The Immunobiology of the Rat Testicular Macrophage

by

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ABSTRACT

Macrophages comprise up to 25% of the interstitial cell population in the testes of mammals including man, monkey, rat and boar, but the significance of this cell population for the apparent immunologically-privileged status of this organ, or for normal testicular function is yet to be established. Macrophages perform a variety of immunological functions, and are present in most organs. However their phenotype usually varies depending on the local environment, presumeably due to the different physiological functions they may perform at any given site.

The immuno-biology of the testicular macrophage is important in this respect because it is likely to contribute to inter-cellular communication in the testis, and the physiological functions it performs may form the basis of immune privilege in the testis. In the studies presented in this thesis the immunobiology of the testicular macrophage has been characterised in an attempt to elucidate its role in normal testicular function, and its role in the apparent immune privilege afforded the testis.

Establishing a novel and effective method of testicular macrophage isolation enabled studies to be undertaken with highly purified cultures, with minimal contamination by the testosterone producing Leydig cells, as verified by screening the cultures for specific macrophage and Leydig cell markers. Characterisation of the testicular macrophage population by macrophage specific monoclonal antibodies and Fc receptor expression showed the cells to be avidly phagocytic and to express surface molecules common to cells of the macrophage lineage as opposed to those of the dendritic cell.

Having characterised the testicular macrophage at the simplest level, a series of experiments designed to investigate the cells immuno-competence were undertaken. The ability of testicular and peritoneal macrophage populations to initiate and promote the proliferation of T-lymphocytes were compared. By culturing the cells with or without the non-specific macrophage stimulant lipopolysaccharide (LPS), the powerful macrophage

activator interferon gamma (IFNγ), and/or the cyclooxygenase inhibitor, indomethacin, the cells themselves, or their secretions as conditioned medium, were cultured with mitogenically (Concanavalin A, ConA) stimulated lymphocytes.

In contrast to the peritoneal macrophages, testicular macrophages consistently inhibited ConA-induced lymphocyte proliferation, even after stimulation with LPS. This inhibitory effect on lympho-proliferation was overcome when cultures of testicular macrophages were simultaneously primed and triggered with IFNγ and LPS. While fractionation of macrophage conditioned medium by microconcentration revealed the presence of several anti-proliferative factors, further investigations revealed that the main inhibition of lympho-proliferation was of low molecular weight and could be blocked by indomethacin, suggesting that they were prostaglandins. Samples of testicular macrophage conditioned medium assayed for prostaglandins E_2 and $F_{2\alpha}$, showed high basal levels compared to medium from the peritoneal populations. Interestingly, IFNγ significantly reduced testicular macrophage $PGF_{2\alpha}$ production without affecting levels of PGE_2 suggesting a differential control in the production of these two prostaglandins.

Macrophages are a potent source of cytokines, factors traditionally viewed as being fundamental for the initiation of and regulation of lymphocytes during an immune response. However, it is now accepted that macrophage derived cytokines such as Interleukin 1 (IL-1), Interleukin 6 (IL-6), Tumor Necrosis Factor α (TNFα) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) can act as paracrine regulators of local tissue function and this role is well documented for the female reproductive tract. Using specific cytokine bioassays and reverse transcriptase-PCR the conditioned medium and cellular mRNA of both testicular and peritoneal macrophage populations were screened for the presence of these cytokines.

Testicular macrophages were found to be a poor source of IL-1, IL-6 and TNF even after LPS stimulation. These results are consistent with observations in the lymphocyte

proliferation experiments and suggest that the refractoriness to LPS of the testicular macrophage reduces the ability of this cell to upregulate cytokine production and hence support lymphocyte proliferation. Furthermore, where the macrophages were primed and triggered with IFNγ and LPS, production of these immuno-stimulatory cytokines was seen to be significantly increased. This again supported the observation of increased lymphocyte proliferation seen under these conditions in the earlier experiments.

The testicular macrophage constituitively produced high basal levels of GM-CSF, yet its production was down regulated by LPS stimulation. The physiological significance of GM-CSF secretion by testicular macrophages remains to be fully investigated but constituitive production of GM-CSF to be associated with macrophages of a suppressor phenotype. GM-CSF has also been implicated as a paracrine regulator of tissue remodelling in the female reproductive tract and it may perform a similar role in the testes.

