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Review

Role of Cytokines in Testicular Function

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Inflammatory disease has been established to affect male reproductive function and fertility. Relevant inflammatory diseases include general and chronic infectious diseases as well as localized acute or chronic infections of the male genitourinary tract. Male accessory gland infections account for almost 15% of all cases of male infertility seen in infertility clinics while fertility usually is not a clinical objective among patients with acute systemic infections such as Gram-negative sepsis. Infections of the male accessory glands frequently are associated with increased counts of white blood cells in semen and elevated levels of proinflammatory cytokines in semen and the testis. There is a mounting body of evidence that demonstrates the importance of cytokines and chemokines in the regulation of testicular and glandular function during pathophysiological states as well as under normal physiological conditions when cytokines act as growth and differentiation factors. The purpose of this review is to examine the role of cytokines in the regulation of steroidogenesis and spermatogenesis in the testis under physiological and pathophysiological conditions and considers clinical investigations that help to improve the evaluation and treatment of male infertility.

Key Words: Steroidogenesis; spermatogenesis; sepsis; inflammatory disease; male accessory gland infections; orchitis; LPS.

Introduction

Cytokines, the "hormones" of the immune system, have a wide range of biological activities in addition to their originally described functions as immune messengers. There are two major roles that cytokines play in the testis: the first is as mediators of pathophysiological outcomes of immune-endocrine interactions during inflammatory disease; the second is as growth and differentiation factors that help to orchestrate cellular interactions during normal physiological functions. A range of cellular mechanisms in testicular cells attributable to cytokines has evolved from various recent studies in experimental animals that facilitate our understanding of reproductive aspects in infectious diseases. The scope of this review is to outline and analyze effects of relevant cytokines on reproductive function, particularly the testis. The review also considers physiological events in the testis due to regulative function of cytokines and includes relevant clinical studies in humans.

Cytokines are a broadly defined group of polypeptide mediators involved in the communication network of cells of the immune system *(1)*. In addition, cytokines have important activities outside the immune system. In particular, cytokines are known to regulate testicular steroid hormone production during inflammation. Cytokines have been implicated as novel growth and differentiation factors involved in the regulation of cells in both compartments of the testis *(2)*. Cytokines are important factors in the integration of the neuroendocrine-immune network that controls testicular function. Recent reviews have examined the control of testis function by locally produced peptides *(3)*, cell–cell interactions *(4,5)*, growth factors *(6,7)*, neuronal signals *(8)*, neuroendocrine mechanisms *(9,10)* and inflammatory mediators *(11)*.

Androgen production by Leydig cells is accomplished in the vascularized interstitial tissue of the testis and testosterone is produced for both the seminiferous tubule compartment and the peripheral circulation for delivery to extratesticular androgen target tissues. Owing to the nature of testicular compartmentalization, both anatomically and functionally (Fig. 1), regulation of interstitial cell numbers and functions is under the control of both extra- and intratesticularly elaborated factors. Androgen production represents the decisive regulator of spermatogenesis, but also supports the function of androgen-dependent tissues. Besides the effects on the seminiferous epithelium, a lack in androgens will ultimately result in failure of reproductive function since the capacitation, transport, and ejaculation of spermatozoa relies on androgen-dependent organs and tissues.

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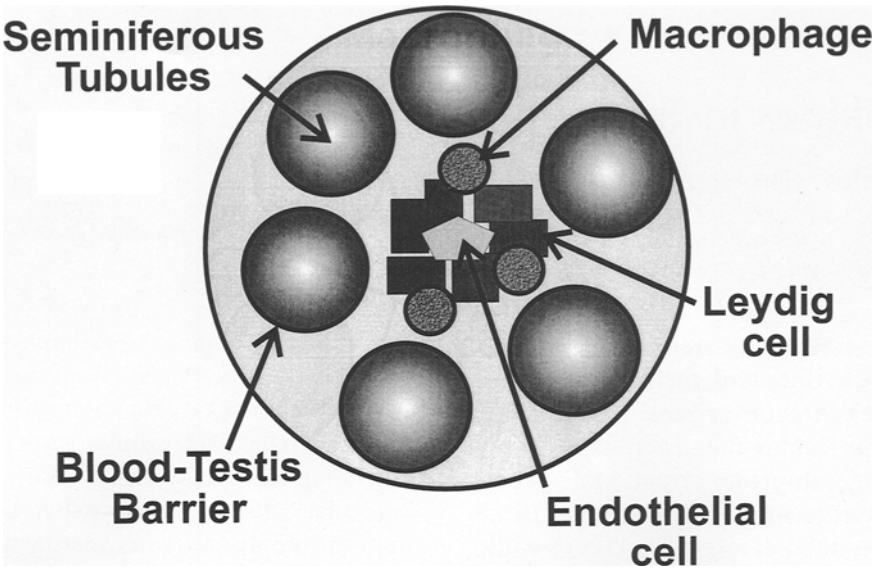

Fig. 1. Functional and anatomical compartmentalization of the testis. The testis is functionally compartmentalized into the gamete and endocrine compartments where spermatogenesis and testosterone biosynthesis take place, respectively. Anatomically, the spermatogenic compartment is formed by the seminiferous epithelium which is composed of Sertoli cells and the various stages of spermatocytes. The blood–testis barrier is formed by tight junctions between Sertoli cells near their basal aspect, plus the basal laminae and peritubular myoid cells that render the seminiferous tubule an immunologically privileged site, sequestered from the peripheral circulation. The endocrine compartment comprises the constituents of the testicular interstitium: Leydig cells, the site of testosterone biosynthesis; resident testicular macrophages, which are closely associated with Leydig cells; the endothelium, which is the distal aspect of the testicular circulation, and the lymphatic space that surrounds the cellular components of the interstitium.

Spermatogenesis is an autonomous process largely under the control of paracrine factors. Growth factors and cytokines that potentially exert effects on cell populations involved in spermatogenesis are produced within the seminiferous epithelium and influence postmeiotic stages of spermatogenesis. While the importance of FSH and androgens for the initiation and maintenance of spermatogenesis is clearly documented, the role of paracrine regulatory factors remains to be elucidated *(2)*. Spermatogenesis is a compartmentalized and continuous process that takes place sequestered within the blood–testis barrier, implicating a need for regulation by locally produced factors *(3)*. Testosterone is essential for the maintenance of spermatogenesis *(12,13)*. Recent studies have demonstrated the importance of high local concentrations of androgens to the development of functional spermatozoa *(14–19)*. Moreover, maintenance of accessory duct function depends on high concentrations of testosterone *(20)*. One mechanism subserving inflammatory disease-associated decreases in male fertility is inhibition of testosterone production.

Performance of testicular cells ultimately depends on the hypothalamic–pituitary–adrenal axis that directs steroidogenesis and spermatogenesis. Cytokines also affect testicular function via this neuroimmune-endocrine pathway. Neuroimmune-endocrine interactions especially are evident during stress related events and have been demonstrated in experimental models that utilize local application of cytokines to the brain. Another neural mechanism directing function of testicular cells involves autonomic nerves in the testis that appear to influence steroidogenesis and spermatogenesis directly or via vascular function. Inhibitory effects of the neuroimmune-endocrine pathway occur over a longer time course while autonomic nerves in the testis appear to be responsible for immediate alterations in testicular function.

Inflammation and Male Reproductive Function

Inflammatory diseases have been established as hazards to male reproductive function and fertility. General acute and chronic infections are associated with disorders in steroidogenesis and spermatogenesis resulting in temporary or permanent male infertility. The reproductive aspect of severe generalized infections has long been neglected since the therapeutic efforts under these conditions are focused primarily on the survival of the patient. The role of impaired steroidogenesis in the pathophysiology of severe infections and for the clinical outcome is under investigation *(21–23)*. Long-term effects on male reproductive function have not been investigated, partly due to the relatively small number of cases that affect patients in the reproductive age. The significance of localized acute and chronic infections, in particular infections of the male accessory glands, have been considered as causes of male infertility for decades *(24)*. Infections of the male accessory glands (MAGI) appear to affect reproductive function as indicated by decreased number, density, and motility of spermatozoa and alterations in seminal plasma markers *(24–26)*. Infec-

tions account for almost 15% of cases of male infertility seen in infertility clinics. Different pathophysiological concepts have been established describing the effects of male accessory gland infections on the reproductive system, and spermatozoa, respectively. These concepts involve direct effects exerted by pathogenic microorganisms in the testis, accessory glands, and spermatozoa *(27)*, alterations in male accessory gland function due to general or localized infections *(24,25)*, and immunological events resulting from the immune defense of the body against invading microorganisms *(28,29)*. Immunological events include the migration of white blood cells to the site of infection due to chemotaxis and the secretion of cytokines that are necessary for the communication between immune-competent cells. Besides this function, cytokines exert a variety of effects on tissues and cells not primarily involved in the immune defense. In fact, cytokines appear to be responsible for most pathophysiological events associated with infection and are the decisive factors that determinate the pathology of an infectious disease.

