation (El-Badawi and Schenk 1967; Norberg et al. 1967). The receptors within the epididymis are of the muscarinic type (considered part of the post-ganglionic parasympathetic system by El-Badawi and Schenk (1967)), post-junctional $\alpha 1$ -adrenoceptors (post-ganglionic sympathetic neurons: Ventura and Pennefather 1991) and purinergic receptors (possibly P_{2X}) (Ventura et al. 1990; Ventura and Pennefather 1991).

The relationship of this innervation with muscle contraction is enigmatic, for spontaneous peristaltic contractions occur more frequently in

the less innervated caput and corpus epididymidis than the cauda *in vivo* and all regions are responsive to neurotransmitters (Pholpramool et al. 1984; Pholpramool and Triphrom 1984; Din-Udom et al. 1985). Even when severed from the nerve supply (*in vitro*) the frequency of spontaneous electrical activity remains higher in more proximal segments of the duct (Talo et al. 1979). The electrical activity is associated with pressure waves (Markkula-Viitanen et al. 1979) and the migration of oil droplets injected into the lumen (Jaakkola and Talo 1982) in the cauda epididymidis *in vitro*. In the caput epididymidis injected oil droplets also display pendular movements *in vitro* (Jaakkola and Talo 1983) and *in vivo* similar experiments indicate that a more directional movement of luminal contents in the proximal regions, with increasing retrograde movements more distally (Jaakkola 1983; Turner et al. 1990). Despite equally rhythmic activity in the duct up to the cauda, there is more rapid movement of oil droplets in the proximal epididymis, most likely reflecting the low viscosity of fluid in this region.

As may be expected in the innervated cauda epididymidis, the low spontaneous electrical and mechanical activity (basal tension, frequency and amplitude) can be modulated by both cholinergic and adrenergic agents *in vivo* (Laitinen and Talo 1981) and *in vitro* (Pholpramool and Triphrom 1984) but there is no effect on spontaneous rhythmic activity.

Estimates of the total transit time of luminal contents through the duct from these experiments agree well with other estimates from completely different approaches. It is clear that propulsive contractions occur at the time of emission and ejaculation (Hib et al. 1982) and so interference with this process should lead to effects on fertility. Pertinent to the present discussion are observations of sperm viability after activity of epididymal peritubular muscles has been modulated.

3.2 Experiments Decreasing Sperm Transport Through the Epididymis

Experimental Ablation of a Sympathetic Plexus

Ablation of the inferior mesenteric plexus results in accumulation of spermatozoa in the epididymis as a result of loss of muscular tone (Bil-lups et al. 1990a). Four to 8 weeks after the operation the percentage of

motile sperm is reduced (Billups et al. 1990a) and straight-line and curvilinear velocities are reduced from 1 week on (Billups et al. 1990b). These changes were not considered secondary to the accumulation of the spermatozoa in the lumen since retention of sperm in the cauda by vasectomy for a similar period increased these motility parameters (Billups et al. 1990b). Whether the drug itself or a hostile luminal environment was responsible for this effect was not clarified.

Effect of Sympatholytics

The systemic administration of guanethidine, an agent that depletes peripheral neurones of endogenous noradrenalin results in the failure of seminal voiding with a concomitant swollen and ruptured epididymal duct and attendant spermatoceles. After long-term treatment and the return of ejaculatory activity the animals are still infertile (Hepp and Kreye 1973). This probably reflects ejaculation of spermatozoa that have degenerated during spermiostasis or reduced sperm numbers following rupture of the vas deferens (Evans et al. 1972). With local administration (intrascrotal silastic rods: Ratnasooriya 1982) oligozoospermic ejaculates from animals with sperm in the epididymis must indicate inhibition of emission or ejaculation.

Neither of the above reports are of interest as models for reversible contraceptives for men, being chemical equivalents of vasectomy, and for neither is the cause of damaged sperm known. After vasectomy there is a fall in intraluminal pH proximally and a rise distally (Cafilisch and DuBose 1990b); toxic products arising from packed spermatozoa, inadequate gas tensions of microcirculation are all possible causes of damage, and when guanethidine is administered, the drug itself could affect the spermatozoa. Understanding this phenomenon of degeneration of sperm within the epididymal canal may permit the creation at will of hostile environments for contraceptive purposes.

Inhibition of Angiotensin Converting Enzyme

The complete renin-angiotensin system is present in the rat epididymis (Grove and Speth 1989; Wong and Uchendu 1990, 1991; Wong et al. 1990). The localization of angiotensin II receptors in the peritubular region of the epididymal duct, especially in the proximal cauda epididymidis, and the powerful *in vitro* effects of angiotensin II on epididymal

muscular contraction raises the possibility that this may be a physiological effector during emission. However, administration of captopril, an inhibitor of angiotensin converting enzyme that catalyses the formation of angiotensin II, at doses sufficient to reduce enzyme activity in serum, has no effect on the fertility of male rats (Wong and Uchendu 1990).

3.3 Experiments Increasing Sperm Transport Through the Epididymis

Administration of Symphathomimetics

When methoxamine was provided in a collar surrounding the vas deferens the drug produced local contractions in the vas deferens (Ratnasooriya et al. 1979a) and infertility was due to azoospermia. That the total sperm content of the epididymis was also decreased was taken as evidence that sperm transport through the organ had been increased (Ratnasooriya et al. 1979b). A decline in total sperm numbers in the epididymis was confirmed when methoxamine was provided in a Silastic implant adjacent to the epididymis in the scrotum (Ratnasooriya et al. 1980). However, the temporary reduction in fertility achieved in these animals (with no effect on mating performance) was a result of oligozoospermic ejaculates. The absence of spermatozoa in the ejaculate could be explained by a lowered extra-gonadal reserve or a disruption of contraction at emission. In either case the proposed enhanced movement of spermatozoa with subsequent ejaculation of immature spermatozoa was not realized.

Depletion of Androgens

Castration is known to reduce the transport time sperm in the epididymis as judged from measurement of epididymal sperm reserves (Dyson and Orgebin-Crist 1973; Foldes and Bedford 1982; Pholpramool et al. 1983) and thymidine labelling of testicular spermatozoa (Sujarit and Pholpramool 1985). Various reasons could account for this; decreased protein secretion (lowering viscosity) or greater muscular activity; after longer periods the reduced volume of atrophic organs may contribute (Foldes and Bedford 1982). Methodological problems probably account for the discrepancies in the literature about whether

epididymal contractions are increased or decreased after castration (see Din-Udom et al. 1985; Chaturapanich and Pholpramool 1990). In the latter studies in the rat short-term castration or anti-androgen treatment was associated with increased amplitude and basal pressure but not with increased contraction frequency in any epididymal region and where spontaneous activity decreased, responses to electrical stimulation remained.

Mating tests reveal that male rats are subfertile 3 days after bilateral castration but it was never demonstrated that the reduced fertility was due to the appearance of immature cells in the ejaculate (Pholpramool and Sornpaisarn 1980; see "Antiandrogens", p. 439).

Administration of Estrogens

Low-dose estrogen administration to mice enhances sperm transport through the epididymis, as judged from the greater numbers of free sulphhydryl groups in sperm obtained from the cauda (characteristic of immature spermatozoa) and confirmed from monitoring the passage of thymidine-labelled spermatozoa (Meistrich et al. 1975). However, the results of these experiments are difficult to interpret because in rodents estrogens are transferred from the male to the female during mating (Banerjee 1968; Ericsson and Baker 1966; Singh et al. 1970) and they can interfere there with other aspects of fertilization. At longer time intervals or with higher doses estrogens can reduce male fertility by blocking testicular and pituitary function or by anti-androgenic effects on the epididymal epithelium. Certain effects of administered estrogens on male fertility related to effects on the epithelium are cited below (see "Estrogens", p. 439).

4 Male Contraception via Action on the Epididymal Epithelium

4.1 Background

There is little chance that the biosynthetic activity of the epididymis could be interfered with specifically since no biochemical pathways are unique to this organ. While it is true that some protein secretion

products are unique to the epididymis, others differ from similar proteins synthesized in other organs with respect to post-translational modification (see chapter by Cooper, this volume) and the relationship of some secreted proteins to phosphomannosyl receptors may differ from other organs (Sosa et al. 1987), the opportunities for interference with normal epithelial functioning reside in interfering with the secretion products themselves, or with the movement of transported molecules.

4.2 Inhibiting Synthetic Activity

The importance of some secreted proteins in the fertilization process makes their elimination, or blocking their interaction with spermatozoa, attractive. Manipulating the hormonal environment to achieve this is a questionable strategy since not all of the epididymal proteins are androgen-dependent (see chapter by Cooper, this volume). Two major non-protein, androgen-dependent osmotic components of epididymal fluid, glycerophosphocholine and inositol are synthesized by the epithelium. It is doubly unfortunate that no inhibitors of their synthesis in the epididymis are known, for one way to elucidate their role in fertility would be to deprive spermatozoa of their presence for the entire time of their epididymal sojourn, and then check the fertility of the animals. Without this knowledge, it is not possible to say whether interference with these compounds would be of contraceptive value.

Because it is not organ-specific, hormonal control cannot be used to advantage in the epididymis. Although the epididymis has a higher androgen requirement than other accessory organs, the concept that it would be more susceptible to alteration in hormonal levels with 'functional sterility' as a consequence (Prasad et al. 1970) was never confirmed (Cooper 1986).

4.3 Inhibiting Epithelial Transport Activities

Both inositol and L-carnitine are transported into the epididymal lumen from the bloodstream and accumulated to high concentrations. *In vitro*

luminal perfusion has indicated that there are no major differences between transport characteristics in the epididymis and in other tissues (Cooper et al. 1986, 1988a). Inhibitors of inositol transport are not available and transport inhibitors of carnitine are expected to affect other organs equally, *e.g.* the heart, that also transport carnitine. Some specificity for carnitine transport by epithelial cells may be possible if it is confirmed that carriers on the basolateral and apical membranes mediate carnitine transport in the epididymis (Yeung et al. 1980) and that there is only a basolateral carrier in non-polarized muscle cells. However, delivery to and a specific action on, the apical membrane carrier would be needed for this to work.

It should be possible to design structural analogues that may block the transport of each of these substances into the epididymis. An alternative approach would be to design a drug, toxic to spermatozoa, that can utilize the carriers and be accumulated in the lumen, in much the way that sulphapyridine (Wong et al. 1987) is thought to utilize the epididymal PAH transporter (Qiu and Wong 1985).

Modification of ion pumps would certainly alter the luminal micro-environment experienced by maturing and stored epididymal spermatozoa, but there would be no tissue selectivity of the process and of great concern would be the action of such drugs on the kidney. However, work has been done on several compounds that interfere with fluid absorption (spironolactone), fluid secretion (reserpine) or fluid acidification (acetazolamide) and no renal side effects were noted; these are summarized below (see Section 4.5).

4.4 Attacking Specific Secretions

An alternative approach to affecting the synthesis or transport of compounds by the epithelium would be to attack specifically the products that are synthesized or transported. It is known, for example, that many proteins in the epididymal lumen are enzymes (see Cooper 1986) but their rôle in sperm maturation is not known. If they are involved in modification of sperm membrane proteins connected with fertilization, inhibiting their activity *in situ* may well have repercussions for fertility (see chapter by Cooper, this volume).

A strategy designed around the activity of luminal enzymes presupposes that the enzymes are active within the epididymal lumen, but it is far from clear whether this is so. There is evidence that proteolytic enzymes are active in the epididymal lumen (Goldin et al. 1989) and protease inhibitors have been demonstrated within the epididymal canal (Djakiew et al. 1985; Suominen and Setchell 1972; Jonakova and Cechova 1985). It is an attractive concept that removing or inactivating these inhibitors would stimulate potent proteolytic activity that could be damaging to sperm cells. Rat epididymal α -mannosidases can be inhibited by swainsonine *in vivo* (Skudlarek and Orgebin-Crist 1988; Tulsiani et al. 1990) but the fertility of these animals is not known.

4.5 Antifertility Agents Acting in or on the Epididymis

The few antifertility compounds that have been shown to have an effect on the epididymis (the epithelium or luminal fluid, rather than just epididymal weight) are given in Table 1. Where an action on the epididymis has been observed, fertility studies have not always been done and *vice versa*.

Adrenalectomy and Spironolactone

Adrenalectomy leads to decreased epididymal resorption of Na^+ and water in the cauda epididymidis *in vivo* with concomitant increase in sperm concentration in the lumen, but it is not known if these animals are fertile. The aldosterone antagonist spironolactone reduces K^+ and sperm concentrations in the cauda, independent of the antiandrogen properties of the compound (Turner and Cesarini 1983), but after treatment objective measures of sperm motility and the fertility of the animals were unchanged. Type I aldosterone receptors are present in the rat epididymis (Pearce et al. 1986) but only clear cells contain a histochemically demonstrable form (Hinton and Keefer 1985). It is uncertain whether the hormone action is mediated *via* clear cells since they represent only 10% of the epithelium (Yeung and Cooper 1982). Studies in which intraluminal pH has been measured in micropuncture samples have indicated that the principal cells may be responsible for luminal acidification (Caflisch and DuBose 1990a).