In order to test whether the secretory profile of the testicular macrophage could influence Leydig cell testosterone production, experiments were set up to measure the concentrations of testosterone and cytokines in conditioned medium after Leydig cell-macrophage co-culture. While the presence of testicular macrophages significantly reduced basal testosterone production in co-cultures, it was also found that the addition of indomethacin to these cultures significantly decreased testosterone secretion. Testicular and peritoneal macrophages inhibited hCG stimulated testosterone production by the Leydig cell except in instances where IFNγ and LPS were also added. In these instances co-cultures of Leydig cells with testicular macrophages had significantly higher testosterone concentrations than cultures of Leydig cells alone or co-culture of Leydig cells with peritoneal macrophages. Similarly, in co-cultures without hCG, testicular macrophages immuno-activated with IFNγ and LPSincreased testosterone secretion by Leydig cells. It is possible to speculate that the increased testosterone synthesis may be due to the change in the ratio of PGE_2 to $PGF_{2\alpha}$ production by the testicular macrophage after stimulation with IFNγ and LPS.

While IL-1 and TNF are unlikely to be involved in Leydig cell steroidogenesis, basal production of IL-6 by the Leydig cell and GM-CSF by the testicular macrophage was altered after hCG stimulation. The concentration of IL-6 in Leydig cell cultures was doubled after hCG stimulation whereas GM-CSF production by the testicular macrophages in Leydig cell co-culture was significantly downregulated. Testicular macrophages in isolation did not downregulate GM-CSF production in response to hCG, testosterone, or Leydig cell conditoned medium suggesting the effect observed in the co-cultures require cell-cell contact.

The findings reported in this thesis suggest that the testicular macrophage exhibits characteristics similar to that of a suppressor macrophage phenotype. The inhibition of lymphocyte proliferation by the testicular macrophage, its unique cytokine profile, high basal production of GM-CSF and prostaglandins, and the refractoriness to LPS all suggests a role that contributes to the immune privilege afforded the testis. However, these aspects of testicular macrophage immuno-biology also support a role for these cells in local cell-cell communication and regulation of the normal physiology of the testis, and macrophages may be directly involved in Leydig cell steroidogeneis. These studies have contributed significantly to our understanding of the immuno-biology of the testicular macrophage and support and extend previous suggestions that these cells might play a role in the physiological regulation of the testis as well as the immunology of the testicular environment.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by anohter person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Stephan Kern

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Finally, I would like to thank the Department of Animal Sciences, both staff and students for providing an excellent environment in which to work, and making my time here an era to remember.

PREFACE

Aspects of the work presented in this thesis have been reported elsewhere:

Abstracts:

Kern S and Maddocks S (1991) Regulation of lymphocyte proliferation in vitro by rat testicular macrophages. Proc. Aust. Soc. Reprod. Biol. 23:67

Kern S, Robertson SA and Maddocks S (1992) Cytokine secretion by macrophages isolated from the rat testis. Proc. Aust. Soc. Reprod. Biol. 24:87

Kern S and Maddocks S (1992) Production of prostaglandin E_2 and $F_{2\alpha}$ by macrophages isolated from rat testis. 8th European Workshop on Molecular and Cellular Endocrinology of the testis. Abs 129

Kern S and Maddocks S (1993) Indomethacin blocks the immunosuppressive activity of rat testicular macrophages in vitro. Proc. Aust. Soc. Reprod. Biol. 25:6

Kern S and Maddocks S (1994) Nitric oxide secretion by macrophages and Leydig cells isolated from the rat testis. Proc. Aust. Soc. Reprod. Biol. 26:67

Kern S, Robertson SA and Maddocks S (1995) Cytokine Secretion by the rat testicular macrophage. 13th Testis Workshop: Cellular and Molecular Regulation of testicular Cells. Abs I-36

Kern S, Robertson SA and Maddocks S (1995) Regulation of testicular macrophage-derived GM-CSF by the Leydig cell. Proc. Aust. Soc. Reprod. Biol. 27:103

Papers:

Kern S and Maddocks S (1995) Indomethacin blocks the immunosuppressive activity of rat testicular macrophages cultured in vitro. J. Reprod. Immunol. 28:189-201

Kern S, Robertson SA, Mau VJ and Maddocks S (1995) Cytokine secretion by macrophages in the rat testis. Biol. Reprod. 53:1407-1416

ABBREVIATIONS

Con A	concanavalin A
LPS	lipopolysaccharide
RT-PCR	reverse transcriptase- polymerase chain reaction
hCG	human Chorionic Gonadotropin
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
IL-1	interleukin-1
IL-6	interleukin-6
INFγ	gamma interferon
TNF	tumour necrosis factor
TGFß	transforming growth factor-ß
GM-CSF	granulocyte macrophage-colony stimulating factor

CHAPTER 1: Literature Review.