It has long been appreciated that chronic inflammation and systemic infection are associated with decreased reproductive capacity [reviewed in ref. *9*]. Men with critical illness, burn trauma, sepsis, and rheumatoid arthritis are reported to have markedly reduced serum testosterone levels resulting at least in temporary infertility *(30–40)*. Experimental adjuvant-induced arthritis *(41)* results in similar dramatic decreases in serum testosterone levels as evidenced in rodents. Injection of lipopolysaccharide (LPS), an endotoxin derived from the cell walls of Gram-negative bacteria, results in the inhibition of gonadal steroidogenesis. Studies from Bosmann et al. demonstrated that LPS-injected male mice have markedly reduced serum testosterone levels *(42)*. Investigations from Sancho-Tello et al. proved that LPS injection also resulted in the inhibition of ovarian steroidogenesis in female rats *(43)*. Induction of sepsis in rats with cecal slurry is typified by a significant decrease in serum testosterone *(44,45)*.

LPS and Endotoxemia

The presence of LPS signals the presence of Gram-negative bacteria. Recognition of LPS triggers gene induction and expression by immune and nonimmune cells, principally monocyte/macrophages of myeloid lineage. These inducible genes include cytokine genes, adhesion proteins and enzymes that produce low-molecular-weight inflammatory mediators responsible for immediate adaptations of various tissues to inflammatory challenge. Together the products of these inducible genes upregulate host defense systems that participate in eliminating the bacterial infection and restore physiological function *(46)*. When purified from Gram-negative bacteria and injected into experimental animals, LPS induces an array of pathophysiological responses. LPS provokes many of the pathophysiological symptoms that are characteristic of Gram-negative infection (e.g., fever, vasoconstriction, hypoglycemia, systemic arterial hypotension, diarrhea, shock and death) without the presence of living pathogens. Host responsiveness to LPS is under genetic control and has been mapped to the "LPS gene" *(47)*.

LPS induces the production of proinflammatory cytokines, principally IL-1, TNFα, and IL-6 by activated immune cells *(48)*. There is a hierarchy of cytokine secretion, with TNFα levels peaking and declining rapidly, followed by IL-1. IL-6 is secreted in a delayed manner and remains elevated. The LPS response is also characterized by stimulation of the hypothalamopituitary–adrenal axis (HPA) *(9,10,49,50)*, activation of the sympathetic nervous system *(51–53)*, increases in peripheral prostaglandin levels *(54,55)*, and rapid increases in levels of the neurohypophyseal hormone arginine vasopressin (AVP) *(56–60)*. LPS stimulates the expression of the inducible form of nitric oxide synthase (iNOS) and causes increase of plasma and tissue levels of NO *(61,62,63)*. NO is responsible for localized vasodilation at the site of infection and contributes to tissue hyperperfusion resulting in the hallmarks of infection. Antagonists of iNOS could partly reverse the effects of LPS injection on testosterone production in animal models indicating that NO derived from testicular macrophages contributes to the impairment of Leydig cell function *(63)*. The systemic response to LPS results in the production of a host of inflammatory mediators and signals that are known to affect Leydig cell steroidogenesis. In addition to soluble, bloodborne messengers released in response to LPS, direct neuronal connections may deliver regulatory signals to Leydig cells *(8,10,64–66)*, as has been shown in the ovary *(67)*. Indeed, LPS itself may exert a direct inhibitory effect on Leydig cells via an unidentified cellular signaling pathway, as has been demonstrated in the ovary *(43,68–70)*.

Leydig Cells and Steroidogenesis

Leydig cells are the sites of androgen production in the testis. The principal and most important androgen produced by Leydig cells is testosterone. Testosterone biosynthesis is primarily under the control of the pituitary gonadotropin LH. LH stimulates the production of cAMP by binding to specific receptors on the surface of Leydig cells that are coupled to the adenylate cyclase second messenger system. Human chorionic gonadotropin (hCG) binds to LH receptors and is used to study LH action in Leydig cells. cAMP is the intracellular second messenger for LH and mediates LH action in Leydig cells by activating the cAMP-dependent protein kinase, PKA [reviewed in ref. *71*]. cAMP has two principal activities in the control of Leydig cell steroidogenesis: The first action of cAMP is the acute stimulation of testosterone biosynthesis via mobilization and transport of cholesterol into the steroidogenic pathway, an

action that takes place *within minutes*. The second action of cAMP in Leydig cells is the chronic and prolonged stimulation of gene expression of the steroidogenic enzymes and upregulation of their activity, a slower process that requires *several hours* [reviewed in ref. *72*].

Testosterone biosynthesis is dependent on the action of two cytochrome P450 enzymes and two flavoprotein dehydrogenase enzymes (Fig. 2). The first and rate-limiting *enzymatic* step in testosterone synthesis is the conversion of cholesterol to pregnenolone that is catalyzed by cholesterol side-chain cleavage (P450scc) enzyme, encoded by *Cyp11A1* that is located on the inner mitochondrial membrane. Pregnenolone diffuses out of the mitochondria to the smooth endoplasmic reticulum where it is further metabolized via the action of 3β-hydroxysteroid dehydrogenase Δ^4-Δ^5-isomerase (3β-HSD) to progesterone. Progesterone in turn is converted by a two-step process to androstenedione via the action of 17α-hydroxylase/C_{17-20} lyase (P450c17), encoded by the Cyp17 gene. The conversion of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) [reviewed in refs. *73* and *74*].

Cholesterol is the precursor for all steroid hormones. Regardless of its source, its mobilization and transport into the inner mitochondrial membrane to P450scc is a cAMP-dependent process. Transfer of cholesterol across the inner-mitochondrial space is regulated by, and dependent on, the action of steroidogenic acute regulatory protein (StAR) *(75,76)*. It has long been appreciated that acute production of steroids was dependent upon a hormone-stimulated, rapidly synthesized and highly labile protein whose function is to mediate the transfer of cholesterol from the outer mitochondria to the inner mitochondrial membrane for further conversion by P450scc. While several candidate proteins have been considered, StAR is the only protein that has all of the necessary characteristics of an acute regulatory protein *(72,75)*. The discovery that a deficiency in StAR accounts for the genetic defect associated with congenital adrenal lipoid hyperplasia has been the essential proof of StAR's critical role in the acute regulation of steroidogenesis *(77,78)*.