Table 1. Antifertility agents for males with a possible action on the epididymis

Species	Substance approach	Dose	Duration	Epididymal fluid	Caudal sperm	Moti- lity	Ferti- lity	Epithelium	References
rat	adrenalectomy		3d		conc↓				Turner & Cesarini 1983
rat	adrenalectomy + aldosterone	25µg/d	3d					resorption	Turner & Cesarini 1983
rat	adrenalectomy		4d					Na ⁺ , H ₂ O↓	Au et al. 1978
rat	adrenalectomy + aldosterone	75µg/d	4d						Au et al. 1978
rat	spironolactone	1 mg/kg/d	3, 14d	Na ⁺ ↑Ca ²⁺ ↑ cap Ca ²⁺ ↑ cor Ca ²⁺ ↑Mg ²⁺ ↑P↑ cau					Jenkins et al 1983c
rat	spironolactone	10 mg/kg/d	3d		conc↓				Turner & Cesarini 1983
rat	spironolactone	10 mg/kg/d	4-17d	K ⁺ ↓	conc↓	OK	OK		Wong & Lee 1982
rat	spironolactone + ligate corpus, vas	10 mg/kg/d	4-17d	K ⁺ ↓	conc OK				Wong & Lee 1982
rat	acetazolamide	50 mg/kg/d	6w			OK	OK		Wong et al. 1987
rat	acetazolamide	20 mg/kg/h	80m					secretion H ⁺ ↓	Au & Wong 1980
rat	acetazolamide	20, 50mg/kg/h	45m	ph OK, pCO ₂ ↑					Caflisch & DuBoise 1990a
rat	reserpine	0.5 mg/kg/d	12-14d	viscos↑ prot conc↑	PCV↑				Wen & Wong 1988
rat	reserpine + EDL	0.5 mg/kg/d	12-14d	viscos↑ prot conc↑	PCV↑				Wen & Wong 1988

Table 1. (cont.)

Species	Substance approach	Dose	Duration	Epididymal fluid	Caudal sperm	Moti- lity	Ferti- lity	Epithelium	References
rat	gossypol (99.9%)	20mg/kg/d	16w	RTF	Na ⁺ , K ⁺ OK	↓	↓		Wong et al. 1984
rat	gossypol (WHO 99.9%)	15 mg/kg/d	3-6w	Na ⁺ , K ⁺ OK	damaged	↓			Wang et al. 1986
rat	gossypol (BMM>98%)	30mg/kg/d	4w	Na ⁺ ↑, K ⁺ , prots OK	conc↓	↓			Wong et al. 1983a
rat	gossypol + ligate corpus, vas	30mg/kg/d	4w	Na ⁺ , K ⁺ , prots OK	conc OK	OK			Wong et al. 1983a
rat	gossypol	7,5 mg/kg/?d	4w	Na ⁺ ↓, K ⁺ ↑ caput			↓		Kaur 1989
rat	gossypol (Leiras)	20,30mg/kg/d	5w	carnitine↓, inositol↓	damaged	↓		vacuolated	Soufir et al.1984
rat	gossypol (Leiras)	20,30mg/kg/d	5w	prot, LDH, ACP, K ⁺ , acetyl carnitine OK	count↓	↓			Soufir et al.1984
				inositol↓, carnitine↓					Soufir et al.1984
		25mg/kg/d	5w	inositol↓, carnitine↓ K ⁺ ↓ cauda					Soufir et al.1984
rat	gossypol (Leiras)	25mg/kg/d	17d	inositol OK, car- nitine OK, K ⁺ OK		↓			Radigue et al. 1988
rat	gossypol	30mg/kg/d	38d	carnitine↓	decapitated, count↓		↓	morphology OK	Giridharan et al. 1987
rat	gossypol	20mg/kg/d	52d	carnitine↓	decapitated, count↓		↓	morphology OK	Giridharan et al. 1987
rat	gossypol	30mg/kg/d	52d	carnitine↓	decapitated, count↓		↓	morphology OK	Giridharan et al. 1987

Table 1. (cont.)

Species	Substance approach	Dose	Duration	Epididymal fluid	Caudal sperm	Motility	Fertility	Epithelium	References
rat	gossypol	30mg/kg/d	4w					transport carn OK	Sun & Shi 1985
rat	gossypol	20, 30mg/kg/d	5w		damaged	↓		ultrastructure OK	Hoffer 1982
rat	gossypol	20mg/kg/d	7w					excludes ^3H -inulin, La^{3+}	Hoffer 1985
rat	gossypol	20mg/kg/d	6w					excludes ^3H -inulin, La^{3+}	Hoffer & Hinton 1984
rat	gossypol	20mg/kg/d	5w			↓		ultrastructure OK	Hoffer 1985
rat	gossypol	30mg/kg/d	3w			↓		ultrastructure OK	Hoffer 1985
rat	gossypol	20mg/kg/d	2-30d		damaged	↓	↓	drug enters lumen	Oko & Hrudka 1982
rat	gossypol (WHO 99.9 %)	15mg/kg/d	3w		damaged	↓	↓		Wang et al. 1988
rat	α -chlorohydrin	8-16mg/kg/d	3-7d	OK		↓			Back et al. 1975
rabbit	α -chlorohydrin	8-112mg/kg/d	3-7d	GPC↑		OK			Back et al. 1975
rat	α -chlorohydrin	9mg/kg/d	7-14d	SDS PAGE OK	surface prots↓	↓	↓		Tsang et al. 1981
rat	α -chlorohydrin	9mg/kg/d	7d					histology OK resorption Na^+ , H_2O ↓	Wong & Yeung 1977

Table 1. (cont.)

Species	Substance	Dose	Duration	Epididymal Fluid	Caudal sperm	Motility	Fertility	Epithelium	References
rat	α -chlorohydrin	10mg/kg/d	30d					secretion K, prot OK excludes ^3H -PEG, ^{14}C -urea synthesis, caput secretion GPC $\downarrow$, GPC $\downarrow$, histological damage	Hinton et al. 1983
rat	α -chlorohydrin	15mg/kg/d	6d					transport	Morris et al. 1976
rat	α -chlorohydrin	90mg/kg/d	2d					30MG $\downarrow$ caput transport	Morris et al. 1976
rat	α -chlorohydrin	500 μmol						DOG OK epithelial damage	Hinton et al. 1983
rat	α -chlorohydrin	10mg/kg/d	30d					endocytosed	Reijonen et al. 1975
rat	α -chlorohydrin	100mg/kg	4h					no damage	Hoffer et al. 1973
rat	α -chlorohydrin	140mg/kg	2-12d						Reijonen et al. 1975
rat	α -chlorohydrin + EDL	100 mg/kg	4h						
rat	α -chlorohydrin	^{14}C -labelled						in cauda, vas > caput	Crabo & Appलगren 1972
rat	α -chlorohydrin							in cauda	Edwards et al. 1975
rat	α -chlorohydrin	15mg/kg/d	8d	pH $\downarrow$					Caflisch & DuBoise 1990d

Table 1. (cont.)

Species	Substance approach	Dose	Duration	Epididymal fluid	Caudal sperm	Motility	Fertility	Epithelium	References
pig	α -chlorohydrin	1mg/kg/d	5d	Na ⁺ , K ⁺ , GPC OK	droplet migration↓	Å			Crabo et al. 1975
pig	α -chlorohydrin	5mg/kg/d	15d	Na ⁺ , K ⁺ , Cl ⁻ ↑	droplet migration↓	↓	caput		Crabo et al. 1979
pig	α -chlorohydrin	30mg/kg/d	15d	Na ⁺ , K ⁺ , Cl ⁻ OK, GPC, prot OK	PCV↓ droplet migration↓	↓	cauda		Crabo et al. 1979
rat	6-chloro 6-DOG	24mg/kg/d	3-4w	SDS PAGE OK	surface prots↓	↓	Å	resorption Na ⁺ , H ₂ O↓	Tsang et al. 1981 Wong et al. 1980
rat	6-chloro 6-DOG	120µmol/kg/d	7-14d					secretion K ⁺ , prot OK	Wong et al. 1980
rat	6-chloro 6-DOG	500µmol						transport 30MG↓ caput	Hinton et al. 1983
rat	sulfapyridine	32.1mg/kg/d	6w		conc↓	OK	↓	enters lumen (> blood)	Wong et al. 1987
rat	sulfapyridine + EDL				conc↓	OK	↓		Wong et al. 1987
rat	castration		1-5d	Na ⁺ , K ⁺ OK					Pholpramool & Sornpaisarn 1980

Table 1. (cont.)

Species	Substance approach	Dose	Duration	Epididymal fluid	Caudal sperm	Motility	Fertility	Epithelium	References
rat	cyproterone acetate	10mg/d	10-50d			↓		resorption Na ⁺ , H ₂ O↓	Wong et al. 1978, 1979
rat	cyproterone acetate	10mg/kg/d	3w	OK			OK		Jones 1977
rat	cyproterone acetate	30mg/kg/d	3-4w	SDS PAGE OK	surface prots OK	OK	OK		Tsang et al. 1981
rat	cyproterone acetate	5.20mg/kg/d	1-5w	AKP↓		↓		histology OK	Back et al. 1977
rat	cyproterone acetate	10mg/rat/d	4-8w	Cl ⁻ ↑K ⁺ ↑Na ⁺ ↑S↑ Mg ²⁺ , Ca ²⁺ , P OK					Jenkins et al. 1983a
rat	¹⁴ C-cyproterone acetate							enters lumen (14% blood)	Cooper 1980
rabbit	cyproterone acetate	400mg/d	35d	OK					Jones 1977
rat	cyproterone acetate	20mg/kg/d	1-5w	Na ⁺ ↑ pH↑, HCO ₃ ⁻ ↑			↓	histology OK	Back et al. 1977
rat	flutamide	25mg/kg/d	15d	pCO ₂ OK, Cl ⁻ OK Na ⁺ ↑Cl ⁻ ↑Caput Cl ⁻ ↑Corpus Ca ²⁺ ↓Mg ²⁺ ↓S↓P↓					Caflisch 1992
rat	estradiol valerate	2.5mg/kg/2d	13d						Jenkins et al. 1983b
rat	estradiol valerate	2.5mg/2d	4d	Caput pH↑, HCO ₃ ⁻ ↑, pCO ₂ OK					Caflisch & DuBose 1990c

Table 1. (cont.)

Species	Substance approach	Dose	Duration	Epididymal fluid	Caudal sperm	Moti- lity	Ferti- lity	Epithelium	References
rat	estradiol benzoate	2.9mg/d	14d					excludes ³ H-inulin	Turner et al. 1981
rabbit	stilbestrol	0.6mg/d	35d	like castrated					Jones 1977
rabbit	medrogestone	60mg/d	35d	OK					Jones 1977
bull	estradiol benzoate	0.03mg/kg/d	36d	Na ⁺ ↑, ↓, K ⁺ ↑, GPC↓	PCV↑				Cupps & Briggs 1965
bull	estradiol benzoate	8-12.5mg/d	?		10-30% bent cauda (not caput, corpus)				Cupps & Briggs 1965
bull	estradiol benzoate	8-12.5mg/d	3w		10-30% bent tails				Cupps & Briggs 1965
bull	+ testosterone	50-200mg/d	3w		56-58% bent tails				Cupps et al. 1960
bull	estradiol benzoate	3.5mg/w	3w	ACE OK	61% bent tails	OK	OK		Wong & Uchendu 1990
rat	captopril	20mg/kg/d	8w		PCV OK, conc OK				

↓, reduced below normal; ↑, increased above normal; BMM, Beijing Materia Medica; EDL, efferent duct ligation; WHO, World Health Organisation; OK, unchanged from normal; conc, concentration; ACE, angiotensin converting enzyme

Carbonic Anhydrase

Another enzyme activity demonstrably present in clear cells is carbonic anhydrase, although principal cells in the corpus epididymidis are also active (Cohen et al. 1976). Although the enzyme inhibitor acetazolamide prevents acidification of the lumen in short-term luminal perfusion experiments, the pH of the epididymal lumen is unchanged *in situ* by administration (Table 1). After long-term treatment rats are fertile and sperm from the cauda are motile (Wong and Lee 1982).

Reserpine

Reserpine, a compound that depletes cellular noradrenalin, serotonin and dopamine reserves, inhibits fluid secretion in the epididymis *via* a reduction in apical chloride secretion (Wen and Wong 1988). This leads to increased luminal fluid viscosity and sperm concentration but spermatozoa from the cauda are motile (Wen and Wong 1988).

Gossypol

The long time that elapses before gossypol-treated rats become infertile is normally interpreted as indicating that its principal site of action is the testis. However, at some doses spermatogenesis appears normal and there have been reports that effects on the epididymis are evident, with concentrations of carnitine, inositol and potassium being reduced and sodium increased (Table 1). The effects of gossypol on serum testosterone is still debated (Hoffer 1985), although no effects on serum potassium or aldosterone have been observed (Tang and Wong 1984). To some extent these difference will reflect the strain of rat used and the source, nature and purity of the gossypol preparations, particularly with respect to vacuolation of the epithelium. The tight junctions between cells remain intact after gossypol treatment.