In accordance with departmental policy regarding the review of the literature, this chapter only contains material published up to and including December 1992.

1.1 Introduction

The mammalian testis has two main functions, the first being the production of spermatozoa and the second being the production of the male sex hormones, the androgens. Both these functions occur within anatomically separate compartments in the testis, yet are very much inter-related. Steroid hormone production in the testis is essential for the continued production of spermatozoa.

The seminiferous tubules and the testicular interstitium form the two distinct compartments of the testis. They are seperated by the Blood-Testis barrier, a partition formed by the tight junctions in the walls of the seminiferous tubules (Fawcett, 1975), which is impervious to cells and high molecular weight substances (Setchell and Waites, 1975). Between the seminiferous tubules lies the interstitial tissue through which the blood and lymph vessels pass (Fawcett et al, 1973), and in which the testicular macrophages are found in close physical association with the testosterone producing Leydig cells (Fawcett et al, 1973). This space is also important in that it is a site of immune privilege and antigen present only at this site will often go undetected by the immune system (Fergusson and Scothorne, 1977).

Macrophages are cells well known for their central role the regulation of the immune response, a task they accomplish by producing a vast array of secretory products (Takemura and Werb, 1984). They also act as regulators of tissue specific functions, by utilising these same factors for non-immune purposes (Rappolee and Werb,1992).

Macrophages are present in most tissues (Gordon et al, 1986) and are found extensively in the gonads of both the male and female (Fawcett et al, 1973; Adashi, 1990). Studies of the macrophage populations resident in the female reproductive tract (Hunt et al, 1985; Adashi, 1990; Hunt and Pollard, 1992) have suggested diverse roles in the areas of cell differentiation and growth, tissue remodelling and steroid production. In comparison, very little is known of the testicular macrophages. There is considerable interest as to the

role the macrophage population might assume in regulating testicular function and how these roles may relate to the immune privilege status afforded the testis.

Elucidating the role of the macrophage in testicular function is of importance in understanding the relationship between cells and factors traditionally associated with immunity and their wider roles in the control of other systems such as reproductive function. The purpose of this thesis is to explore the capabilities of the testicular macrophage with particular respect to the immuno-biology of the cell and how this may relate to testicular function both immunologically and physiologically.

1.2 The Testis

1.2.1 Structure and Function

There is little variation in gross anatomy of the testis between the majority of mammalian species. All testes consist of a series of elongated convoluted tubules, the seminiferous tubules, in which the Sertoli cells and the developing sperm reside (Setchell, 1978). The Sertoli cells rest upon the basement membrane of the tubule, which usually consists of several layers, an innermost layer of non-cellular material, a smooth layer of myoid cells, a layer of collagen fibres and an outermost layer of epithelial cells which line the lymphatic sinusoids (reviewed by Setchell, 1978).

At puberty, the Sertoli cells and myoid cells form tight junctions in the walls of the seminiferous epithelium, thereby creating a highly selective barrier, the blood-testis barrier, which separates the contents of the tubules and the cells of the testicular interstitum (reviewed by Setchell and Brooks, 1988). The barrier effectively prevents the transfer of cells and larger hydrophilic substances such as protein across the seminiferous tubules and helps to maintain the unique environment in which the developing germ cells mature within the tubules (Setchell and Waites, 1975; Setchell and Brooks, 1988).

The presence of the Blood-Testis barrier is vital for the production and growth of the sperm. From an immunological perspective the seminiferous tubules are avascular and contact with blood-borne antigen is greatly limited in this region. Consequently the dangers of a disease or viral attack upon the developing germ cells is minimized (Setchell and Waites, 1975; Setchell and Brooks, 1988).

Another aspect of the barrier's function may be to shield the germ cells from the immune system. Germ cells are haploid and most develop post-pubertally in the testis. Because of this developing germ cells are not recognised as 'self' by the body's immune cells (Setchell et al, 1990) and if exposed to the immune system may ellicit an immune response. In order to prevent the initiation of an immune response against these cells, the germ cells must be physically separated from the body's immune system (Setchell et al, 1990). The disruption of the blood-testis barrier after puberty by diseases such as mumps orchitis or microbial infection (Bartak et al,1968; Morgan, 1976; Wicher and Wicher, 1990), has been implicated in the production of anti-sperm antibodies and the subsequent sterility.