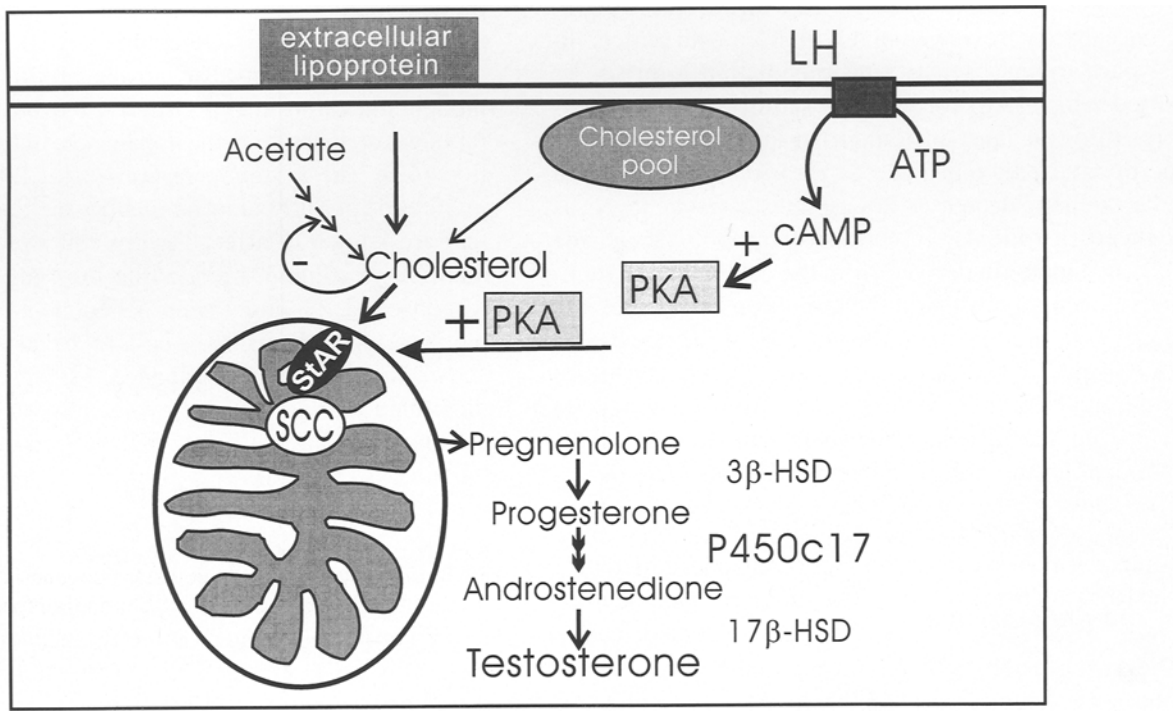

Fig. 2. Testosterone biosynthetic pathway in mouse Leydig cells. Testosterone biosynthesis in Leydig cells is under the control of *LH*, which binds to 7-*trans*-membrane G protein-coupled receptors on the plasma membrane and activates adenylate cyclase which catalyzes the conversion of ATP to *cAMP*, the intracellular second messenger for LH in Leydig cells. cAMP has two major roles in testosterone biosynthesis: acute stimulation of the mobilization and transport of *cholesterol* into the steroidogenic pathway (illustrated in this cartoon), and transcriptional regulation of steroidogenic enzyme gene expression. cAMP action is mediated via activation of PKA. PKA in turn activates cholesterol mobilization from intracellular cholesterol pools, extracellular lipoprotein sources, or from *de novo* cholesterol synthesis from acetate. Regardless of its origin, cholesterol transfer into the inner-mitochondria membrane is a cAMP-dependent process requiring the action of the steroidogenic acute regulatory protein (*StAR*). Cholesterol is converted to pregnenolone via the action of cholesterol side-chain cleavage P450 (*P450scc*) which resides on the inner face of mitochondrial inner matrix membrane. Pregnenolone diffuses to the smooth endoplasmic reticulum where it is converted to progesterone via the action of 3β-hydroxysteroid dehydrogenase-Δ^4-Δ^5 isomerase (*3β-HSD*). Progesterone in turn is converted to 17α-hydroxyprogesterone, then androstenedione by the action of 17α-hydroxylase/C_{17-20} lyase (*P450c17*). Androstenedione is then converted to testosterone via the action of 17β-hydroxysteroid dehydrogenase (*17β-HSD*).

Immune-Endocrine Control of Leydig Cell Function

While there is some controversy about the effect of cytokines on basal testosterone synthesis, the overwhelming consensus is that proinflammatory cytokines inhibit gonadotropin or cAMP-stimulated steroidogenesis in Leydig cells [reviewed in ref. *11*]. Many factors have been implicated in mediating the pathological effects associated with endotoxemia and sepsis. Cytokines are believed in general to be the decisive factors in determining the pathology in sepsis *(79–81)*. The cytokines most strongly associated with LPS endotoxemia and Gram-negative sepsis are TNF, IL-1, and IL-6. Considerable attention has been paid to the effects of TNF and IL-1 on Leydig cell function while the effects of IL-6 have not been as extensively studied *(10)*. Cytokines are released in a sequential manner following exposure of bacterial endotoxin to immune competent cells resulting in initiation of the cytokine cascade *(79)*. IFNγ and IL-2 are also elevated in amounts that correlate with the severity of illness. With the exception of IL-6, each of these cytokines (TNF, IL-1, IFNγ, and IL-2) has been demonstrated to inhibit cAMP-stimulated steroidogenesis in Leydig cells at the level of expression of different steroidogenic enzyme genes *(11)*. Recently, IL-6 was shown to have similar effects on Leydig cell steroidogenesis and inhibit cAMP-stimulated testosterone and P450c17 mRNA expression *(82,83)* (Fig. 3). IL-6 reveals a unique mechanism here since it also compromises the cAMP-induced expression of 17β-HSD (type 3) in Leydig cells. It is noteworthy that the distal cytokine in the proinflammatory cascade inhibits the last enzyme in the testosterone biosynthetic pathway *(83)*.

Leydig Cell–Macrophage Interactions

Leydig cells and macrophages in the interstitial tissue of the testis are closely associated. This close physical association suggests that testicular interstitial macrophages and Leydig cells are functionally related *(11,84)*. Resident macrophages have specialized functions in addition to the classical macrophage activities. The structural, phagocytic, and immunological functions of resident testicular macrophages appear to be normal *(85–87)*. They express major histocompatibility complex class II (MHC II) molecules characteristic of normal antigen presenting cells *(88,89)*. Immune-activated testicular macrophages have been shown to express mRNA for IL-1β, IL-6, TNFα and granulocyte macrophage-colony stimulating factor (GM-CSF) *(90–93)* and secrete bioactive IL-1, IL-6, TNFα and GM-CSF protein *(91,93–95)*. While testicular macrophages have numerous characteristics common to macrophages from other sources, they have unique and highly specialized properties, presumably important for their interaction with Leydig cells *(84)*. Although testicular macrophages have been shown to secrete bioactive cytokines, as described above, it has been suggested that they have a

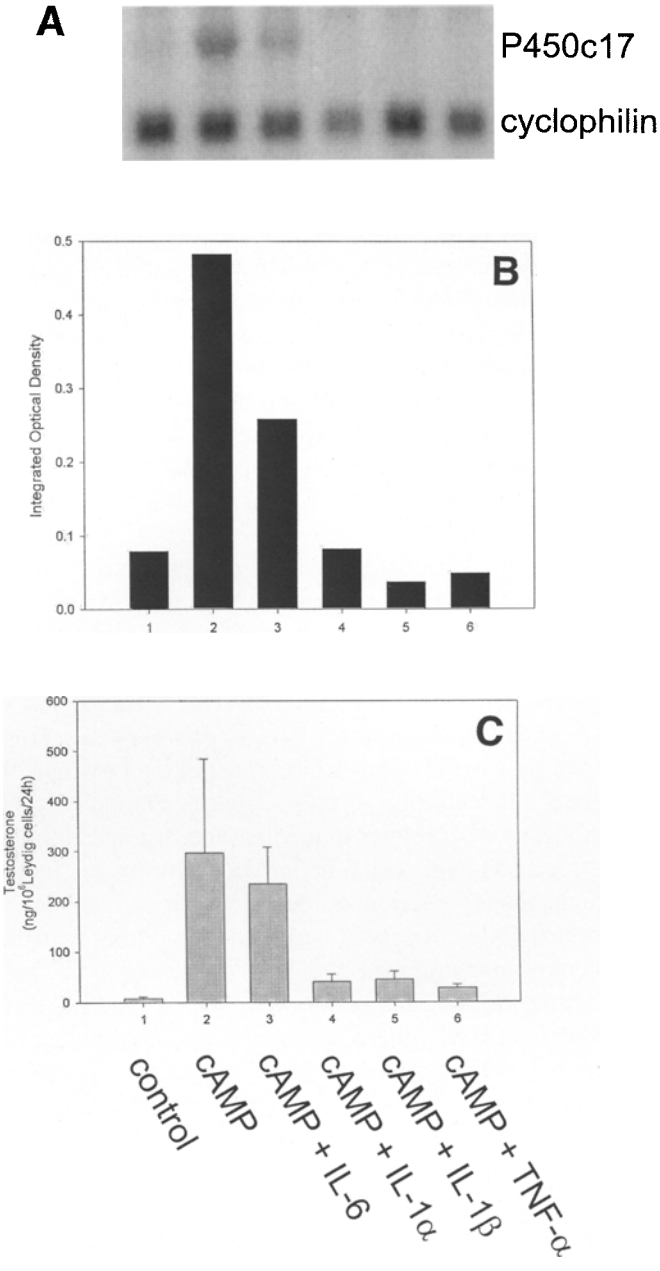

Fig. 3. Inhibition of Leydig cell steroidogenesis by pro-inflammatory cytokines. Leydig cell testosterone biosynthesis is cAMP-dependent and inhibited by the proinflammatory cytokines interleukin-1 (IL-1α, IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNFα). Of all of the steroidogenic enzymes, P450c17 is the most sensitive to cytokine inhibition. Mouse Leydig cells in primary culture were treated for 24 h with 50 m*M* 8-Br-cAMP (cAMP), or cAMP plus 100 ng/mL mrIL-6, 10 ng/mL hrIL-1α, 10 ng/mL mrIL-1β, or 10 ng/mL mrTNFα. Media were collected for testosterone RIA and RNA extracted for Northern blot. **(A)** The blot was probed simultaneously for P450c17 and cyclophilin. **(B)** Data were quantitated by Phorsphorimaging and expressed as ratio of P450c17/cyclophilin. **(C)** Testosterone was determined by RIA and expressed as ng/10^6 Leydig cells/24 h (n = 3). [Data are representative of previously published studies *(83,128,141)*.]

blunted secretory response compared to peritoneal macrophages *(93,95)*. In addition, testicular macrophages demonstrate a pattern of protein secretion distinct from peri-

toneal macrophages *(96)*. Moreover, testicular macrophages have been reported to express FSH receptors that may be related to their specialized functions in the testis *(97,98)*.