In a micropuncture study, increases in intraluminal Na^+ and decreases in K^+ were found only in caput fluid of rats given low doses of gossypol (Kaur 1989). In yet another study decreases in intraluminal K^+ , carnitine and inositol were found in the cauda (Soufir et al. 1984). At earlier times of treatment, however, this group found no such effects of gossypol on these markers (Radigue et al. 1988) and they argued that damaged spermatozoa entering the lumen mediated the belated effects of gossypol on the epididymis. The movement of severely

damaged spermatozoa through the epididymis after gossypol administration has certainly been documented (Oko and Hrudka 1982; Baccetti et al. 1986) but there is still debate about whether the cells are damaged within the testis or after they enter the epididymis.

Other reports have shown that the ionic composition of cauda epididymal fluid is unchanged after application of an antifertility dose of gossypol that reduces sperm motility (Wong et al. 1983a; Wang et al. 1986). A raised Na^+ concentration, indicative of inhibition of resorption of this ion, was not found when ligatures were placed on the corpus epididymidis, suggesting that proximal structures are involved in this effect. Ligation would deprive the epithelium not only of the normal intraluminal Na^+ concentration, but also of any gossypol and spermatozoa entering the lumen *via* the testicular fluid. It may be these damaged sperm cells that influence the epithelium, since gossypol does not affect the secretion or composition of rete testis fluid (Wong et al. 1984; Wang et al. 1986). On the other hand, the drug enters testicular fluid more readily than epididymal fluid in acute studies (Wang et al. 1989; Qiu et al. 1989) and could affect the luminal aspect of the epididymis. After chronic treatment gossypol is found in epididymal fluid (Wang et al. 1988) but it could be concentrated from that entering the testis and affect the epididymis after sufficient time for it to accumulate there.

α -Chlorohydrin and 6-Chloro 6-Deoxysugars

Alpha-chlorohydrin is a good example of a compound that itself, or through metabolites, interferes with the metabolism of spermatozoa. It is surprising that, in view of its effects on the transporting functions of the caput and cauda epididymal epithelium demonstrated by sensitive techniques *in vivo* and *in vitro* (Table 1), the composition of epididymal fluid from the cauda is largely unaltered in treated animals (Table 1). Specific surface proteins are absent from epididymal spermatozoa (Tsang et al. 1981). At high doses spermatocoeles are formed in the caput region following ductal occlusion as a consequence of its concentration in the lumen or ingestion by epithelial cells and subsequent epithelial cell damage (Reijonen et al. 1975).

The action of chlorosugars closely resembles that of α -chlorohydrin in that effects on the epididymal epithelium are documented (Table 1),

certain sperm-coating proteins are missing (Tsang et al. 1981) and effects on sperm metabolism have been noted (Ford and Waites 1986).

Antiandrogens

Subfertility in mated rats 3 days after castration is associated with an unchanged ionic composition of epididymal fluid (Pholpramool and Sornpaisarn 1980). These results are complicated by the reduced sexual drive and smaller accessory organs after androgen withdrawal, both of which may reduce the effectiveness of sperm transfer to the female. However, that a lack of epididymal secretions was responsible for the reduced fertilizing capacity of sperm from castrated animals was demonstrated when the effects of increased transport were taken into account and mating effects obviated by *in vivo* insemination (Dyson and Orgebin-Crist 1973).

The antiandrogen cyproterone acetate has effects on epididymal epithelial resorption (Na^+ , water) and secretion (K^+ , proteins) (Wong et al. 1978) but the only significant difference in the composition of epididymal fluid from these animals and controls was a decrease in activity of alkaline phosphatase (Back et al. 1977). This steroid enters the epididymal lumen by binding to luminal proteins (Cooper 1980) and thus may act intraluminally. In some studies the motility of epididymal spermatozoa was retained (Tsang et al. 1981). Unesterified cyproterone increased luminal Na^+ in the rat in one study (Jones 1977) and in the rabbit medrogestone was far less effective than stilbestrol in influencing the composition of rabbit cauda epididymal fluid, although sperm function was not analyzed.

Estrogens

The antifertility effects of estrogens have been discussed above (p. 426) with respect to increased ductular transport of spermatozoa in mice. In the rabbit Jones (1977) showed that stilbestrol was extremely effective in inducing a castration-like composition of epididymal fluid. In the rat great changes in the composition of epididymal fluid have been shown in micropuncture studies after estrogen administration (Table 1), but the fertility of these particular animals was not examined. The fact that epididymal fluid was altered is of interest in light of earlier reports that morphological changes in epididymal spermatozoa, similar to those

found in naturally occurring infertility in domestic animals, can be induced in bulls by injection of estrogens (Cupps et al. 1960; Cupps and Briggs 1965; Gustafsson 1966) but not boars (Linde et al. 1975) (see Section 6.). In order to see if the effect was due to reduced androgens, Cupps and Briggs (1965) gave testosterone in addition to the estrogen implant in bulls but found, without comment, even more deformed spermatozoa in the ejaculates. They argued that estrogen was not acting as an anti-androgen but directly on the epithelium. An abnormal, local, conversion to estrogen was not considered and the role played, if any, by estrogen receptors in this epididymis remains unknown (Cooper 1986).

Sulphapyridine

Many sulphasalazine derivatives have been tested in rats for antifertility properties but the time for infertility to appear is consistent with an action on the testis (Pholpramool et al. 1991). The rapid return of fertility to male rats fed sulphapyridine suggested to Pholpramool et al. (1991) an epididymal action, although the earliest onset of an effect was observed at 3 weeks. Sulphapyridine decreases sperm concentration in the epididymis by an unexplained action and it also enters the epididymal lumen, in some cases to exceed blood levels (Wong et al. 1987). The authors suggested that the antifertility effects depended on the luminal concentration of the drug; the time before an antifertility effect was exerted would reflect the time required for sufficient drug to be accumulated.

Captopril

The products of the renin-angiotensin system in the rat epididymis are not only active in eliciting peritubular muscular contractions (see "Inhibition of angiotensin converting enzyme", p. 424) but can also induce fluid secretion by epithelial cells (Wong and Uchendu 1990 1991; Wong et al. 1990). This represents an interesting and complex interplay between luminal spermatozoa and the epithelial cells lining the duct. Angiotensin I is an androgen-dependent secretion product of the epithelial cells and is converted to angiotensin II by angiotensin-converting-enzyme (ACE) which is present in spermatozoa. The enzyme has been localized by Vanha-Perttula et al (1985) in cytoplasmic dro-

plets, some of which are known to undergo lysis within the epididymal lumen (Hermo et al. 1988). This enzyme could be responsible for the luminal angiotensin II. It has not been demonstrated whether this source of angiotensin II can affect the peritubular muscle (see likewise p. 424) but the luminal product can stimulate fluid secretion by epithelial cells in culture (Wong et al. 1990). The failure of the ACE inhibitor captopril to affect sperm motility and male fertility may be due to its inability to reduce the intraluminal enzyme activity (Wong and Uchenda 1990).

5 Male Contraception *via* Action on Spermatozoa

For a compound to have a direct effect on the luminal spermatozoa it must first gain access to the tubule lumen. Antifertility drugs can be thought of as entering every tissue in the body but, by being target-specific, affecting only spermatozoa. On the other hand, less target-specific compounds could be accumulated by tissue-specific carriers and concentrated in the epididymal lumen. A target-specific drug concentrated by tissue-specific mechanisms would offer the greatest hope of specificity with minimal side effects.

5.1 Access to the Epididymal Lumen

The Paracellular Route

The presence of occluding junctions between cells of the epididymal canal has been well documented (Suzuki and Nagano 1978; Hoffer and Hinton 1984). They are not as tight as those between Sertoli cells but they largely contribute to the maintenance of different fluid compositions either side of the epididymal epithelium and restrict the movement of large molecules by the paracellular route. However, there are albumin-like proteins in the epididymis (see Cooper 1986) and after castration the protein composition of epididymal fluid changes to resemble serum (Jones and Dott 1980), suggesting a gross breakdown of the normal barrier to circulating proteins. The question of how drugs may enter the lumen in normal animals requires a brief review of the mechanisms operating to maintain the luminal environment.

Transcellular Pathways

There is ample evidence for fluid-phase and adsorptive endocytosis from the apical surface of rat epididymal cells; to date, though, a transcellular vesicular bypass of tight junctions has been demonstrated only for cationic ferritin (taken up by adsorptive endocytosis) in the rete testis (Morales and Hermo 1986) and vas deferens (Hermo and De Melo 1987). The extent of breach of the occluding junctions in the rat epididymis is minimal in the case of horse radish peroxidase provided to the serosal surfaces of the epithelium both *in vitro* (Cooper et al. 1987) and *in vivo* (Cooper et al. 1988b). In short-term experiments there is no detectable pro-luminal transport of infused immunoglobulin (Wong et al. 1983b) or specific transport of IgG *in vitro* (Yeung et al. 1991).

Notwithstanding these results, sperm antibodies appear to accumulate in epididymal tissue after their systemic injection in mice (Naz and Bhargava 1990) and other studies have suggested that proteins may gain access to the epididymal tubule lumen of rats (antibodies to specific epididymal proteins: Cuasnicú et al. 1984); rabbits (epididymal extracts: Orgebin-Crist and Jahad 1979) and hamsters (epididymal zona-binding proteins: González Echeverría et al. 1984) in quantities sufficient to modify sperm fertilizing capacity. These studies have involved somewhat longer incubations of intact epididymal tubules but the properties of the incubated tubules with respect to entry of macromolecules was never validated. Although the tubules were not closed, it is extremely unlikely that sufficient proteins entered the cut ends to affect luminal spermatozoa. From evidence that upon even short term incubation with peroxidase the enzyme is detectable deep within the interstitium of rat epididymal tubules (Cooper et al. 1987), it is probable that in these experiments contact of spermatozoa with the proteins occurred upon termination of the incubation when the tubules were minced to release the spermatozoa for insemination.

Smaller molecules cross epididymal cell membranes by free or facilitative diffusion or active transport processes (see Cooper 1986; Sutarit et al. 1986). Lipid soluble drugs may well depend on binding to intraluminal proteins for their intraluminal accumulation as is the case for steroids (Cooper 1980; Turner and Roddy 1990) and water soluble compounds may utilize carrier-mediated transport of facilitative diffu-

sion (Brooks 1979). The possibility of using carriers of active transport systems in the epididymis has been discussed in Section 4.3.

5.2 Antifertility Agents Acting on Spermatozoa

Various examples of actions on spermatozoa can be contemplated: effects on motility (adrenergic and cholinergic agents, carnitine), metabolism (*e.g.* chlorosugars) or cell viability (spermicides), but full discussion of these is beyond the scope of this article. Spermicidal agents should, of course, be specific for some peculiarity of the sperm cell membranes in order to avoid damaging the epididymal epithelium. Perhaps the generation of an active metabolite at the lower pH of the epididymal lumen from a drug permeating the epithelium in uncharged form could be considered. Analogues of carnitine which block the activity of sperm carnitine acetyltransferase *in vitro* have no effect on subsequent fertilization events in the mouse (Boots et al. 1980).

Ornidazole

Designing a compound to attack the only haploid, free-swimming flagellated cells in the healthy body (spermatozoa) should be no more difficult than targetting microbes, against which a wide array of therapeutic agents have been designed in the past. It is intriguing that one such antimicrobial agent compound has antifertility effects in the male rat and apparently acts on spermatozoa at the level of the epididymis. Ornidazole is a 5-nitroimidazole compound with antifertility action in males that, in contrast to most others, has no effect on the testis (McClain and Downing 1988a,b). Its rapid action and the equally fast return to fertility after drug withdrawal indicated an action on the epididymis. In the original work stroboscopic studies of flagellar movement revealed a change in the beating pattern of sperm recovered from the infertile rats (McClain and Downing 1988b). Whether this is an action of the drug which enters the epididymal lumen or a change in the epididymal environment is as yet unknown. Further studies *in vitro* (Fig. 2) indicate that whereas ornidazole does not reduce the percentage of motile cells as much as the structurally similar metronidazole at equivalent doses, it severely curtails the velocity of the remaining

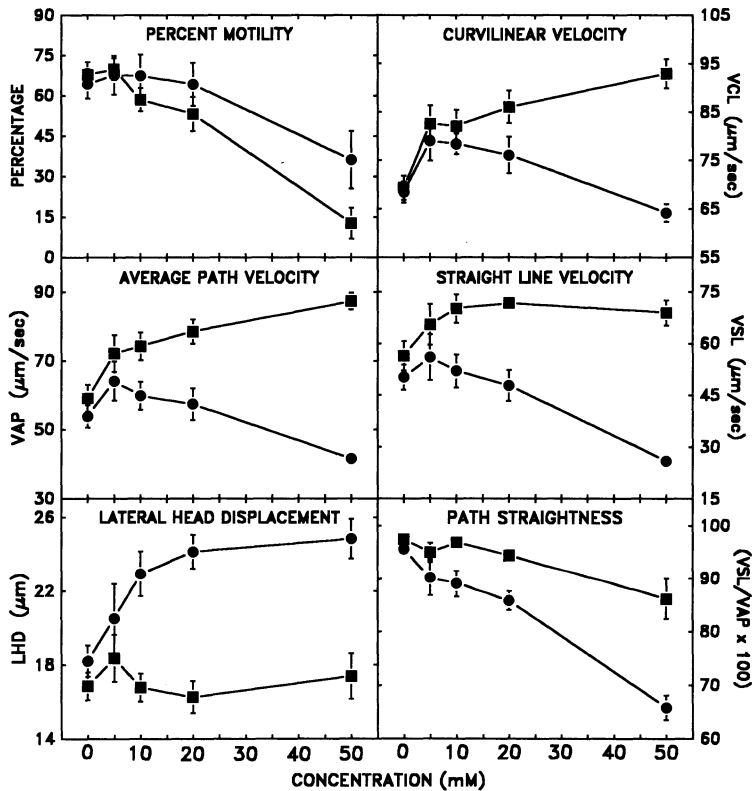


Fig. 2. The effects of ornidazole (circles) and metronidazole (squares) on motility characteristics of rat epididymal spermatozoa *in vitro*. Sperm from the distal cauda epididymidis were transferred to bicarbonate buffered medium containing the drugs (concentrations in abscissa) and motility characteristics (ordinate) was assessed by the Hamilton-Thorn CASA system as described by Yeung et al. (1992). Unpublished data from G. Oberländer and C. H. Yeung

sperm cells; by contrast, metronidazole increases motility parameters. The concentrations of these compounds required *in vitro* to obtain these effects are high and are unlikely to be achieved in the epididymis of treated animals. An action *in situ* on the epididymal epithelium or secretions thus seems likely.