Thus it is very important to protect the sperm from both the risk of infection and the immune system to maintain a viable and fertile status. As a result the sperm develop within the tubules in an enviroment free of blood and lymphatics which form the normal channels through which the immune system extends its afferent and efferent arms.

1.2.2 The Interstitial Tissue

The interstitial tissue is formed by the stacking of the seminiferous tubules in such a way that a three sided space usually occurs (Fig. 1.1). The blood and lymph vessels, nerves, fibroblasts, Leydig cells and macrophages fill this space (Fawcett et al, 1973). While the proportions in which these cells occur vary from species to species, the design of the interstitial tissue remains essentially the same. The Leydig cells and macrophages cluster

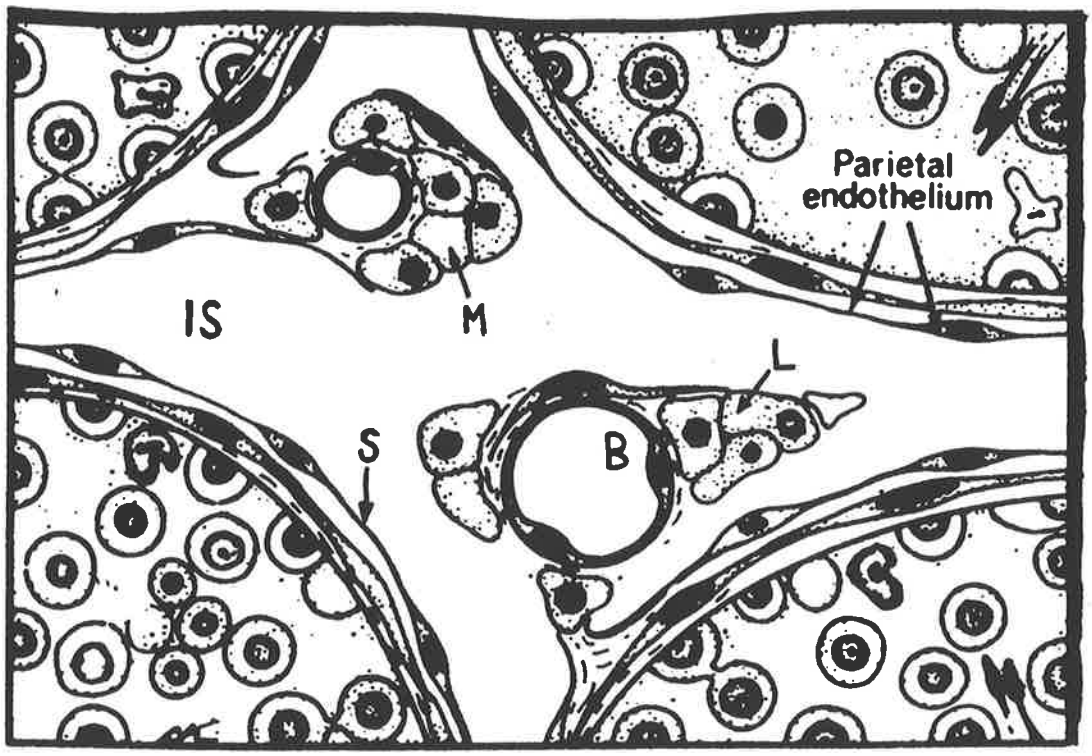

Figure 1.1

The interstitial space in the rat testis. The interstitial space is bounded by the seminiferous epithelium (S). Between the tubules, in the lymphatic sinusoids of the interstitial space (IS) lay the blood vessels (B), macrophages (M) and Leydig cells (L).

around the blood and lymph vessels amidst variable amounts of connective tissue (Fawcett et al, 1973)

The Leydig cell is the predominant source of testosterone in the mammalian testis as well as a number of other steroids. In addition to these androgenic products, the Leydig cell also produces other bioactive substances such as oxytocin, vassopressin, neurophysin, renin and ß-endorphin (reviewed by Maddocks and Setchell, 1988).