Morphological examination of the adult rat testis indicates that there is a direct structural interaction between these cells *(99)*. Cytoplasmic processes of Leydig cells were observed that extended to membrane invaginations of adjacent macrophages *(85,99)*. These specialized membrane associations have been termed "digitations" *(86)*. During development, changes in Leydig cell and macrophage morphology are coordinated *(100,101)*. Macrophages are present in the rodent testis throughout postnatal and prepubertal development and they appear to be regulated by Leydig cell function during development *(84,102)*. Macrophages also have been identified in the interstitial tissue of the human testis *(103)*.

LPS injection stimulates the local expression of TNFα by testicular interstitial macrophages [reviewed in refs. *11* and *86*]. TNFα expression can be induced in isolated testicular macrophages . *(91)* In addition, testicular macrophages have been shown to produce IL-1 *in vivo (90)* and *in vitro (93,95)*. Testicular macrophages express and secrete TNF and IL-1 in the microenvironment of the Leydig cell. The local concentration of these cytokines is likely to be very high and will produce immediate and sustained inhibitory effects on Leydig cell function. In addition to testicular macrophages as paracrine sources of cytokines, many other testicular cells have been shown to produce cytokines in the microenvironment of the Leydig cell [reviewed in ref. *74*]. In particular, Sertoli cells are known to secrete IL-1 and IL-6 *(82,104–109)*. Thus, there are several potential paracrine sources of cytokines, in addition to resident testicular macrophages that may influence the performance of testicular cells. Furthermore, there is a large peripheral response to LPS [reviewed in refs. *48,80,110–114*]. LPS injection results in a rapid peripheral increase in TNFα *(92)*. Systemic increases of proinflammatory cytokines are of sufficient magnitude to contribute to the inhibition of Leydig cell function. Moreover, Leydig cells themselves are a potential source of proinflammatory cytokines. Leydig cells express and produce IL-1, TNF, and IL-6, suggesting a possible autocrine regulatory role of these cytokines on Leydig cell function *(115–118)*. It is obvious from these studies that Leydig cells are exposed to cytokines derived from the peripheral circulation, from adjacent testicular cells, and from Leydig cells themselves that direct their steroidogenic function. Leydig cell function therefore may be impaired by infections far from the testis as well as close to their localization.

Effects of Cytokines on Leydig Cell Steroidogenesis

Interleukin-1

Interleukin-1 (IL-1) consists of a family of three distinct, single-chained glycosylated 17 kDa proteins: IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1Ra). IL-1α and β share only 22% amino acid homology but bind to the same receptors and have similar biological activities. Both are important in the initiation of inflammatory response, and coordinate the proliferation and activation of T-lymphocytes, B-lymphocytes, and monocytes. IL-1Ra is a naturally occurring antagonist that modulates the activity and toxicity of IL-1α and IL-1β. IL-1α and β are secreted by a variety of differentiated cells, but most characteristically by activated macrophages and monocytes [reviewed in ref. *119*]. IL-1 and IL-2 are also produced and secreted by testicular Sertoli cells and a variety of regulatory factors have been identified *(120,121)*. Interleukins derived from Sertoli cells may contribute significantly to the physiological levels that are evident in interstitial fluids of the testis *(109,121)*.

A review of the literature on the effects of IL-1 on basal testosterone production reveals that IL-1 is either stimulatory or has no effect *(122–124)* Table 1. However, there is general agreement that IL-1 inhibits LH/hCG and/or cAMP stimulated testosterone production *(92,122,123,125–129)*. The major site of inhibition is at the level of the 17α-hydroxylase/C_{17-20} lyase enzyme. IL-1 causes a dose-dependent inhibition of cAMP-stimulated induction of P450c17 gene expression in macrophage-depleted mouse Leydig cells in primary culture. Thus, IL-1-mediated inhibition of testosterone biosynthesis is primarily mediated by the inhibition of P450c17 *(128)*. IL-1 inhibited cAMP-induction of P450c17, P450scc, and 3β-HSD, but did not inhibit the basal or constitutive expression of these enzymes *(128,130)*. Interleukin-1 appears to affect rat Leydig cell steroidogenesis at the level of P450ssc, while StAR gene expression and protein synthesis is unaffected *(131,132)*.

The interstitial fluids of the testis contain biologically relevant concentrations of IL-1 that appear to regulate immune responses in the testis *(133)*. In particular, IL-1 has been shown to inhibit T-cell responses via induction of another not yet identified protein of high molecular weight that is not identical with TGFβ *(134)*. An important role of IL-1 in the physiological control of steroidogenesis and spermatogenesis is questioned by the fact that mice lacking a functional type I interleukin-1 receptor have normal sexual function and are fertile *(135)*.

Tumor Necrosis Factor alpha

TNFα is a 17-kDa glycosylated polypeptide secreted principally by activated monocytes and macrophages *(80)*. It binds as a trimer to either of the two TNF receptors that are found on most cells in the body, and plays a central role in the initiation of the inflammatory response due to invading microorganisms *(48)*. TNFα stimulates the release of IL-1 and IL-6 from activated monocytes and macrophages, and its synthesis and release is enhanced by interferon-gamma (IFNγ) secreted by activated T-lymphocytes *(81)*. The sequela following immune activation *in vivo* is complex but the initiation of the network of cytokines that are produced depends on the secretion of TNFα *(48)*.

There are a number of reports in the literature describing the effects of TNFα on Leydig cell steroidogenesis *(92,136–143)*. Although one group concluded that TNFα stimulates steroidogenesis *(142)*, the majority of these reports describe inhibitory effects and a decrease in the production of testosterone. These studies have been performed in a variety of systems, including whole animal studies *(138,139)*, isolated primary cultures of Leydig cells *(140,141,143)* and in MA-10 tumor Leydig cells transfected with *Cyp17*-reporter constructs *(137)*. TNFα-mediated inhibition of hCG binding to its Leydig cell receptor has been reported *(140)*, but the majority of reports suggest that TNFα inhibition of steroidogenesis occurs downstream of cAMP production at the level of steroidogenic enzyme gene expression. This inhibition appears to be exerted via alterations in AP-1 activity as evidenced in MA-10 cells *(144)*. In mouse Leydig cells from primary cultures, TNFα caused a decrease in P450scc, P450c17, and 3βHSD expression (141,145). TNFα has no effect on the basal expression of P450scc but does inhibit basal expression of 3βHSD *(130)*.

Interleukins

IL-2 is primarily a T cell growth factor that enhances the activity and formation of cytotoxic cells. IL-2 also stimulates TNFα, IL-1, and IFNγ secretion. IL-2 inhibits gonadotropin-stimulated testosterone production by rat Leydig cells at the level of the P450c17 enzyme, similar to TNFα, and IL-1 *(146)*. In a clinical study male patients treated with high doses of IL-2 as immunotherapy for metastatic cancer had significantly reduced serum testosterone levels *(139)*. In general, IL-2 causes a robust peripheral response resulting in elevation of numerous serum cytokines, in particular, TNFα, IL-1, and IFNγ *(147,148)*. The inhibitory effects of IL-2 therefore could be directly on Leydig cells, or indirect, mediated by activation of testicular macrophage TNFα and IL-1 secretion. IL-2, in similarity to other cytokines, stimulates the hypothalamic–pituitary–adrenal axis, resulting in increased ACTH and corticosteroid levels. Glucocorticoids have direct inhibitory effects on Leydig cell steroidogenesis as can be expected from the overall inhibition of protein synthesis; thus, elevated glucocorticoids may also contribute to the in vivo inhibitory effects of IL-2 in Leydig cells *(149)*.