The marked differences in motility response towards ornidazole and metronidazole must be a consequence of the sole chemical difference between the two compounds, a chloromethyl group in the former. Although metronidazole also impairs male fertility, it renders rats azoospermic through an action on the testis (McClain et al. 1989). Of relevance for human contraception is that metronidazole is non-toxic and given routinely to men suffering from lower male tract infections. It is secreted into the semen (Eliasson and Dornbusch 1980) but has, unlike ornidazole, no effect on the motility of human sperm *in vitro* (Cooper and Krüsemann, unpublished data). It certainly should not be beyond the wit of interested pharmaceutical companies to design a similar drug with a specific action on spermatozoa that can be used at lower doses.

Antibodies

The most specific agents for spermatozoa would be antibodies, but access to the epididymal lumen is the problem (see p. 442). In long-term immunization studies in rabbits there was an accumulation of antibodies in the epididymal lumen, but the time taken for a reasonable titre (6 weeks: Weininger et al. 1982; Bedford et al. 1982) is contrary to the requirements for a contraceptive acting on the epididymis. In mice retention of anti-sperm antibodies in the lumen have been detected as early as 72 h after injection (Naz and Bhargava 1990), but mating tests were not performed on these animals. Where active immunization against specific epididymal sperm-coating proteins produced infertility at 3 months (30 % of males) and 6 months (100 % with protein SEP: Fournier-Delpech et al. 1985) or at 3-4 months (40 - 100 % with protein "DE": Cuasnicú et al. 1990), the fertility of Freund's adjuvant-injected rats was not tested. In the latter report the infertility was reversible for 60 - 70 % of males after 6 months.

6 Infertility and Contraception: Two Sides of the Same Coin

Clues to the development of an antifertility agent for males may come from clinical and veterinary findings on stud animals or infertile men.

6.1 Epididymal Dysfunction in Domestic Species

There are reports of distinct changes in the flagellum of ejaculated spermatozoa from certain infertile bulls (Swanson and Boyd 1962; Cupps and Briggs 1965; Blom 1966; Gustafsson 1965 1966; Koefoed-Johnsen and Pederson 1971; Bech and Koefoed-Johnsen 1973; Gustafsson et al. 1972 1974; Wenkoff 1978; Kojima 1978), boars (Einarsson and Gustafsson 1973; Holt 1982; Kunavongkrit et al. 1988), and stallions (Hellander et al. 1991). The common feature of spermatozoa in these animals was a bend at the annulus (mid-piece/principal piece junction) such that when motile, the sperm appear to swim "backwards" (*i.e.* with the head pointing away from the direction of motion). Such spermatozoa are thus clearly viable but presumably unable to progress to the site of fertilization *in vivo*. The percentage normal progressive motility is low in the samples that display abnormal morphology. In many of these studies exhaustive, consecutive ejaculation studies in bulls showed an improvement in progressive motility and a decrease in abnormal forms in semen (Swanson and Boyd 1962; Hopwood et al. 1963; Gustafsson et al. 1972 1974; Gustafsson 1965). This was interpreted as the voiding, with each successive ejaculate, of spermatozoa that had spent less time in an unfavourable caudal environment, rather than a change in the properties of the seminal fluid suspending the cells. The observation that a high percentage of abnormal forms and low progressive motility characterized the ejaculate obtained after a sexual rest of only 10 hours after previous multiple ejaculations (Gustafsson 1965) supports the contention that the caudal environment was in some way hostile to the stored sperm.

Where the epididymides of these animals have been studied, changes in the spermatozoa were found to occur in more proximal regions of the duct in boars (Einarsson and Gustafsson 1973; Holt 1982) and bulls (Koefoed-Johnsen and Pederson 1971; Wenkoff 1978) but it was nevertheless confirmed that a shift to bent forms was occurring within the epididymis. These coiling changes are reminiscent of that achieved simply *in vitro* by immersion of spermatozoa in hypotonic media (Drevius 1963; Drevius and Eriksson 1966) and are most likely a consequence of abnormal epithelial functioning. The osmolality of epididymal fluid has been measured in animals that ejaculate sperm

Table 2. Differences in composition of epididymal fluid from infertile domestic animals ejaculating spermatozoa with bent tails^a

Species Reference ^b	Pig 1	Bull 2	3	4	5	6
Spermatozoa		↑	OK			
Dry weight	↓		↓			
Osmolality	↓	OK	↓			
Na ⁺	OK	OK	↓	↓	OK	↓
K ⁺	OK	↓	OK	↓	↓	↓
Cl ⁻		OK			OK	
Glutamate		OK				
Protein	OK	OK	OK		OK	
Acid phosphatase			OK			
Alkaline phosphatase	OK					
Glycerophosphocholine		OK	OK	OK	OK	↓

^a OK, unchanged from values found in normal animals; ↑ above normal values; ↓ below normal values

^b 1, Einarsson and Gustafsson 1973; 2, Bech and Koefoed-Johnsen 1973; 3, Gustafsson et al. 1974; 4, Gustafsson et al. 1972; 5, Gustafsson 1966; 6, Gustafsson 1965

with bent tails, but reports are conflicting about whether an increase or decrease is found (Table 2). In no case, though, has the composition of the fluid at the site of the defect been measured. The changes in other parameters confirm that epididymal function is abnormal in these animals. Since the epididymal tubule is continuous, an osmotic insult in only a small portion of the duct would ensure that all sperm passing through this region are effected. The cause of this sperm tail anomaly, recognized for many years, remains completely unknown.

An important observation that has yet to be explained is the maintenance of luminal osmotic pressure in the epididymis after castration, when all androgen-dependent secretions have declined (Jones and Glover 1973). Both GPC and inositol are compounds employed by other cell types (including the kidney) to maintain intracellular osmolality in face of large and rapid changes in extracellular tonicity (Nakanishi et al. 1988). The epididymis would appear to maintain extracellular osmolality for the benefit of luminal spermatozoa. Perhaps an

antifertility agent could be developed to exploit the difference in osmotic response of epithelial cells and spermatozoa.

6.2 Epididymal Dysfunction in Man

Examples are few and far between for direct epididymal involvement in human male infertility. Reduced epididymal markers in semen from azoospermic men with normal testicular size is now interpreted as evidence of ductal obstruction (Cooper 1990) and the reduction found in epididymitis (Cooper et al. 1990; Guérin et al. 1990) most likely represents the response of the tissue to the disease rather than its cause. Where a human epididymal specific protein is found in infertile men on the post-acrosomal, rather than the acrosomal, region (Blaquier et al. 1987), the secretion of the protein clearly occurs in the epididymis. It is not yet known if the abnormal location of the protein reflects an abnormality of the protein structure, caused by an epididymal defect, or an abnormality in the sperm membrane domain to which the protein binds. The latter situation could arise from an inherent defect occurring during spermatogenesis or epididymal dysfunction during remodelling of the sperm plasma membrane.

There is one report that human infertility can be caused by epididymal dysfunction (Wilton et al. 1988). In this condition (epididymal necrostermia) poor sperm motility is associated with low sperm viability and in two such men biopsies revealed well-formed testicular spermatozoa. It was inferred that the sperm were damaged in the epididymis since seminal plasma from three of these men was not hostile to donor sperm viability when examined in long term crossed incubation studies. In support of this view, frequent ejaculation to deplete the extra-gonadal sperm reserve (twice a day for 4 to 5 days) marginally improved the motility and vitality of sperm recovered in the first ejaculate of four of these men. Unfortunately, examination of these semen samples for epididymal markers was not made to confirm epididymal dysfunction.

7 Summary

There are two complementary approaches to discovering if contraception *via* the epididymis can be realized. If one assumes that epididymal function is normal in fertile males then it is clearly of interest to establish the fertility status of males in which epididymal function is in some way altered. As indicated above, however, there are currently too few inhibitors of specific epididymal functions for them to be used in this way. The alternative approach is to discover whether epididymal function is altered in males rendered infertile by certain drugs. The literature indicates that, in addition to compounds acting solely on the testis, there are compounds that may act on the epididymis, either directly, or indirectly as a consequence of previous action on the testis. The literature reviewed above indicates that such compounds fall into 3 main categories with respect to action on the epididymal epithelium (or fluid composition) and fertility:

1. compounds or treatments that affect the epididymis but do not affect sperm motility or fertility (adrenalectomy; spironolactone; acetazolamide; reserpine). The changes in epididymal function may be too marginal to influence the spermatozoa.
2. compounds that affect the epididymal epithelium and reduce fertility (gossypol; cyproterone acetate; estradiol; α -chlorohydrin, 6-chloro 6-deoxysugars). These compounds may also affect the testis (estrogens) and other accessory organs (anti-androgens) as well as the sperm cells (chloro-compounds, gossypol). Such drugs may enter the epididymal lumen and affect spermatozoa only when sufficient time has passed for them to accumulate.
3. compounds that reduce fertility rapidly, but whose action, if any, in the epididymis is unknown or unclear (sulphasalazine; ornidazole).

In 1980 Hinton published a review with a similar title to this one, pointing out the advantages of attacking the epididymis for contraceptive purposes. In the same year Patanelli argued that "so little [is] known of epididymal dysfunction and so few compounds known to safely interfere with epididymal function, that [contraception *via* the epididymis] does not seem to be a promising approach for the immedi-

ate future". Although this comment is also relevant today, there is now a far greater appreciation of the secretions of the epididymis and their regulation, and of intracellular events in spermatozoa during their maturation. The link between the two has yet to be made in detail, but when it is possible to start mimicking epididymal function *in vitro*, new avenues for epididymal contraception will be open. At present the most promising of infertile males, those producing ejaculates of sperm with bent tails, display what is required of an ideal male contraceptive; they are otherwise healthy individuals, reproductively active but infertile. If Nature can make such mistakes, it should be possible to mimic her.

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Risks and Benefits of Immunological Contraception

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1 Introduction

We now stand at the beginning of a decade when we expect the global population to rise by around 500 million. This increase will mean that the world will have to house, clothe and feed an additional population the size of China's. Moreover, because the rates of population growth in the developing countries of Africa, South America and Asia are so much higher than in the rest of the world, the distribution of this dramatic population growth will be uneven. The result of this imbalance will be that by the year 2000 approximately 80% of the world's population will be living in developing countries. When this pattern of population growth is translated into terms of environmental destruction, famine and disease, it is clear that whatever methods of contraception are developed for the future, they must take into account the needs of developing countries.

In this context the most appropriate contraceptive modality would undoubtedly be a contraceptive vaccine. Vaccines have the obvious advantages of offering a prolonged protection against pregnancy while being cheap and easy to administer through the infrastructure for the administration of vaccines against disease that exists in most developing countries. Moreover, it should be possible to engineer such specificity into a contraceptive vaccine that this approach should be free from harmful side-effects. The fact that there are clinical models for contraceptive vaccines, for example, in the otherwise healthy patients whose infertility appears to be due to auto- or iso- immunity against sperm surface antigens, also lends weight to the feasibility of this approach. The major disadvantage of a vaccine is that its efficacy will be dependent on the magnitude and duration of the immune response, which we can expect to vary considerably from individual to individual. It may therefore be difficult to predict the reversibility of a given vaccine or to be certain of its efficacy. However, it would be wrong to suppose that a vaccine has to be reversible to be acceptable. Our primary goal should be to engineer vaccines that are efficient, even if this is achieved at the cost of reversibility. If the end result of this approach were to be the development of an injectable means of sterilization, the resulting vaccine would still be extremely useful. There is a major need for a nonsurgical means of sterilizing men and women in both developed and developing countries, and an immunological approach is probably the most likely to achieve this objective successfully. If, on the other hand, a vaccine could be developed that were found to provide reliable protection against pregnancy for a period of 1-2 years, this would also be an extremely useful addition to our contraceptive armamentarium. Clearly the behaviour of any given vaccine will depend, to a large extent, on the immunogenicity of the material used for vaccination and the nature of the target antigen.