In addition to the Leydig cells there are appreciable numbers of macrophages in the testicular interstitium of the rat (Fawcett et al, 1973; Christensen, 1975; Miller et al, 1983; Neimi et al, 1986). A close developmental correlation between the Leydig cell and testicular macrophage has been noted (Bergh, 1985; Bergh, 1987; Hutson 1990). These studies have described the differentiation and growth of macrophages in the pre-pubertal rat testis, and changes in macrophage and Leydig cell size, mass and number, in response to cryptorchidism and hCG in the adult. These two cell types also have a close physical relationship in which the macrophage appears to both endocytose Leydig cell cytoplasm and interdigitate with the Leydig cell (Miller et al, 1983; Hutson, 1992). The functional significance of this relationship has yet to be established and the existing literature on this subject will be examined more closely in section 1.7.

Another component of the interstitial space is the protein-rich interstitial fluid, which acts as the primary source of nourishment for the avascular seminiferous tubules. All the cells in the interstitial tissue are bathed in this fluid and it appears that this might be the way in which cellular communication via biologically active factors (eg. hormones) is mediated (Setchell and Maddocks, 1988).

1.3 Testicular Immunology

1.3.1 Immune Privilege in the Testis

The term "immune privilege" as applied traditionally to the classic immunologically-privileged sites, the brain and the anterior chamber of the eye, describes the absence or apparent inability of the body to formulate an immune response against antigens presented at these sites. The testis has also long been recognised as possessing an immune-privileged status and has a long history as a site for transplantation (Maddocks and Setchell, 1990).

The first recorded experiments using the testis as a site for transplantation were by Sand (1919). The results of this work showed that in most cases grafts of foreign tissue placed into the testis were not rejected. Subsequent work (Green 1940; Medawar and Russell 1958) confirmed that foreign tissue grafts placed in the testis enjoyed a prolonged and sometimes indefinite survival.

This however, is not to say that all intratesticular grafts escape rejection and that the testis has total immune privilege. Fergusson and Scothorne (1977) showed that a single intra-testicular graft of skin or parathyroid tissue, rarely induced systemic immunity in the rat testis. However, where the recipents had been pre-sensitised to the donor tissue by previous skin grafts, the subsequent intra-testicular grafts were rejected (Fig. 1.2). From this it appears that while it is possible to induce a pre-sensitised immune response in the testes, the immune system will not usually be activated against antigens present only at this site. As a result the testes should be considered as being sites of incomplete immune privilege.

Most immune privilege sites posess three specific features wich afford the organ immune privilege status. The first involves an abnormal or deficient lymphatic drainage pathway, the second is the presence of a blood-organ barrier and the third is the creation of a local