Interferons

Interferons (IFNs) are a group of structurally and functionally related polypeptides that comprise three main groups, IFNα, IFNβ, and IFNγ. The best known effects of IFNs are their antiviral, antiproliferative, and immunomodulatory actions. The major cellular sources for interferons are different: IFNα is produced by monocyte/macrophages, IFNβ is produced by fibroblasts and epithelial cells, and IFNγ is produced by T-lymphocytes [reviewed in ref. *150*]. IFNs have also been shown to affects the endocrine system. In the testis INFα and γ is produced in resident macrophages, Sertoli cells and Leydig cells and the same cell types have been identified to express IFN receptor subunits *(151,152)*. In particular, both IFNα and IFNγ have been demonstrated to inhibit testosterone production in primary cultures of porcine Leydig cells *(153,154)*. IFNγ exerts its inhibitory effect on testosterone production at the level of cholesterol transport into the mitochondria. IFNγ inhibits the expression of both P450scc and P450c17 *(155)*, similar to the effect of TNFα on mouse Leydig cells *(141)*. Normal healthy men who were treated with human leukocyte-derived IFN (IFNα) had significantly decreased serum testosterone levels *(156)*. The observed decrease in testosterone was most likely due to a direct inhibition of Leydig cell steroidogenesis because serum levels of gonadotropins were unaffected by the treatment. Dejucq et al. recently have shown that Leydig cells in the rat testis strongly express IFNα and γ during infection with Sendai virus. This elevation in IFN expression interestingly was associated with an increase in testosterone production in this model *(151)*. In other experimental studies, steroidogenesis in rat ovarian cells and testicular Leydig cells was compromised by human and murine IFNα and the effect could be reversed by addition of specific IFN antibodies *(157)*.

Other Cytokines and Growth Factors

Transforming growth factor (TGF)β belongs to a family of at least five distinct dimeric proteins, of which, three are expressed in mammals (β1, β2, β3). These proteins are part of the superfamily of dimeric proteins that includes inhibin, activin, and anti-Müllerian hormone *(158)*. TGFβs are multifunctional growth and differentiation factors involved in many aspects of tissue remodeling and repair as well as interacting with cytokines in the regulation of the immune system *(159)*. Nearly all cells synthesize a form of TGFβ and possess functional membrane receptors for this family of polypeptides. In vitro studies have shown that TGFβ is a potent inhibitor of Leydig cell steroidogenesis. TGFβ inhibits steroidogenesis at the level of LH receptor number and signaling as well as distal cAMP generation at the level of P450c17 gene expression [reviewed in ref. *160*]. TGFβ 1 and 2 have also been identified as inducing agents of apoptosis in gonocytes during developmental and proliferative stages but do not exert the same effect on Sertoli and Leydig cells *(161)*. Fetal testes of rats also express and produce TGF-β1 during development in a cAMP-dependent pattern. Sertoli cells and Leydig cells apparently regulate the concentration of TGF to physiological levels to prevent effects, such as apoptosis of gonocytes, that have been observed with nonphysiological levels in experimental studies *(161,162)*.

Epidermal growth factor (EGF) is another cytokine that appears to be involved mainly in the regulation of spermatogenesis in the testis *(163)*. Leydig cells have been shown to be a rich source of EGF, but this cytokine is also expressed by Sertoli cells and spermatogenic cell popula-

tions within the seminiferous epithelium *(164)*. A correlation has been described between EGF expression in Sertoli cells and defects in spermatogenesis in infertile patients *(165)*.

Pathology and Testis Function

The question arises, then, why is there a mechanism in place for the cytokine-mediated inhibition of testosterone production? It is widely recognized that in most species studied, males have weaker immune responses than females *(166–168)*. The weaker immune response contributes to higher susceptibility to infection, and poorer survival of males compared to females. One consequence of the increased immune response in females in comparison to males is an increased incidence of autoimmune disease *(32,166)*. Studies in many experimental models have established that the underlying basis for this sex-related difference in susceptibility to disease is due to differences in gonadal steroids. Androgens indeed are immunosuppressive and have been applied for the experimental treatment of autoimmune disorders such as rheumatoid arthritis *(32)*. The effects of androgens on peripheral blood monocytes collected from patients with rheumatoid arthritis indicated that testosterone inhibited IL-1 secretion indicating that men with lower androgen levels might be more susceptible to the disease *(169)*. Moreover, dihydrotestosterone depresses the production of IL-4, IL-5, and IFNγ by activated murine T-lymphocytes suggesting that androgens are important in the regulation of certain aspects of the immune response *(170)*. Administration of testosterone decreases thymus weight and causes a depletion of cortical lymphocytes, while castration causes thymic hyperplasia due to withdrawal of androgenic steroids *(171)*. The mechanism of androgen-induced thymolysis is unknown, but it has been suggested that nonlymphoid cells in the thymus are targets for androgens and that these nonlymphoid cells mediate the androgen-induced depletion of thymocytes *(171)*. The testis has long been considered as an immunologically privileged site and it has been suggested that locally produced androgens are immunosuppressive and important to testicular immune privilege *(88)*. These observations, together with the data demonstrating that cytokines are elevated during conditions associated with decreased serum testosterone, provide the basis for the hypothesis: in order for the animal to wage the maximum possible immune response, products of the immune reaction (cytokines) inhibit the production of immunosuppressive androgens.

Nature provides an example that elegantly supports this hypothesis. The male marsupial mouse, *Antechinus stuartii,* is overtly preoccupied with copulation and dies abruptly at the conclusion of the mating season *(172)*. By contrast, the female *A. stuartii* is longer lived and survives several mating seasons. To determine the mechanism behind the difference in longevity between males and females, autopsies of males revealed a variety of disease states, all associated with suppression of the immune system and inflammatory responses. It was observed that castrated males survived in the field well beyond the period of natural mortality. Males who were captured and raised in a pathogen-free environment lived up to 3 years, equivalent to the life span of females *(173)*. Thus, these observations support the hypothesis that androgens are immunosuppressive. It follows, then, that inhibition (or removal) of androgens allows the animal to mount an effective immune response.

Autoimmune Disease of the Testis

When the immune system encounters testis-specific autoantigens, they are recognized as foreign despite their endogenous origin. Thus, antibodies to sperm antigens are elicited in animal models of experimental autoimmune orchitis (EAO) when animals are immunized with autologous sperm or testicular cells. Animal models of EAO have provided important insights into human pathological incidence of autoimmune orchitis that results from testicular injury associated with biopsy, vasectomy or an obstructed vas deferens *(174)*. An apparent correlation is evident between elevated antisperm antibodies and testicular lesions after vasectomy *(175)*. Similar inflammatory changes have been reported in the epididymis after vasectomy that results in elevated pressure and extension of the epididymial duct *(176)*.

Historically, the sole mechanism believed to be necessary to protect autologous sperm antigens against immune destruction was their sequestration behind the blood–testis barrier. However, evidence has emerged suggesting testis autoantigens are exposed to circulating immune cells and that active immunoregulatory mechanisms are involved in preventing autoimmune responses to antigens present on testicular cells *(174)*. The blood–testis barrier is comprised of tight-junctions between adjacent Sertoli cells and the basal laminae of the seminiferous epithelium and separates germ cells in the basal compartment from those in the adluminal compartment. This barrier prevents antibody and lymphoid cells from reaching the adluminal compartment and under physiological conditions, lymphocytes and macrophages are not detected on the adluminal side of the seminiferous tubules. Germ cell stages sequestered within the tubule include pachytene spermatocytes, spermatids, and testicular spermatozoa. All known orchitogenic antigens are present within the protected compartment *(174)*. It is presumed that mechanisms subserving the pathogenesis of EAO are responsible for the etiology of autoimmune or granulomatous orchitis in humans. The progression of the disease is first accompanied by activation of testicular macrophages surrounding the tubules, demonstrated by a 20-fold increase in Ia^+ immunoreactivity. Stimulation of macrophages and upregulation of Ia+ is dependent on circulating interferon-γ and requires $CD4^+$ lymphocytes. Evidence suggests sperm antigens are presented by IFNγ stimulated MHC class II cells (Ia^+ macrophages) to activated $CD4^+$ lymphocytes that in turn initiate lesions in seminiferous tubules and the vas deferens *(174)*. Once the blood–testis

barrier is breached, immune complexes are formed outside the barrier on the peritubular basal lamina. The T cell response is then potentiated by the ensuing inflammatory response resulting from activation of macrophages that remove the exposed intra-tubular germ cells. The significance of IFNγ in this pathophysiological concept is supported by the finding that IFNγ blocking antibodies can prevent the development of EAO when applied 20 days after induction of EAO *(177)*.