Given the potential of an immunological approach to contraception, what are the target antigens being considered to achieve this end? The control of reproductive function is an extremely complex process involving intricate interactions between releasing factors and gonadotropins generated in the brain and the germ and somatic cells of the gonads. In principle, an immune attack on any component of this system should lead to a disruption of reproductive function for the purpose of

contraception. In practice, our choice of potential targets is constrained by considerations of immunogenicity, specificity, antigen concentration and location. Such factors have led to the identification of three primary candidates for the development of a contraceptive vaccine comprising: i) human chorionic gonadotropin (hCG) ii) the zona pellucida and iii) the sperm surface. In this brief review I shall consider the risks and benefits associated with each of these approaches, the current status of product development and the likelihood of a successful outcome to this avenue of endeavour.

2 Human Chorionic Gonadotropin (hCG)

The fundamental principle behind this approach to contraceptive vaccine development is to prevent the maternal recognition of pregnancy by inducing a state of immunity against hCG, the hormone that signals the presence of the embryo to the maternal endocrine system. This gonadotropin-like protein is secreted by the trophoblast of the implanting human blastocyst and passes in the maternal circulation to the ovary, where it serves to stimulate the continued production of progesterone by the corpus luteum. In this way the embryo ensures that the cyclical demise of the corpus luteum that precipitates menstruation is prevented. The induction of immunity against hCG should lead to a sequence of normal or slightly extended menstrual cycles during which any pregnancies are terminated at around the blastocyst stage of development, as a consequence of a failure in the maternal recognition of pregnancy. The elegance of this approach is emphasized by its potential reversability. In theory this should be readily achieved by the administration of a long-acting progestin over that critical period of time when the endometrium is dependent on the corpus luteum as a source of progesterone, before this function is taken over by the placenta.

The immunogens that have been developed to generate antibodies against hCG have been shaped by the existence of considerable homology in the primary amino acid structure of hCG and the pituitary gonadotropin LH (luteinizing hormone). These molecules consist of two subunits, one of which, the α , is identical in the two molecules while the remaining β -subunit, is identical except for a short sequence of 37

amino acids at the carboxyl terminal of hCG (Mitchison 1990). In order to minimize immunological cross reactivity with the pituitary gonadotropin, candidate vaccines have been developed which are based on synthetic peptides representing this hCG-specific carboxyl sequence, conjugated to immunogenic carriers of bacterial origin such as diphtheria or tetanus toxoid. Vaccines incorporating the carboxyl terminal peptide coupled to diphtheria toxoid have been used in phase 1 clinical trials (Jones et al. 1988) and have been shown to produce significant antibody titres in the absence of any detectable side-effects. The antibody titres were thought to be adequate to neutralize hCG action but definitive proof that vaccination with this peptide will result in a contraceptive effect will have to await the outcome of phase 2 clinical trials.

One of the major disadvantages of this approach is the poor immunogenicity of the C-terminal peptide, as a consequence of which powerful muramyl peptide adjuvants have had to be used to promote the immune response. Even so, the antibody responses to this peptide were found to be too short-lived to be clinically useful. This shortcoming has stimulated research into the use of long-acting delivery systems, such as biodegradable microspheres, for the delivery of vaccines based on the C-terminal peptide of hCG (Stevens et al. 1990).

An alternative approach to enhancing the immune response to hCG has been to use the entire β -subunit of this molecule as the immunogen, rather than the C-terminal peptide. This particular approach has been promoted by Talwar's group in India and has already reached the stage of clinical trials (Talwar et al. 1990). Initial studies were based on the conjugation of the β -subunit of hCG to tetanus toxoid. These trials demonstrated that it was feasible to raise antibodies against hCG using this approach and that the response was reversible and free from any harmful side-effects (Kumar et al. 1976; Hingorani and Kumar 1979; Nash et al. 1980; Shahani et al. 1982). The most significant shortcoming of this approach was that the antibody responses were variable from recipient to recipient, with the result that the poorest responders would not have experienced a contraceptive effect with the titres of anti-hCG antibodies obtained. In order to improve the immunogenicity of the β -subunit, hybrid hCG molecules have been generated in which the β -subunit of hCG has been annealed to the α -subunit

of ovine LH. This heterospecies dimer resulted in the generation of conformational antibodies that exhibited greater bioactivity than the isolated β -subunit (Talwar et al. 1988).

Using such formulations all of the 101 women involved in phase 1 clinical trials generated high anti-hCG antibody titres, with high avidity and enhanced bioactivity. A curious feature of this study was that although the subjects participating in this trial raised antibodies that cross reacted with endogenous LH, there was no apparent disruption of the menstrual cycle. Another interesting finding in these phase 1 clinical trials was that some patients showed signs of induced hypersensitivity and immunosuppression following repeated administration of tetanus toxoid-based immunogens. This problem has been addressed by using vaccines conjugated to two different carriers, tetanus and diphtheria toxoid, in an alternating sequence (Gaur et al. 1990).

In terms of safety, the hCG vaccines appear to be free of overt adverse side-effects in the phase 1 clinical trials that have been conducted. Even in the case of the vaccines incorporating the entire β -subunit of hCG, which clearly generate antibodies against LH, there has been no evidence of autoimmune reactions at the level of the pituitary gland. However, unexpected cross reactivity has been observed against pancreatic and pituitary cells when the the carboxyl terminal of the β -subunit was used as the immunogen (Rose et al. 1988). Clearly, long-term *in vivo* studies will be needed to determine whether such cross reactivities are significant. Where such studies have been conducted in non-human primates, the results have been very encouraging, even when the immunization protocols have involved the long-term (7 year) induction of hyperimmunity against the β -subunit of LH (Thau et al. 1987).

Hence the current position with respect to anti-hCG vaccines is that they appear, in the short term, to be safe and reversible. On the one hand, there is no evidence that hCG generated in early pregnancy can have a self boosting effect on antibody titres while on the other, the problem of hyporesponders may be overcome through the use of diverse carriers. The major problems with the approach are the questions that remain concerning the specificity of the response, and hence its long-term safety, and the fact that this particular approach is associated with an abortifacient mechanism of action. In contrast, the alterna-

tive approaches to contraceptive vaccine development serve to prevent rather than disrupt pregnancy, through the inhibition of sperm-oocyte recognition during the early stages of fertilization.

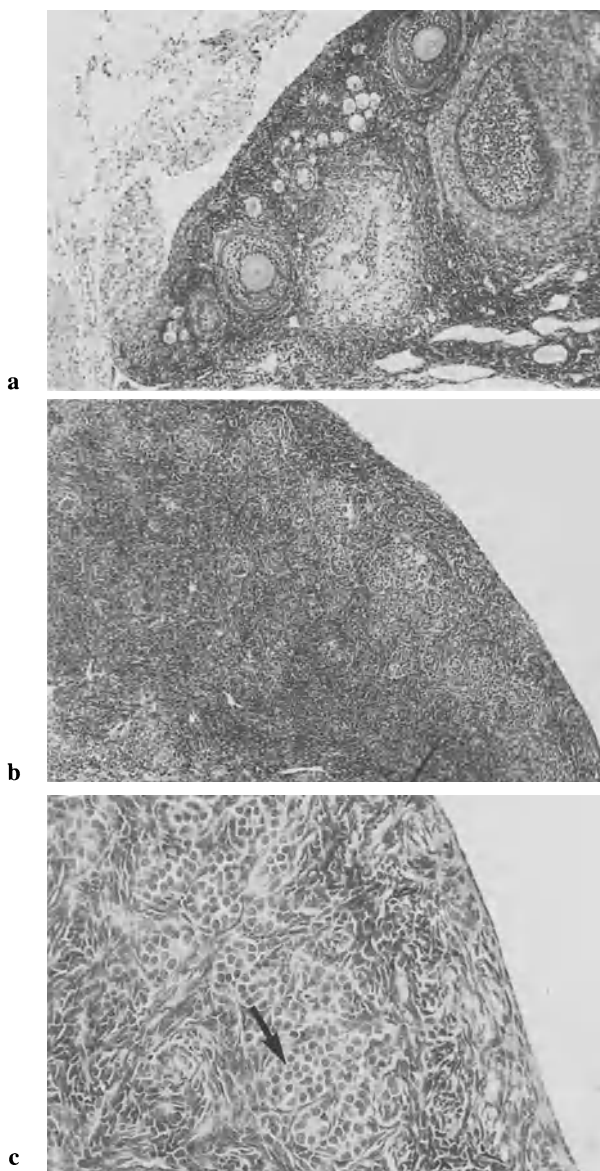
3 Zona Pellucida

The ability of human spermatozoa to recognize and bind to the human ovum during fertilization is dependent upon a highly species- and cell specific-interaction between the sperm surface and a glycoprotein component of the zona pellucida, ZP3 (Wassarman 1988). This molecule serves as a recognition site for human spermatozoa and also as an activating stimulus, triggering the acrosome reaction (Wassarman 1988). The notion of using this molecule as the target for a contraceptive vaccine stems from experiments dating back over twenty years, indicating that antibodies raised against the zona pellucida have the capacity to inhibit the fertilization of mammalian ova *in vitro* (Aitken et al. 1990). Despite the species specificity of sperm-egg interaction, analysis of the primary amino acid structure of this molecule has revealed considerable homology between species as disparate as the mouse, human and hamster (Chamberlin and Dean 1989,1990). As a consequence of this homology, *in vitro* fertilization of the ova of one species can be inhibited by the use of antibodies against the zona pellucida of another. Of particular importance to vaccine development has been the fact that antibodies against the porcine zona pellucida can inhibit the fertilization of human ova *in vitro* (Trownson et al. 1980).

It was subsequently shown that active immunization of primates with porcine zona antigens could inhibit fertility *in vivo* (Sacco et al. 1983; Gulyas et al. 1983; Henderson et al. 1988; Paterson et al. 1992). Such inhibition of fertility could be observed with the glycosylated porcine ZP3 molecule and with the fully deglycosylated ZP3 peptide backbone (Paterson et al. 1992). The infertility induced was potentially reversible and was of prolonged duration (Aitken et al. 1990). However in the course of these active immunization studies a major problem has emerged involving the disruption of normal ovarian cyclicity. Although this side-effect was originally thought to be associated with the use of glycosylated ZP3 as the immunogen (Keenan et al. 1991),

recent evidence indicates that a similar disruption of ovarian function can be observed when the deglycosylated peptide backbone is used for immunization purposes (Paterson et al. 1992). When the immunogen consists of deglycosylated peptide, the onset of this pathology is delayed and may take two years to manifest itself. However, when the cessation of ovarian cyclicity does become apparent, the pathology does not reverse as antibody titres decline, it is permanent. Histological examination of such ovaries reveals that the reason for this lack of reversibility is that the primordial follicle population has been completely destroyed, with the result that the animals experience what is effectively a premature menopause (Aitken et al. 1990; Paterson et al. 1992). The loss of the primordial follicles is associated with the appearance of follicular clusters (Skinner et al. 1984) which are thought to represent the hypertrophied remnants of the somatic components of the original primordial follicles (Fig. 1).

The mechanisms responsible for this loss of primordial follicles is currently the subject of intense speculation and clearly represents a key issue in the viability of the zona approach to contraceptive vaccine development. The current hypothesis concerning the mechanisms underlying this pathology is that the disruption of ovarian function is due to a cell mediated immune response stemming from T-cell epitopes within the ZP3 sequence. If this is the case then the solution to the problem of ovarian pathology would be to identify B-cell epitopes in the primary amino acid sequence of ZP3, which could then be used to engineer peptides capable of disrupting fertility without adversely influencing ovarian function. This objective has been achieved by Millar et al. (1989) who used a monoclonal antibody directed against murine ZP3 to screen an epitope library created by the expression of ZP3 cDNA fragments in the lgt11 expression vector. The result of this analysis was the cloning of the cDNAs for series of peptides which overlapped to give a seven amino acid sequence that was common to all structures and which must, therefore, encode for the epitope targeted by the monoclonal antibody. A synthetic 16-mer peptide including this B-cell epitope was then generated, conjugated to keyhole limpet hemocyanin and used to actively immunize female mice. Following this treatment all of the animals experienced a prolonged period of infertility. In 75% of the animals a spontaneous return to fertility



was observed 4 to 10 months after the initiation of the trial, while the remaining animals remained infertile for the entire 10 month duration of the study (Millar et al. 1989). The lack of any significant pathology in these animals was confirmed by histological examination of the ovaries, encouraging the belief that epitopes may be found within the primary structure of the ZP3 molecule, that are viable candidates for the development of a contraceptive vaccine. Extension of these studies to human ZP3 and assessment of peptides encoding B-cell epitopes in primate models is clearly a high priority task for the future.

4 Sperm Surface Antigens

Of all the approaches to contraceptive vaccine development, the concept of generating antibodies against the sperm surface is the one which is most heavily supported by clinical data. About 5% of male and female patients attending infertility clinics appear to be infertile as a result of generating auto- or iso- antibodies against spermatozoa. Since such patients appear to suffer no other consequences of their immunity apart from the loss of fertility, their existence is a perfect demonstration of the feasibility of developing contraceptive vaccines in general, and anti-sperm vaccines in particular. Unfortunately this potential has been slow to translate into a scientific reality. An obvious route to pursue would be to characterize the epitopes targeted by patients exhibiting infertility associated with spontaneous immunity against sperm surface antigens. Where this approach has been adopted the results have been disappointing. Most patients expressing immunity against sperm antigens according to the conventional sperm-ag-

Fig. 1 a-c. **a** Ovarian cortex of a control marmoset immunized with vehicle alone. Note the presence of primordial and primary follicles (x600). **b** Ovarian cortex of a marmoset monkey immunized against deglycosylated porcine ZP3. Note absence of primordial follicles (x600). **c** High power micrograph of the ovarian cortex of a marmoset monkey following the induction of immunity against deglycosylated porcine ZP3. Note follicular clusters (*arrow*), which are thought to represent the hypertrophied somatic cell components of degenerated primordial follicles (x1000)

glutination assays appear to generate a broad spectrum of antibodies directed against multiple antigenic targets, and not against any single dominant surface determinant (Aitken et al. 1987). A possible exception to this generalization was the high frequency of samples which possessed antibodies against a 90kDa antigen associated with the sperm plasma membrane (Aitken et al. 1987). Analysis of sperm antigens interacting with antisera from the World Health Organization's serum bank also revealed a high incidence of samples containing antibodies directed against a 90 kDa component (Lee et al. 1983). Such results are of interest in view of recent studies in the mouse (Leyton and Saling 1989) and human (Naz et al. 1991) of a sperm surface antigen with a molecular mass of just over 90 kDa, which exhibits tyrosine kinase activity and appears to be involved in the activation of spermatozoa during fertilization.

Another sperm surface antigen which has received attention is SP10 (Herr et al. 1990), which has also been designated by the World Health Organization as MHS-10 or S20. This particular antigen was identified as a result of an international research effort orchestrated by the World Health Organization's Task Force on Contraceptive Vaccines. A monoclonal antibody against this antigen was selected on the basis of its cross reactivity with testicular germ cells and mature spermatozoa, its lack of reactivity towards somatic cells and its biological activity. On Western blots this monoclonal antibody identified a series of peptides with molecular masses ranging from 18-34 kDa, while immunocytochemical analyses indicated that the target antigen has an intra-acrosomal localization (Herr et al. 1990). Although the fact that this is an internal antigen may limit its usefulness in a contraceptive context, Herr et al. (1990) emphasize that the antigen does become exteriorized during the acrosome reaction and in the fully acrosome reacted cell remains localized around the equatorial segment of the sperm head. Whether or not an internal antigen such as SP10 can be used as the basis for engineering a contraceptive vaccine will only be ascertained when active immunization trials in non-human primates are initiated. The presence of testicular antigens cross reactive with SP10 antibody in baboons suggests that such trials should be feasible.

Where internal sperm antigens have been employed in previous studies to examine their potential for the development of contraceptive vaccines, the results have been disappointing. Hence *in vivo* trials involving the induction of immunity against hyaluronidase or acrosin

(Morton and McAnulty 1979; DeIoannes et al. 1990) have failed to demonstrate an efficient contraceptive effect.

A possible exception is the 95 kDa antigen identified by Moore et al. (1987) which is held to prevent the binding of human spermatozoa to the zona pellucida despite the fact that the target antigen is located intracellularly.

Another product of the World Health Organization's efforts to identify sperm surface determinants with contraceptive activity has been the antigens targeted by the monoclonal antibodies HS-11 and HS-63 (Lee et al. 1990). These antibodies react specifically with the acrosomal region of spermatozoa from several mammalian species but not with any somatic cells investigated. Biochemical studies carried out on the cross reactive antigens from the murine testis indicated that HS-63 interacted with 3 soluble proteins with molecular masses of 40-50 kDa while HS-11 targeted a group of membrane-associated antigens with molecular masses in the range 35-50kDa (Lee et al. 1990). *In vitro* studies have demonstrated that HS-11 and HS-63 antibodies inhibit fertilization by interfering with the acrosome reaction induced either by the ionophore, A23187 or, more physiologically, by the zona pellucida. In view of the contraceptive potential of the antigens corresponding to the HS-11 and HS-63 antibodies, efforts are now being made to generate recombinant versions of these peptides for evaluation as contraceptive agents *in vivo*.

PH20 protein is a sperm surface antigen involved in sperm-zona binding in the guinea pig (Primakoff et al. 1985). When male or female guinea pigs are immunized with this particular protein a contraceptive effect is observed in all of the treated animals (Primakoff et al. 1988). Moreover, the contraceptive consequences of inducing immunity against this particular antigen are reversible in males and females. As a model to demonstrate the feasibility of developing a contraceptive vaccine directed against sperm surface antigens, these studies by Primakoff et al. (1988) on PH20 are extremely important. Whether or not the human spermatozoon possesses an antigen equivalent to PH20 is a key question which has not yet been answered.

5 Conclusions

In conclusion there are three major approaches currently being pursued with the objective of developing a contraceptive vaccine. Immunogens

based on human chorionic gonadotropin have reached the most advanced stage of development. Phase 1 clinical trials have already been completed and phase 2 studies are about to commence. The advantage of this approach is that the molecule involved is extremely well characterized and its relationship to the pituitary gonadotropins accurately defined. As a consequence of this knowledge it has been possible to identify a limited domain of the hCG molecule that is unique to this structure. Synthetic vaccines based on this hCG-specific region of the β -subunit of hCG are currently being evaluated by the World Health Organization in clinical trials. The Indian Government is also supporting clinical trials with vaccines based on the β -subunit of hCG or a hybrid molecule involving the β -hCG and the α -subunit of ovine LH. The major problems with the hCG approach remain those of specificity and efficacy, both of which will ultimately be resolved by the data generated in the ongoing clinical trials. What cannot be resolved by clinical trials is the fact that this particular approach to the development of a contraceptive vaccine involves an abortifacient mechanism of action which will render it unacceptable in many areas of the world.

In contrast, the remaining two approaches to contraception aim to prevent conception by interfering with the intricate cascade of interactive events that characterize the union of male and female gametes at fertilization. The zona glycoprotein ZP3 is a prime candidate for engineering such a vaccine in view of its important role in the recognition and activation of spermatozoa and its unique antigenic composition. The biochemical composition of this molecule has been particularly well characterized and the major epitopes within the polypeptide backbone have been mapped and synthesized. A major problem with this approach involves the loss of ovarian function observed in the many *in vivo* studies in which active immunity against this protein has been induced. The possibility that this problem can be overcome by identifying B-cell epitopes that will avoid the T-cell responses thought to be responsible for the appearance of ovarian dysfunction is now being actively investigated.

Disruption of fertilization through the induction of immunity against sperm surface antigens is a third approach for which there is clinical support in the form of patients exhibiting infertility associated with the appearance of spontaneous immunity against sperm antigens.

Biochemical characterization of candidate antigens has traditionally been a problem in this area, largely because of a lack of material for analysis. The introduction of molecular biology techniques has largely overcome these problems and we are now beginning to see sequence data on a growing number of sperm surface antigens. The impact of such technology on reproductive science will be so profound that, in the next decade, we are likely to see an explosion of information on the biochemical composition of proteins and glycoproteins involved in key reproductive processes. With the availability of this information will come many new and exciting possibilities for the development of contraceptive vaccines.

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Hormonal Male Contraception: A Real Chance?

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1 The Need for Male Contraception

Health and prosperity of individuals and communities alike are linked to population growth. European and North American countries are now in the late phase of a demographic transition and have achieved largely stable populations where birth rates equal or only slightly exceed death rates. This balance depends to a paramount extent on the availability of methods for birth control. This is illustrated by representative opinion polls performed in Germany 1958 and in 1989 (Allensbach 1958 and 1989). At both time points the ideal family size, in the opinion of the majority of couples of reproductive age, comprised two children.

While in 1958 the real number of children per family was still 3, in 1989 the actual number of children matched the desired family size due to the fact that new methods for contraception had become available. In contrast, the developing countries are now in an early phase of the demographic transition which European and North American countries passed through when industrialization began. Since in developing countries the population explosion endangers any medical and economic progress, intensified family planning appears mandatory (Diczfalusy 1987).

Thus, developed countries depend on methods for birth control to maintain their populations in balance and developing countries require birth control in order to curb their population explosion.

Although practiced in all countries to varying degrees, abortion is nowhere considered an ideal method of birth control. Notwithstanding, in many places it is the only method available in the absence of functioning family planning. Modern family planning depends on the availability of contraceptive methods. At present, the major burden of fertility regulation is carried by women since most available methods are female methods. Oral contraceptives, implants and injectables, intrauterine devices, tubal ligation, diaphragms and spermicides are all methods practised by the female.

There is, however, an increasing demand that men should share not only the benefits but also the burden of contraception. The existing male methods offer comparatively little choice and are characterized by high failure rate, in the case of condom, or - for practical purposes - by irreversibility, in the case of vasectomy (Nieschlag 1987). Despite their shortcomings these two methods enjoy increasing popularity, indicating the demand for male methods and the willingness of men to accept them. The readiness of men to accept new male methods is underlined by repeated opinion polls. While in 1977 only 14.8% of German men would have accepted a hormonal male contraceptive, by 1991 the percentage had increased to 56% (König 1991).

These considerations led to a search for new male methods for contraception. In particular, a pharmacological, fully reversible method appears desirable. In this search serendipity plays a role that should not be ignored. For example, research on gossypol as a possible male contraceptive was precipitated by a food-poisoning epidemic in China

(Segal 1985); the antifertility effects of sulphasalazine (Giwerzman and Skakkebaek 1986) or, more recently, of the traditional Chinese drug made from *Tripterygium wilfordii* (Quian 1987) were discovered as side effects of drugs long in use. In addition, however, a more systematic approach based on our understanding of male reproductive physiology appears mandatory. Therefore, basic research on cellular and molecular events in spermiogenesis was initiated by WHO with the aim of finding new avenues towards male contraception (Hamilton and Waites 1990). While this systematic approach yields a host of new information, it may take some time until viable methods for contraception emerge. In this book, Aitken et al. (1992) describe approaches to contraception based on immunological methods and Cooper (1992) discusses the possibilities for male contraception by interference with epididymal function. However, both approaches are still in a preclinical phase of experimentation and a long road must be travelled before clinical application becomes possible. Of the factors controlling reproduction, endocrine mechanisms are best understood so far and as a consequence, endocrinology has provided the most intensive systematic approach to male contraception, including clinical testing.

2 The Principle of Hormonal Male Contraception

The testes have an endocrine and an exocrine function: the production of androgens and of male gametes. Suppression of gamete production without affecting the endocrine function is the goal of endocrine approaches to male fertility regulation. However, since the two functions of the testes are closely interdependent, it has remained impossible so far to suppress spermatogenesis exclusively and reversibly without significantly affecting androgen synthesis.

FSH and LH/testosterone are responsible for the maintenance of fully normal spermatogenesis (for review see Weinbauer and Nieschlag 1990 and 1992). If only one of the two is eliminated, spermatogenesis will be reduced, but only in quantitative terms, i.e. fewer but normal sperm will be produced and azoospermia will not be achieved. This has been demonstrated by the elimination of FSH by immunoneutralization, resulting in reduced sperm numbers but not in complete

azoospermia (Srinath et al. 1983), which – at least until quite recently – was considered to be required for an effective male method. Therefore, even if new modalities for the selective suppression of FSH should become available, it remains doubtful whether they would lead to a method for male contraception (Nieschlag 1986) unless they would affect sperm function as well as sperm production (Moudgal et al. 1991).

In order to achieve azoospermia it appears necessary not only to suppress FSH, but also to deplete the testes of testosterone. Since testosterone alone can maintain spermatogenesis and much lower testosterone concentrations appear to be necessary for maintenance of spermatogenesis than previously considered, the depletion of intratesticular testosterone must be quite complete (Weinbauer and Nieschlag 1990 and 1992). In order to maintain androgenicity, including libido, potency, male sex characteristics, psychotropic effects, protein anabolism, bone structure and hematopoiesis, testosterone levels in the general circulation have to be replaced while the testes themselves are depleted of testosterone.

This leads to the general principle of endocrine regulation of male fertility, namely the suppression of FSH and LH, resulting in a depletion of intratesticular testosterone and cessation of spermatogenesis, while at the same time, peripheral testosterone is substituted with an androgen preparation. This can be achieved by testosterone (or another androgen) alone, or by the combination of testosterone with other substances suppressing pituitary gonadotrophin secretion. As in female hormonal contraceptives, gestagens as such pituitary-suppressing agents have been tested in men in combination with androgens. GnRH agonists as well as antagonists are being considered as further possible combinations with androgens.

3 Endocrine Approaches to Male Contraception

3.1 Testosterone Alone

According to the principle outlined above, testosterone should be the first choice for hormonal male contraception since it not only suppresses pituitary LH and FSH secretion, but also replaces testosterone

in the general circulation. Consequently, a number of clinical trials based on testosterone application have been performed in the past. However, it is only recently that the first efficacy study for hormonal male contraception using testosterone injections has been completed under the auspices of the WHO (1990).

In all previous studies sperm parameters were the end point of the investigation and the couples had concurrently used another form of contraception. The WHO efficacy study which was undertaken in ten centres in seven countries involved 217 healthy fertile men. Each subject received 200 mg testosterone enanthate weekly by intramuscular injection. Of these, 157 became azoospermic and entered a 12-month contraceptive efficacy phase during which the testosterone injections were the only form of contraception. Only one pregnancy occurred during 1486 months of the efficacy phase (0.8 conceptions (95% confidence interval 0.02-4.5) per 100 person-years).