Orchitis is the most common complication of mumps that affects between 15% and 35% of mumps infected men after puberty. Mumps orchitis is characterized by severe fever, scrotal swelling, decreased serum testosterone, and impaired Leydig cell responsiveness to gonadotropin that usually follows systemic infection in an interval of 1 to 2 weeks *(178)*. The etiology of orchitis associated with mumps has not been determined, but it has been suggested that autoantigens from the salivary gland cross-react with testicular antigens resulting in autoimmune orchitis. This pathophysiological concept is challenged by the clinical fact that mumps orchitis also occurs simultaneously with or precedes mumps parotitis. The classical pathophysiological understanding includes that testicular cells are targeted directly by the mumps virus resulting in localized immunological events that leads to impairment of testicular function. This concept is supported by the current standard for the treatment of mumps orchitis that is based on systemic application of IFN. INF injections in case of mumps orchitis have been reported to ease the symptoms and improve the post-infectious pathology which is frequently typified by tubular sclerosis and testicular atrophy in untreated cases *(179,180)*. Infection by the mumps virus results in elevated IFN secretion and IFNγ activates a number of macrophage functions including upregulation of MHC class II antigens and cytokine secretion *(150)*. Presumably the accompanying inflammatory response observed in autoimmune orchitis results in the elaboration of a cascade of proinflammatory cytokines. The resultant sequela of cytokine secretion in the micoroenvironment of the testis likely affects Leydig cell function and spermatogenesis.

Other events compromising testicular integrity are supposed to induce similar reactions. Indeed, a characteristic consequence of vasectomy is a marked temporary depression of testicular androgen output with concomitant increase in LH secretion as an immunological response to the surgical lesion *(181–184)*. In addition, it has been demonstrated that testicular biopsies performed to isolate spermatozoa from testicular tissue in azoospermic patients result in decreased levels of serum testosterone *(185)*. This condition is maintained for several weeks and is more likely due to the posttraumatic secretion of testicular cytokines rather than to the loss of testicular tissue and Leydig cells.

Immune-Endocrine Interactions in the Testis

In addition to macrophages, lymphocytes and other immune cells are found in the rodent *(186)* and human testis *(89)* and thus can also serve as potential sources for immune regulatory molecules such as lymphocyte derived cytokines (IFNγ and IL-2). Leydig cells are also known to interact directly with lymphocytes, as well as with macrophages. In fact, Leydig cells are the only normal highly differentiated endocrine cells that spontaneously form rosettes with lymphocytes, macrophages, and eosinophils, consistent with specific receptors and recognition molecules encoded on the surface of Leydig cells *(187)*. Following depletion of testicular Leydig cells by EDS injection (see below), precursor Leydig cells are recruited, proliferate, differentiate, and, within a month, repopulate the testis with normal adult Leydig cells. Mast cells proliferate and differentiate in parallel to Leydig cells and are subject to the same regulatory influences *(188)*. It has been suggested that Leydig cells mediate local regulation of testicular leukocyte populations *(189)*. Indeed, Leydig cells, macrophages and Sertoli cells are all sources for immunoregulatory polypeptides [reviewed in refs. *3* and *190*].

The testis is considered to be an immunologically privileged site. Allografts and xenografts have been shown to survive in the testes of rodents *(88)*. Despite the presence of normal components of the immune system (macrophages and lymphocytes), the regulation of these cells in the testis must be altered to allow alloplastic tissues to survive. Testicular macrophages comprise approximately 25% of the cells of the interstitium and have numerous characteristics of macrophages from other tissues, including structural, phagocytic, bactericidal functions, and express major histocompatibility complex class II (MHC II) molecules and Fc receptors [reviewed in ref. *86*]. Macrophages participate in the initiation of the immune response by antigen presentation and costimulation of T lymphocytes, and by secreting proinflammatory cytokines, in particular, IL-1 *(191)*. In addition to proinflammatory cytokines, macrophages also secrete antiinflammatory cytokines such as TGF-β, and IL-1 receptor antagonist. Thus, there exists a dynamic balance between immune activation and suppression. Androgens are also known to be immunosuppressive, in particular toward T-lymphocyte-mediated responses *(170)*. It is possible that immunological suppression of the testis is due, in part, to the high local concentration testosterone in the interstitial tissue. Indeed, androgens have been shown to inhibit IL-1 secretion by blood mononuclear cells *(169)*, and orchidectomy markedly increases the number of macrophages in the adrenal cortex, suggesting that androgens regulate macrophage numbers as well as secretory activities *(192)*. Therefore, it is entirely plausible that macrophage activation of T lymphocytes is inhibited in the androgen rich milieu of the testis. Leydig cells may also secrete other factors, in addition to steroid hormones, that regulate macrophages and/or affect the immune reactivity of the testis *(189,193,194)*. Among these potential immune regulatory factors, proopiomelanocortin (POMC) and derived peptides *(195)*, inhibin *(196)*, and activin *(197)* have been

shown to be produced by Leydig cells. Immunoreactive AVP is expressed in the testis and has been show to be secreted in vitro by Leydig cells *(198)*. Other immunomodulatory factors shown to be secreted by Leydig cells include gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GHRH), corticotrophin-releasing hormone (CRH), oxytocin (OT), proenkephalin B and dynorphin *(74,199–201)*. Recently Meinhardt et al. demonstrated that Leydig cells produce macrophage inhibitory factor (MIF) and presented evidence for its role in the regulation of testicular function *(202)*. In addition to Leydig cell elaborated immune-regulatory factors, the testis is a rich milieu of locally produced growth and differentiation factors *(3)*. Immune–endocrine interactions in the testis are complex and are the subject of intensive current research.

Physiological Immune–Endocrine Interactions

Macrophages–Leydig Cell Interactions During Development

Macrophages play important roles in the regeneration of many cell types after tissue injury. For example, macrophages secrete several growth factors and are central to the wound-healing response required for proliferation of several regenerating cell types. Testicular macrophages are potential sources for several growth and differentiation factors and are closely associated, both physically and developmentally, to Leydig cells. Macrophages are, therefore, ideally located to provide some of the regulatory factors that govern Leydig cell proliferation and differentiation.

The cytotoxic drug ethane 1,2-dimethylsulfonate (EDS) causes the acute and selective destruction of Leydig cells and has been used extensively to study Leydig cell regeneration [reviewed in refs. *203* and *204*]. After EDS treatment, there is extensive phagocytosis of dead Leydig cells by testicular macrophages. Following the destruction of the Leydig cells, a wave of proliferative activity is evident and new Leydig cells repopulate the interstitial space. During this time an increase in the number of resident macrophages occurs as the first morphological signs of inflammation. These observations are consistent with the involvement of the inflammatory response in the stimulation of the proliferative activity of interstitial cells *(204)*. It is probable that phagocytosis of atretic Leydig cells, and/or other signals, activate testicular macrophages to secrete growth factors that stimulate the proliferative activity of interstitial cells.

Macrophage-secreted factors such as IL-1 are known mitogens for lymphocytes. These same factors also have been shown to stimulate the proliferation of immature Leydig cells. Khan et al. reported that interleukin-1β caused a dose-dependent stimulation of the incorporation of (^{3}H)-thymidine into the DNA in Leydig cells from 10- and 20-day old rats, but had no effect on DNA synthesis in Leydig cells from adult rats *(205)*. They also reported that IL-1α and TNFα also stimulated DNA synthesis in immature Leydig cells, but that these cytokines were much less potent than IL-1β. The close association of macrophages with Leydig cells, the invasion of the testicular interstitium by macrophages during prepubertal development, and the detection of IL-1-like activity in the testes from 20 days of age coincides with the development and differentiation of Leydig cells. Since IL-1 has been shown to stimulate proliferation of a variety of cells, these data are consistent with the role of IL-1, or other macrophage-secreted cytokinelike growth factors, in signaling Leydig cell growth and differentiation both during normal development and during Leydig cell regeneration following EDS treatment. Another macrophage-secreted cytokinelike factor, TGFβ, also stimulates DNA synthesis in immature, but not adult, Leydig cells *(205)*.