Discontinuations from the study occurred mainly because azoospermia was not achieved within six months and because subjects disliked the injection schedule. The mean time for subjects to become azoospermic was 120 days; reappearance of spermatozoa was detected in 11 men and in no case led to discontinuation from the study or to pregnancy. After the testosterone injections had been stopped, the estimated median time from azoospermia to recovery (sperm concentration of at least 20 million/ml) was 3.7 months and to the subject's mean baseline sperm concentration was 6.7 months. The study provides convincing evidence that, once the laboratory diagnosis of azoospermia is achieved, normal men are rendered infertile and able to sustain safe, effective and reversible contraception for at least 12 months.

The experience acquired in this study has been invaluable in many respects. The motivation for a couple to be involved often arose because of the female partner's wish to discontinue pill usage, which underlines the need for a male pill. Recruitment of volunteers was slow or non-existent in two centres when physician contacts were used, but achieved momentum within one month of a public radio programme describing the study and its purpose.

The large multicentre trial demonstrated that testosterone enanthate can provide effective contraceptive protection once azoospermia has been achieved. However, to reach this goal, testosterone enanthate had

to be injected every week. The pharmacokinetics of testosterone enanthate, producing a high initial serum peak and a rapid decline thereafter, explain the necessity for relatively frequent injections (Behre et al. 1990). Earlier multicentre trials sponsored by the NIH had shown that sperm counts recover if the injection intervals are longer than 7-10 days (Patanelli 1978). Furthermore, peak testosterone concentrations may be high enough to stimulate spermatogenesis directly, thus overriding the effects of pituitary suppression. Therefore, other preparations are required that produce more constant serum testosterone levels.

In a search for a longer-acting androgen ester, 19-nortestosterone hexyloxyphenylpropionate was identified as a potential steroid for male contraception (Schürmeyer et al. 1984a). This ester has been in clinical use as an anabolic for many years without serious side-effects. Besides its androgenic property, 19-nortestosterone has a stronger pituitary suppressing effect than does testosterone. In a study involving 12 volunteers, half were rendered azoospermic and half severely oligozoospermic, even when 19-nortestosterone injections were given at only three-week intervals (Knuth et al. 1985). Since no serious side-effects were encountered, this steroid should be further considered if other new options should fail.

Such an option emerged from a WHO-initiated synthesis programme for a long-acting testosterone preparation: testosterone buciclate (20-Aet-1) (Fig. 1) after single intramuscular injection showed effective serum testosterone levels up to 3 months, first in preclinical studies in monkeys (Weinbauer et al. 1986) and more recently in hypogonadal men (Behre and Nieschlag 1992c). Pharmacokinetic data of this testosterone ester in comparison to other preparations is shown in Fig. 1. The potential of this preparation for male contraception is currently undergoing clinical testing.

Further possibilities are provided by testosterone incorporated into microspheres which, after some initial experimentation (Burris et al. 1988), have now been tailored to provide constant serum testosterone levels over several months (Bhasin et al. 1992). It should be mentioned here that microencapsulated testosterone propionate has proved quite effective in suppressing fertility in feral stallions (Turner and Kirkpatrick 1982). Constant serum levels are also achieved with testosterone

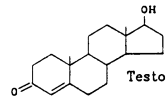
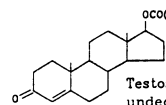
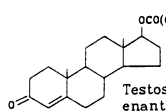
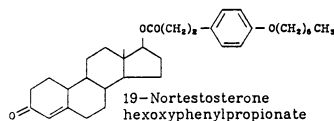
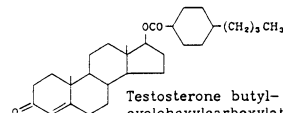
Androgen preparation	MRT	Terminal half life
 Testosterone		
 Testosterone undecanoate	3.7 h	1.6 U
 Testosterone enanthate	12.1 d	8.0 d
 19-Nortestosterone hexoxyphenylpropionate	29.1 d	20.1 d
 Testosterone butylcyclohexylcarboxylate	65.0 d	29.5 d

Fig. 1. Pharmacokinetic data of different androgen preparations used in trials for male contraception (MRT = mean residence time)

pellet implants (Handelsman et al. 1990). However, although implants have been in clinical use for several decades they have not gained much popularity, in part because their insertion requires minor surgical procedures. Whether they would be acceptable for contraceptive purposes remains to be determined.

While the question of a suitable testosterone preparation appears to be resolvable, another problem remains: only about two thirds of all volunteers who entered the WHO study responded to the testosterone injections with azoospermia. Notwithstanding ethnic differences, i.e. eastern Asian men showing a higher rate of azoospermia than Caucasians, the overall percentage of azoospermia achieved remained unsat-

isfactory. In order to increase the rate of azoospermia, substances which could be administered in combination with testosterone have been and are being searched for.

3.2 Testosterone Plus Gestagens

Gestagens are potent inhibitors of LH and FSH secretion and therefore have been tested, in combination with testosterone, for male fertility regulation. Norethindrone, medroxyprogesterone acetate (MPA), depot-MPA (DMPA), 17-hydroxyprogesterone capronate and megestrol acetate have been used in clinical trials initiated by the WHO (1972-1983) and the Population Council (Schearer et al. 1978). The most favourable combination was the monthly intramuscular injection of 200 mg DMPA plus 200 mg testosterone enanthate or testosterone cypionate; this combination gave the best results in suppressing spermatogenesis and the incidence of untoward side effects is low. No effects on libido and potency were observed. However, even this combination does not produce azoospermia uniformly; therefore its efficacy is uncertain. Patients who have been treated with DMPA for offensive sexual behaviour for long periods have shown weight gain and increased blood pressure, as well as an increased insulin response to glucose tolerance testing and gall bladder dysfunction (Meyer et al. 1985). However, because DMPA was given without testosterone and the dosage of DMPA was higher than would be required for fertility regulation, these side effects might not occur when contraceptive doses are utilized.

To develop a method that could be self-administered, 20 mg of MPA was given orally, together with 50 to 100 mg of testosterone percutaneously via a cream for one year (Soufir et al. 1983). Sperm counts in the six volunteers were suppressed to less than 5 million/ml for most of the study time, but azoospermia was not achieved. A further limitation of this approach was shown in another study in which the wives of five of the twelve men treated developed hirsutism secondary to testosterone being transferred to their skin from their husband's abdomen or buttocks (Dalanoe et al. 1984).

Animal studies and studies in sexual delinquents have shown that the antiandrogen cyproterone acetate, which can be considered a potent

gestagen, suppresses spermatogenesis, an effect exerted through the suppression of pituitary gonadotropin secretion. In clinical trials using 5 to 20 mg cyproterone acetate per day for up to 16 weeks, sperm counts and motility were reduced markedly (Fogh et al. 1979; Moltz et al. 1980; Wang and Yeung 1980). Thus, cyproterone acetate appeared to be a possibility for male fertility control. However, decreases in serum testosterone levels to below normal also were observed. Some of the volunteers complained of fatigue, lassitude and decrease in libido and potency attributable to the diminished testosterone levels. Thus, although cyproterone acetate produces infertility, clinical trials have not been continued as the lowered testosterone levels were considered intolerable. The consequence would have been to combine with testosterone. The combination of an androgen with an antian-drogen, however, appeared somewhat illogical and has therefore never been tested.

Danazol, a derivate of ethinyltestosterone stongly inhibits LH and FSH secretion when given orally but is devoid of other significant estrogenic or gestagenic activities. Various combinations of daily and oral doses of danazol with monthly injections of testosterone enanthate have been tested (Leonard and Paulsen 1978). A daily oral dose of 600-800 mg danazol, in combination with 200 mg of testosterone enanthate monthly, induced azoospermia or severe oligozoospermia. However, since the rate of azoospermia was not significantly higher than that achieved with testosterone alone and since the daily steroid load was quite high and liver toxicity had been reported by female users (for the treatment of endometriosis), these studies were not continued.

Since monotherapy with the long-acting androgen ester 19-nortestosterone injected every three weeks resulted in effective suppression of spermatogenesis to azoospermia in about 70% of the volunteers, we tested the combination of 19-nortestosterone with DMPA to see whether it would result in even more complete suppression of spermatogenesis (Knuth et al. 1989b). 12 volunteers were injected weekly with 200 mg 19-nortestosterone hexyloxyphenylpropionate followed by injections with the same dose every three weeks up to week 15. In addition, the volunteers were injected with 250 mg DMPA in weeks 0, 6, and 9. Azoospermia was achieved in 9 of 12 volunteers during the study course, while in 3 of the remaining 4 volunteers spermatogenesis

was suppressed to single sperm, and in one volunteer to a sperm concentration of 1.3 mill/ml.

These promising results prompted the WHO Task Force on the Regulation of Male Fertility to launch a large-scale multicentre trial in five centres in Indonesia comparing the effectiveness of testosterone enanthate or 19-nortestosterone hexyloxyphenylpropionate in combination with DMPA for a treatment period of six months. Preliminary results indicate a very effective suppression of spermatogenesis with more than 97% azoospermia in both study groups (WHO 1991). These surprisingly favorable results in Indonesian men underline the importance of studying the contraceptive efficacy of androgens and androgen/gestagen combinations in different ethnic groups.

Recent studies with DMPA in combination with testosterone enanthate in 10 Caucasian men indicated an additional antifertility action of the gestagen/androgen combination on the functional capacity of the residual spermatozoa in those volunteers with spermatogenesis suppressed only to severe oligozoospermia (Wu and Aitken 1989). These studies support the view that even severe oligozoospermia, which is achieved in all volunteers whose spermatogenesis is not suppressed to azoospermia after injection of DMPA/androgen combinations, might be a tenable target for reversible male contraception.

3.3 Testosterone Plus GnRH Agonists

After a transient stimulation of the pituitary, GnRH agonists, when given in appropriate doses, lead to a down regulation of GnRH receptors in the pituitary and, in consequence, to a decrease in gonadotropin secretion, followed by suppression of testicular function. This approach is being used advantageously in the treatment of testosterone-dependent prostate carcinoma. Similarly, it has been tested in trials for male contraception.

As summarized in Table 1, to date results from 104 healthy men participating in 12 trials for male fertility regulation have been published. In these studies the GnRH agonists decapeptyl, buserelin and nafarelin were administered at daily doses of 5-500 µg/volunteer for periods of 10 to 30 weeks. In about 30% of men sperm production could be

suppressed below $5 \times 10^6/\text{ml}$ and in a few men also to azoospermia. In another 30%, sperm counts were lower than $30 \times 10^6/\text{ml}$ while in the remaining volunteers, the sperm number was only slightly reduced or remained unaffected.

These results are not as good as those generated by steroid trials for male contraception and therefore GnRH agonists are no longer considered useful for male contraception. Two important general findings, however, emerged which were first described in monkeys serving as models in trials for male contraception which are important pointers for future investigations.

First, constant infusion of the GnRH agonist is much more effective in suppressing gonadotropins and testicular functions than single injections (Schürmeyer et al. 1984b; Michel et al. 1985; Pavlou et al. 1986; Bhasin et al. 1987). Since this principle is also of advantage for patients with prostatic carcinoma, pharmaceutical companies have been working successfully on the development of slow-release preparations of GnRH agonists. From these, in turn, clinical trials for male contraception also benefit since the various implants developed obviated the cumbersome wearing of extracorporal pumps by the volunteers (Bouchard and Garcia 1987; Behre et al. 1992b).

Secondly, as in monkeys (Akhtar et al. 1983), testosterone supplementation appeared to attenuate sperm-suppressing effects of GnRH agonists, since lower sperm counts were achieved in men receiving GnRH agonists without simultaneous testosterone supplementation (Linde et al. 1981, Frick et al. 1986).

Although the latter observation cannot be translated into the design of clinical trials with GnRH agonists as it would be impossible to leave volunteers without testosterone substitution i.e. with serum levels in the hypogonadal range, the question remains whether GnRH agonists would be more effective if combined with slow-release androgen preparations not producing supraphysiological testosterone levels. On the other hand, when we tried to give androgens first and then added buserelin at a later stage, similarly disappointing results were achieved as when testosterone was given simultaneously with buserelin (Behre et al. 1992b).

Table 1. Twelve clinical trials with GnRH agonists for male contraception (104 subjects)

Reference	Subjects (n)	Agonist	Daily $\mu\text{g/day}$	Route of dose	Weeks administration	Androgen	Effect on sperm counts
Berquist et al (1979)	4	buserelin	5	sc injection	17	none	none
Linde et al (1981)	8	D-Trp ⁶	50	sc injection	6-10	none	azoospermia in 1/8
Doelle et al (1983)	6	D-Trp ⁶	50	sc injection	20	TE ^a 100mg	12 $\pm$ 4 ^b biweekly
Rabin et al (1984)	8	D-Trp ⁶	100	sc injection	20	TE 100 mg	5.5 ^b biweekly
Bhasin et al (1985)	7	nafarelin	200	sc injection	16	TE 200 mg	17 $\pm$ 6 ^b biweekly
Schürmeyer et al (1984b)	7	buserelin	118	sc infusion	12	TU daily	18 $\pm$ 5 ^b biweekly
Michel et al (1985)	4	buserelin	230	sc infusion	12	TU daily	10 $\pm$ 3 ^b
Pavlou et al (1986)	7	buserelin	440	sc infusion	12	TU daily	44 $\pm$ 14 ^b
Frick and Aulitzky (1986)	6	D-Trp ⁶	500	sc infusion	16	TE 100 mg	4.5 $\pm$ 11 ^b in 5/8
Bouchard and Garcia (1987)	4	buserelin	3x50	nasal	20	biweekly	none
	4	buserelin	3x100	nasal	24	none	azoospermia
	4	buserelin	3x200	nasal	20	fluoxymest	none
	5	D-Trp ⁶ microspores	100	im	15-60	TE 125 mg	azoospermia in 0/5
	5	D-Trp ⁶	100	im	15-60	biweekly	azoospermia in 0/5

Table 1. (cont.)