Colony stimulating factor-1 (CSF-1) stimulates the survival, proliferation, and differentiation of mononuclear phagocytes and their precursors [reviewed in ref. *206*]. Osteopetrotic (*op/op*) mice are characterized by an autosomal recessive mutation in the CSF-1 gene, resulting in the absence of CSF-1. Consequently, *op/op* mice have impaired mononuclear phagocyte development, resulting in a deficiency of both macrophages and osteoclasts. Only bone marrow macrophages show some restoration with age. All other populations of macrophages, including those in the testis, do not recover with age. The importance of macrophages in the gonads is clearly demonstrated with *op/op* mice, which have markedly reduced fertility [reviewed in refs. *206* and *207*]. Notably, *op/op* mice have severely reduced numbers of testicular interstitial macrophages *(206)*. Serum testosterone levels in *op/op* mice are significantly reduced compared to *op/+* mice. Leydig cells in testes of *op/op* mice have structural abnormalities such as disruption of organelles and may not be fully differentiated *(208,209)*. The lack of CSF-1 primarily affects morphology and function of Leydig cells and the resulting lack of testosterone leads to hypoplasia of the seminiferous epithelium *(209)*. Together these observations suggest that testicular macrophages secrete factor(s) necessary for the proliferation and differentiation of Leydig cells. The *op/op* mice have a phenotype similar to the IGF-1 null mutation, suggesting that one factor secreted by testicular macrophages may be IGF-1.

Other models of testicular macrophage depletion further demonstrate that in the absence of testicular macrophages, the development and normal functioning of Leydig cells is impaired. This suggests that the role of macrophage–Leydig cell associations during development and under noninflammatory conditions is to provide an appropriate microenvironment and extracellular milieu for the support of Leydig cell functions *(11)*.

Recently, Hutson and colleagues have identified a novel testicular macrophage-secreted factor that stimulates androgen biosynthesis by Leydig cells *(210,211)*. This macrophage-elaborated factor is lipopophilic and acutely stimulates Leydig cell steroidogenesis in a StAR-independent

manner. The identification of this macrophage factor that can positively regulate Leydig cell function further illustrates the importance of bidirectional interactions between macrophages and Leydig cells during normal physiological function.

Cytokines and Spermatogenesis

Historically, much emphasis has been placed on understanding the roles of gonadotropins and androgens in the control of spermatogenesis *(212)*. In recent years the emphasis has shifted to elucidating paracrine control mechanisms in the regulation of spermatogenesis *(5)*. Mammalian spermatogenesis encompasses three phases: proliferation and renewal of spermatogonia by mitosis, meiosis, and metamorphosis of spermatids to mature spermatozoa by spermiogenesis. Spermatogonial proliferation, meiosis, and spermiogenesis are found continuously throughout the testis, so that spermatogonia, spermatocytes, and spermatids coexist in the seminiferous tubule. These cohorts of cells form specific associations during their development resulting in defined stages of differentiation referred to as the wave of the seminiferous epithelium *(212)*.

Paracrine control of spermatogenesis by peptide growth factors and cell-cell interactions has been reviewed *(3,5,6)*. Cytokines have pleiotropic actions and act as growth and differentiation factors within the seminiferous tubule. An interleukin-1-like activity was observed in rat testis *(213)*, and this activity was shown to be produced coincident with the initiation of active spermatogenesis *(214)*. Gerard et al. demonstrated that an IL-1-like factor was IL-1α and that Sertoli cells were the site of its synthesis *(104)*. They suggested that Sertoli cell-derived IL-1α was important in the paracrine regulation of germ cells by Sertoli cells. Subsequently, phagocytic activity of Sertoli cells has been shown to stimulate IL-1 production *(107,108)*. Sertoli cells are also known to produce IL-6 *(82,105,106,108)*. IL-6 is produced in the seminiferous epithelium in a stage-dependent manner with highest levels at stages XIII-XIV-I-V. FSH stimulated IL-6 production has been observed in most stages, especially at stage VII. Exogenous IL-6 inhibited the onset of meiotic DNA synthesis of spermatocytes and to a lesser extent of spermatogonia. These results underscore that IL-6 is a stage-specific paracrine regulator of the seminiferous epithelium exerting specific inhibitory action on meiotic DNA synthesis *(215)*.

Cytokines have been shown to modulate Sertoli cell transferrin release *(216)* supporting the hypothesis that there is a cytokine-mediated bidirectional communication between testicular cells. De et al. have outlined that pachytene spermatocytes and round spermatids express TNFα mRNA and that Sertoli cells, but not spermatogenic cells, express TNFα receptors *(217)*.

Interferon (IFN)-α and -γ expression also has been investigated in the rat testis. IFNs are well known for their antiviral and immunoregulatory activities. Several studies have suggested an involvement of IFNs in the spermatogenic processes, but Dejucq et al. were the first to demonstrate that IFN α/β and γ mRNA and protein were produced by testicular cells *(218)*. Sertoli cells produced the highest concentrations of IFN α/β, followed by peritubular cells. By contrast, IFNγ was found only in early spermatids, but not in Sertoli cells, peritubular cells, or pachytene spermatocytes *(218)*. However, studies on the effects IFNγ revealed that male mice treated chronically with IFNγ had delayed sexual development, reduced testis and epididymal weights, reduced sperm counts, abnormal sperm morphology and reduced mating performance and fertility *(219)*. In contrast to these results obtained with IFNγ, systemic application of IFN-α resulted in a profound increase of daily sperm production and motility in rats so that this therapy has been suggested for the treatment of male infertility *(220)*. Cytokines have been reported to upregulate the expression of inducible nitric oxide synthetase (iNOS) in Sertoli cells resulting in high levels of nitric oxide. Nitric oxide is known to affect mitochondrial function so that the induction of iNOS may represent one molecular effector of cytokine activity in the testis *(221)*.

Macrophage-stimulating protein (MSP), a member of the hepatocyte growth factor (HGF) family, is a ligand for receptor tyrosine kinases in the HGF receptor family. In situ hybridization revealed that MSP mRNA was localized to spermatogonia and early spermatocytes in the testis, and in the epithelial lining of the epididymis. This localization suggests that MSP may be involved in germ cell–germ cell interactions during spermatogenesis *(222)*.

Infectious diseases of the testis and male accessory glands in men have been demonstrated to profoundly affect spermatogenesis and posttesticular spermatozoa, respectively *(24,25)*. Testicular concentrations of cytokines in human testes cannot be determined in states of acute infections, since infectious diseases of the testis usually do not justify a testicular biopsy. Much emphasis has been placed on investigating different infectious mechanisms that affect spermatogenesis and spermatozoa in in vitro studies. Human spermatozoa incubated with the supernatants from activated lymphocytes and monocytes revealed significantly reduced motility *(223)*. Similar results were obtained when spermatozoa were incubated with IFNγ and TNFα. In addition both cytokines compromised the fertilizing ability of spermatozoa as determined by the zona-free hamster egg penetration test, indicating the important role of cytokines in posttesticular stages of reproduction *(28,224)*. The difficulty in interpreting such experimental data is the lack of studies that define physiological concentrations and cutoff points for cytokines in the testis and in seminal fluid of humans. Recently, Fujisawa et al. have shown that patients suffering from oligoozoospermia and azoospermia demonstrate elevated plasma levels of IFNα and -γ, but thus far, these findings have not been correlated to other parameters of infection *(225)*. IL-8 levels in human seminal plasma

have been investigated indicating significant differences of levels between normal subjects and patients with male accessory gland infections. The origin of IL-8 in seminal plasma and its role in spermatogenesis and steroidogenesis has not been elucidated, but this cytokine might be useful as a seminal plasma marker for infections *(180)*. In the future, the availability of reliable methods to measure various cytokines in seminal plasma will facilitate the study and understanding of the role of cytokines in infectious diseases of the genitourinary tract.

In conclusion, these observations further support the role of cytokines as paracrine regulators of testicular function. Nonlymphoid cells secrete cytokines in a stage-specific manner during spermatogenesis, presumably as growth and differentiation factors, whereas in the adult, macrophages and lymphocytes secrete cytokines under conditions causing immune-activation, such as inflammation.

Role of the Neural-Immune-Endocrine Axis in the Control of Testicular Steroidogenesis

A hallmark of immune-endocrine interactions is immune activation of the hypothalamic–pituitary–adrenal axis, resulting in the activation of the "stress response" [reviewed in refs. *226* and *227*]. The interaction between the stress and reproductive axes has been studied extensively. The reproductive axis can be suppressed by hormones from all levels of stress related events [reviewed in refs. *9* and *228*]. Glucocorticoids have been shown to inhibit LH and FSH secretion. Such suppression requires days to become evident, suggesting a more chronic role for adrenal corticosteroids in the inhibition of gonadotropin secretion and suppression of other reproductive functions such as spermatogenesis and steroidogenesis. Similarly, the direct inhibitory effects of glucocorticoids on Leydig cell steroidogenesis are manifested over a longer time-course, and act at the level of transcriptional steroidogenic enzyme repression [reviewed in ref. *73*]. In contrast, the hypothalamic hormone CRH appears to repress the reproductive axis more acutely [reviewed in ref. *9*]. Rivest et al. demonstrated that intracerebroventricular (icv) injection of IL-1β into the rat inhibited GnRH release into the median eminence *(228)*. Recently, Battaglia et al. have shown that injection of the bacterial endotoxin LPS into ovariectomized sheep inhibited pulsatile GnRH secretion into the hypothalamic-pituitary portal blood, further supporting the hypothesis that inflammatory stimuli inhibit the reproductive axis by acting centrally *(229,230)*. Previously, Rivier et al. *(231,232)*, and subsequently Kalra et al. demonstrated that IL-1 inhibits LH secretion *(233,234)*. While perturbation of LH secretion by central mechanisms would certainly result in a concomitant inhibition of gonadal steroidogenesis, evidence suggest that in addition, a neural pathway exists through which icv IL-1 directly inhibits steroidogenesis. During stress conditions that lower LH levels, decreased testosterone levels are undoubtedly due at least in part to a pituitary-mediated event. However there are many stresses that lead to low testosterone levels in the absence of decreased LH secretion. These observations lead Rivier et al. to postulate that there may be a direct neuronal connection between the brain and the testis that is activated by cytokines derived from the central nervous system (CNS) *(10)*. Recent support for this hypothesis comes from the demonstration that icv IL-1β decreases testicular responsiveness to hCG in rats pretreated with a GnRH antagonist and therefore lack LH secretion *(235)*. This inhibitory effect of centrally injected IL-1β precedes elevation of peripheral cytokine levels or decreases in plasma LH. Recent studies support the existence of a direct neural link between the ventral medial hypothalamus and the testes which is influenced by IL-1 and regulates testicular responsiveness independently of the pituitary *(236)*. The possible involvement of prolactin and opiods as mediators of the IL-1β effect has been ruled out; however, it appears that central catecholamine pathways may be involved. Of pharmacological interventions tested, propranolol effectively reversed the inhibitory effect of icv-injected IL-1β. Recently, Olgivie et al. have demonstrated that icv IL-1β-mediated decreases in testicular responsiveness to hCG are not due to decreased number of LH receptors or binding activity of Leydig cell, but result from a rapid inhibition of StAR protein expression, further supporting the existence of direct neuronal control of Leydig cell function *(237)*.

In light of the large body of evidence demonstrating the existence of neuronal peptide signaling pathways that affect Leydig cell function [reviewed in refs. *3* and *160*], it is tempting to speculate that these neuronal peptides are the efferent effectors of the direct brain-to-gonad signal produced in response to immune activation. In support of this hypothesis, surgical or pharmacolgical denervation of the testes blocks the effects of oxytocin on steroidogenesis. Oxytocin has been shown to stimulate steroidogenesis in immature rat testis. Serotonergic elements were destroyed by treatment with 5,6-dihydroxytryptamine. Transsection of the inferior testicular nerve by vasectomy resulted in similar effects. Electrical stimulation of spermatic nerves results in a significant increase of testosterone in venous blood from the spermatic vein in cats *(238)*. These results support that testicular innervation is involved in the control of local peptide effects *(239)*. Acute spinal cord injury in experimental animals is followed by immediate decrease of serum testosterone levels and profound reductions in spermatogenesis indicating the important role of efferent nerves for testicular function. These events are accompanied by elevation of gonadotropins. Defects in spermatogenesis and steroidogenesis can be rescued by external administration of testosterone *(240,241)*. However, lesions in spermatogenesis after spinal cord injury are not evident throughout the testis and appear to recover in a period of months. Local regulatory mechanisms of the testis appear to be able to regulate spermatogenesis and steroidogenesis without

neuronal assistance after a period of adaptation *(240)*. These mechanisms evident in experimental animals are supported by findings among patients with chronic spinal cord injury where spermatozoa for assisted reproduction can be retrieved from the testis or the ejaculate after electroejaculation although disorders in spermatogenesis also are evident in many patients *(242)*.

The testis lacks somatic nerves and is supplied only with autonomic nerves, and the bulk of testicular nerves are sympathetic. The neurotransmitter associated with the sympathetic fibers innervating the testis appears to be norepinephrine. Other neuropeptides are coexpressed with norepinephrine in nerve fibers innervating the testis [reviewed in ref. *8*]. Considerable evidence demonstrates the importance of catecholaminergic control of Leydig cell function and development, notably, pharmacological denervation by intratesticular injection of 6-hydroxydopamine inhibited LH-responsiveness and testosterone production in the hamster *(64)*. Surgical denervation of the rat testes induced a decline in gonadotropin responsiveness and resulted in a decrease in LH receptor numbers on the surface of Leydig cells *(243)*. However, high concentrations of catecholamines are correlated with decreased androgen production *(64)*. These findings suggest that local intratesticular actions of catecholamines acting in concert with other central and peripheral mechanisms maybe involved with suppression of testicular functions during times of acute stress and activation of the sympathetic nervous system *(8)*.

Under normal physiological conditions, pituitary gonadotropins, direct neural innervation, local neuroactive peptides and catecholamines act in concert to control testicular function, especially androgen biosynthesis in testicular Leydig cells. During stress and inflammation, perturbation of pituitary gonadotropin secretion, activation or suppression of direct neural connections, production of local inflammatory mediators, and inhibition or activation of local neuroactive peptide pathways act in concert to suppress testicular androgen production. The highly complex integration of the neural-immune-endocrine signals that control testis function awaits elucidation.

Conclusions and Prospects for Future Studies

Our understanding of the neural circuitry that controls testicular function is in its infancy. The proposed direct neuronal control of Leydig cell steroidogenesis needs to be elucidated before we can fully understand the integration of neural-immune-endocrine regulatory mechanisms that control the testis. Classic neurological approaches that identify nerve fibers involved in control of Leydig cells coupled with molecular biological techniques are being applied to the problem. The use of gene knockout and transgenic approaches will be invaluable when candidate regulatory genes have been identified.

The role of the testicular microcirculation in the control of steroidogenesis and spermatogenesis needs further evaluation. The recent demonstration that Leydig cells secrete angiogenic factors such as vascular endothelial growth factor (VEGF) is a compelling observation *(244,245)*. Leydig cell stimulation of endothelial cell proliferation suggests that the testicular microvasculature is constantly being remodeled. Classic studies by Desjardins et al. and others have emphasized the importance of the testicular microcirculation in the local control of cells in the interstitial and tubular compartments of the testis *(246,247)*. It will be important for our understanding of the integrative process that controls testis function to elucidate the role of vasoactive factors such as endothelin, nitric oxide, atrial natriuretic peptide, arginine vasopressin, oxytocin, and prostaglandins, all of which have been implicated as potential regulators of testicular function.

Much has been learned about paracrine and cell–cell interactions in the testis, yet our understanding of the integration of these processes into the control of testicular function is inadequate. Epithelial cell biology approaches, such as those recently described by Jegou and colleagues *(105)* who examined the vectoral secretion of IL-1 and IL-6 from Sertoli cells in culture, will offer important insights into control and integration of testicular processes.

In the era of the genome project, much emphasis has been placed on the use of homologous recombination, transgenic animal studies, gene-knockout and antisense knock out approaches. Clearly, these experimental approaches, which are in active use in the study of testicular function, are essential tools. It is important however, to continue to conduct more traditional, even descriptive studies, which are absolutely required to define all of the players involved in the control and integration of neuroimmune-endocrine regulation of testicular function.

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