Reference	Subjects (n)	Agonist	Daily µg/day	Route of dose	Weeks adminis- tration	Androgen	Effect on sperm counts
Bhasin et al (1987)	7	nafarelin	400	sc infusion	16	TE 200 mg biweekly	4.5±1.1 ^b in 5/8
Behre et al (1992b)	8	buserelin	(3.3 mg)	implant	24	19 NT 200 mg every 3 wk	azoospermia in 2/8
	8	buserelin	(6.6 mg)	implant	24	19 NT 200 mg every 3 wk	azoospermia in 2/8

^aAbbreviations: TE: testosterone enanthate; TU: testosterone undecanoate; fluoxymest: fluoxymesterolone

NT: 19-nortestosterone-hexyloxyphenylpropionate. ^bLowest mean sperm counts

3.4 Testosterone and GnRH Antagonists

GnRH antagonists suppress gonadotropin secretion through preventing GnRH action at the pituitary level by competitive GnRH receptor occupancy (Clayton et al. 1982; Heber et al. 1982; Marshall et al. 1986). Unlike the GnRH agonists, the antagonists induce an immediate decline of gonadotropins and testosterone (Weinbauer and Nieschlag 1985) and produce azoospermia very effectively in the non-human primate model (for review see Weinbauer et al. 1990 and 1991). Therefore, GnRH antagonists are potential male contraceptives but, for clinical use, similar to GnRH agonists, testosterone supplementation is necessitated by the precipitous cessation of testicular androgen production under GnRH antagonist administration.

Preclinical studies in monkeys indicated that *concomitant* testosterone replacement using testosterone-buciclate prevented the induction of azoospermia (Weinbauer et al. 1988) whereas a *delay* of androgen substitution permitted the induction of complete and sustained suppression of sperm numbers (Weinbauer et al. 1989).

In contrast, Bremner et al. (1991) reported induction of azoospermia during GnRH antagonist and *concomitant* testosterone administration using a different mode of androgen substitution and animals with rather low sperm numbers at initiation of the study.

Clinical trials with GnRH antagonists for male contraception started only about 2 years ago since many of the GnRH antagonists caused histamine release and skin reactions at the injection sites (Karten et al. 1990). The model of GnRH antagonist and delayed testosterone administration, developed by us in the monkey (Weinbauer et al. 1989) has been applied in two clinical studies (Table 2). The GnRH antagonist Nal-Glu was administered at a daily dose of either 7.5 (Tom et al. 1991) or 10 mg/volunteer (Pavlou et al. 1991). Androgen supplementation with testosterone enanthate started 2 weeks after inception of GnRH antagonist treatment. Among the 16 men participating in the two studies, 14 developed azoospermia within 6-12 weeks of GnRH antagonist administration. This demonstrates the potential of the combined GnRH antagonist and testosterone regimen for male fertility regulation.

The Nal-Glu antagonist used in these two studies, however, induces formation of erythema and pruritus at the injections sites and will not

Table 2. Male contraceptive trials with a GnRH antagonist and delayed testosterone substitution

Reference	STUDY 1 Tom et al. 1991	STUDY 2 Pavlou et al. 1991
GnRH antagonist	Nal-Glu	Nal-Glu
Mode of administration	sc	sc
Dose (mg/d)	7.5	10.0
Subjects (n)	8	8
Duration of treatment (weeks)	16	20
Androgen-substitution (TE, start in week 3) ^a	150 mg/2 weeks	25 mg/week
Rate of azoospermia (n/n)	7/8	7/8
Recovery of semen parameters	100%	100%

^aTE = Testosterone enanthate (im injection)

become available for widespread clinical use. Promising results were recently obtained with the new GnRH antagonist cetrorelix (Behre et al. 1992a). In the latter investigation a single sc injection of 1 - 5 mg cetrorelix/volunteer significantly suppressed testosterone. In the following study daily injections of up to 10 mg for 8 days suppressed gonadotropins and testosterone consistently and effectively. All injections of cetrorelix were well tolerated. No local induration or pruritus nor any adverse systemic side effect was noted in any volunteer. This GnRH antagonist was similarly potent to the Nal-Glu compound but was clearly superior in terms of side-effects. Recently another LH-RH antagonist, RS 26306, given as a single sc injection, was reported to inhibit gonadotropin and testosterone secretion with minimal erythema seen at the injection site (Gaitan et al. 1991). These first clinical evaluations render GnRH antagonists as promising agents for male fertility regulation.

3.5 Summary

In summary, the first efficacy study for male hormonal contraception demonstrated that weekly injections of testosterone-enanthate can provide safe and effective contraceptive protection. The practicability of this approach may be improved by the use of longer-acting testosterone preparations which are under development. While almost all Eastern Asian men in China and Indonesia participating in these studies responded to the testosterone injections with azoospermia, the problem remains to be resolved why only about two thirds of Caucasian study subjects reacted with azoospermia. If an ongoing WHO multicenter trial reveals that severe oligozoospermia induced by testosterone leads to impaired fertility, similarly effective in terms of contraception as azoospermia, then the extent of suppression of spermatogenesis achievable by testosterone may be sufficient for contraception. If not, studies combining testosterone with other agents suppressing pituitary function may solve the problem. Of these agents, GnRH antagonists appear most promising, as model studies in monkeys and initial clinical trials have shown.

4 Risks and Chances

The possible realization of hormonal male contraception is met with enthusiasm by the general population, as reflected in the media and by individual response, but is also accompanied by scepticism on the part of some scientists.

Some concerns are expressed regarding the reversibility of hormonal methods for male contraception. If the hormonal methods are not fully reversible, one of the prime aims of any pharmacological method for male contraception would not be met. Thus it is important to emphasize that long-term studies in monkeys, including careful histological examination of the testes (Weinbauer et al. 1987) and fertility testing (WHO 1991), revealed that all effects of GnRH analogue administration with or without testosterone were fully reversible. All other studies in monkeys quoted in this review also resulted in full reversibility of all effects seen. Even more important, in none of the hun-

dreds of volunteers who have participated in trials for hormonal male contraception using testosterone, gestagens, other steroids and/or GnRH analogues for hormonal male contraception were any irreversible effects observed. From all existing evidence it can therefore be concluded that reversibility is not a real problem.

Since all hormonal male methods proposed to date are based solely on or include testosterone, possible side-effects of testosterone have to be considered. Of these, the possible effects on the cardiovascular system may be mediated through lipid metabolism. In properly controlled clinical studies testosterone enanthate did not alter high-density-lipoprotein (HDL) cholesterol levels (Friedl et al. 1990). While the combination of testosterone enanthate with DMPA caused a slight decrease in HDL cholesterol (Hedman et al. 1988; Wallace and Wu 1990). Therefore, DMPA does not appear to be suitable for male contraception. As the comparison in Fig. 2 shows, the addition of DMPA does not induce a markedly higher rate of azoospermia than androgens alone and thus does not appear necessary.

Neither in other subjects exposed to testosterone treatment, in particular hypogonadal men treated with testosterone on a long-term basis (Gooren and Polderman 1990) nor in young men treated with testosterone in high doses for reduction of a predicted overall stature have similar effects been reported. Athletes and bodybuilders may use testosterone and other anabolic androgens in excessively high doses for extended time periods (Knuth et al. 1989a), but serious short- and long-term side-effects have not been reported unless 17 alpha-alkylated steroids were used which are obsolete in clinical practice. Nevertheless, only long-term surveillance of men on hormonal male contraception will ultimately clarify the potential risks for the cardiovascular system.

Another possible risk of testosterone treatment frequently quoted (see Nieschlag et al. 1990) is the effect on the prostate, in particular, of elderly men. Those conducting trials for male fertility control are aware of these problems and have therefore frequently consulted experts and held formal consultations and meetings related to this and other issues (Nieschlag and Behre 1990; Bardin et al. 1991). All discussions have concluded "that it is extremely unlikely that androgens play a role in the initiation of prostate cancer, the pathogenesis of dys-

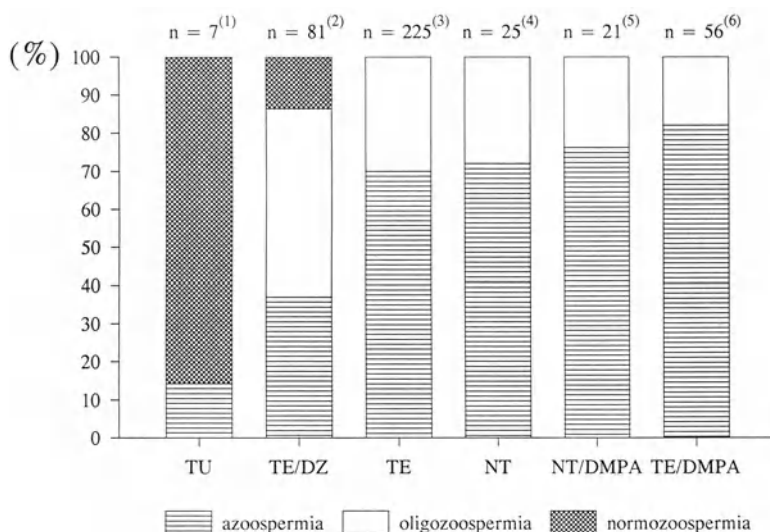


Fig. 2. Comparative efficacy in representative clinical trials of male contraception with androgens or androgen/gestagens combinations. Bars show the percentage of volunteers achieving azoospermia (*horizontal-hatched bars*), oligozoospermia (*open bars*), and the percentage of volunteers with no suppression to subnormal values (normozoospermia; *cross-hatched bars*). TU: testosterone undecanoate; TE: testosterone enanthate; DZ: danazol; NT: 19-nortestosterone hexyloxyphenylpropionate; DMPA: depot-medroxyprogesterone-acetate. Data based on: ⁽¹⁾ Nieschlag et al. 1978; ⁽²⁾ Paulsen et al. 1982; ⁽³⁾ WHO 1990; ⁽⁴⁾ Schürmeyer et al. 1984a, Knuth et al. 1985, Behre et al. 1992b; ⁽⁵⁾ Knuth et al. 1989b, Moeloek 1990; ⁽⁶⁾ Alvarez-Sanchez et al. 1977, Wu and Aitken 1989, Pangkahila 1991, Moeloek 1990

plasia or focal lesions" (Schröder 1990). Aware, however, that testosterone may act as a promoter of existing prostate cancer, that the incidence of cancer of the prostate increases after the fifth decade of life and that there is no marker so far to detect those individuals in whom prostatic carcinoma is likely to develop, men over the age of 45 to 50 years should be excluded from trials and should not be considered the first users of testosterone-based male contraceptives. In addition, function and morphology of the prostate should be carefully monitored in

clinical trials by uroflow, rectal sonography and prostate specific antigen (PSA) until this issue has been resolved.

While the possible risks of hormonal male contraception should be carefully monitored and evaluated in appropriate clinical studies, possible benefits of testosterone application should also not be overlooked (Nieschlag and Behre 1990; Bardin et al. 1991). Long-term use of androgens at a younger age may prevent the development of osteoporosis in senescence, a growing medical problem in the light of increasing life expectancy. The anabolic effects of testosterone may be beneficial in populations with low protein intake e.g. in developing countries. Its hematopoietic effects could contribute to vigour and well-being. All these possibilities require scientific evaluation in ongoing and coming studies, so that a clear picture of the pattern of risks and benefits may emerge. In general, any deviation from normal male physiology may be kept small if the goal of testosterone application is the imitation of serum testosterone levels as close to normal as possible avoiding supra- or infra-physiological peaks and troughs.

If compared to female contraception, development and acceptance of a male method for fertility regulation suffer from an inherent disadvantage. In female contraception any risks of the methods must be weighed against the risks of pregnancy and childbirth, while in men such a balance cannot be established. This emphasizes that male methods must be characterized by highest standards of safety. However, this inherent disadvantage of male methods represents a rocksolid argument why pharmaceutical companies which have already given up active research for new female methods are very reluctant to seriously consider investing in the development of male methods under the current liability regulations. This is why governmental institutions such as WHO, NIH and family planning authorities in China have assumed the responsibility of developing new contraceptive methods, in particular, those to be used by the male. Application of new methods is likely to occur first in countries where contraception requirements override liability issues. In addition, changes in legal systems appear mandatory before the pharmaceutical industry, as the final distributor of male contraceptive methods, will reenter the scene. While these developments depend largely on the will of the public and of politicians, research has

made considerable progress towards a hormonal male contraceptive which may be ready for wide clinical use soon.

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