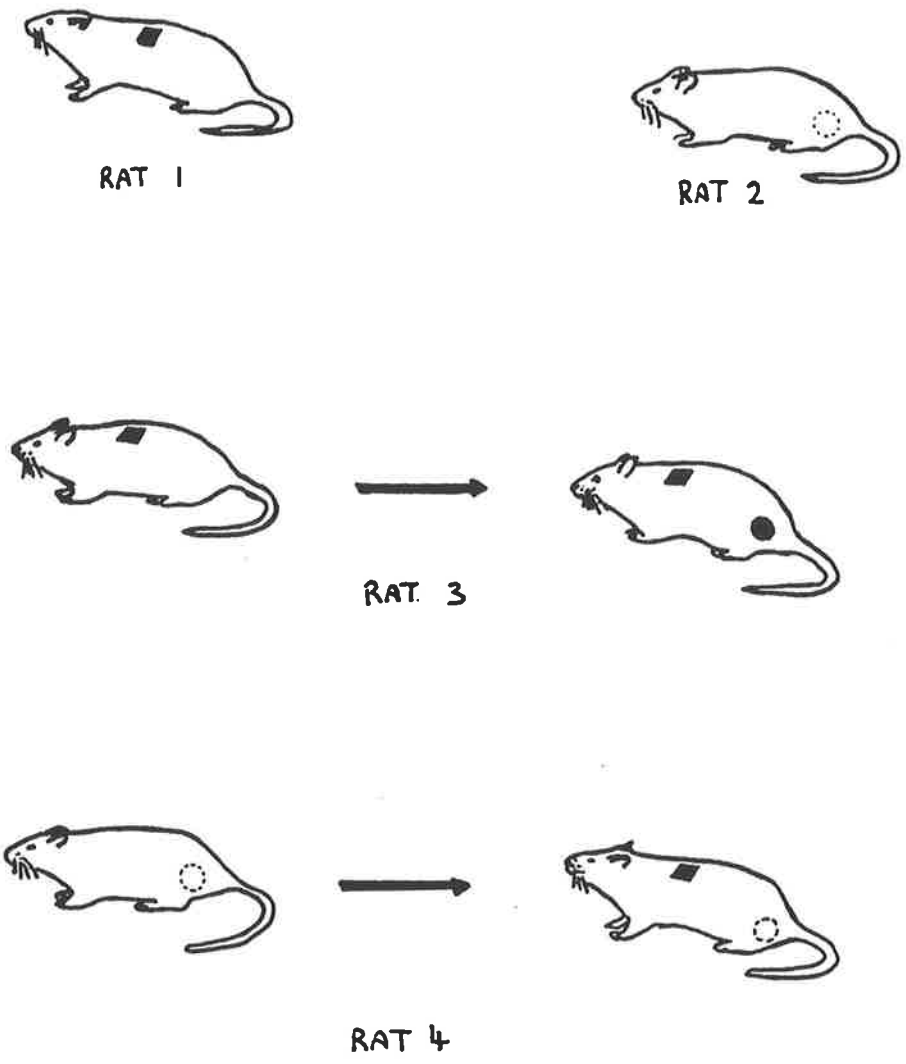

Figure 1.2

The foreign skin graft recieved by Rat 1 is rejected (dark patch), but in Rat 2, the singular intra-testicular graft does not initiate an immune response (hatched circle). However after Rat 3 is immunologically pre-sensitised by a foreign skin graft (rejected, dark patch), the presence of an antigenically-comparable graft to the testicular interstitium now invokes an immune response and the graft is rapidly rejected (dark circle). In Rat 4, a skin graft is rejected (dark patch) when grafted after an initial intra-testicular graft (which survives, hatched circle) has been made. The slow rate of rejection of this skin graft indicates that the intra-testicular graft has not induced pre-sensitisation of the host (adapted from Fergusson and Scothorne, 1977).

immunosuppressive environment that somehow minimises antigen presentation and accessory cell function.

In view of this the testes appear to be somewhat unique in their provision of immune privilege. While the testes share certain characteristics with other immune privileged sites such as the brain and eye, there are important differences. The presence of a blood-organ barrier in other organs may contribute to the apparent shielding of the organs cells from antigenic contact with the body, but the testicular barrier only protects cells within the tubules (Setchell and Waites, 1975). The interstitial tissue however is the site for most intratesticular transplants and lays unprotected outside the blood-testis barrier. Therefore in the testes it is unlikely that the presence of any immune privilege status can be related directly to the presence of a blood testis barrier.

An additional and equally important difference between the classic immune privileged sites and the testes is that of lymphatic drainage. Initial theories for the immune privilege of the testis centred around the concept of poor lymphatic drainage (Medawar and Russell 1958); a situation which was thought to apply in the classic sites. This theory was disproved in the testis by Fawcett, Heidger & Leak (1969) and Fawcett, Neaves, & Flores (1973) who described high lymphatic drainage through the testicular interstitial tissue and additional studies by Tilney (1971), McCullough (1975) and Head, Neaves & Billingham (1983) have confirmed that the testes possess very efficient lymphatic drainage. So efficient is this drainage that it might in fact be expected to assist in the activation of the immune system within the testis. Furthermore, the migration of T-cells and other leukocytes through the testicular interstitum (El-Demiry et al, 1987; Pollanen and Niemi, 1987; Pollanen and Maddocks, 1988) should enhance the immune response at this site.

Given the presence of effective lymphatic drainage, and the known ability of the testis to produce an immune response in some circumstances it seems most likely that some sort

of localised immune suppression is present in the testis. It has been shown (Pollanen et al 1988; Pollanen, 1989) that testicular immunosuppressive activity in interstitial fluid exists in rats after puberty most likely as the result of the production of immunosuppressive factors by the seminiferous tubules.

These peptides, thought to be produced to protect the developing germ cells from immune surveillance, may pass through the tubule wall and into the interstitial fluid as is the case for a number of other seminiferous tubule products (Sharpe,1988). Although the presence of these peptides in the interstitial tissue may facilitate immune privilege, there are some inconsistencies in the observed immune reactions which suggest that these factors can not entirely account for the privileged state of the testis.

The presence of immunosuppressive factors in the interstitium should affect all local immune responses, but this is not the case. That an immune response can be initiated at this site if the host has been pre-sensitised to the antigen suggests that the primary immune reaction in the testis may normally be suspended somewhere in the early phase of the afferent response, ie. just prior to lymphocyte activation. Hence, this suggests that the refractoriness of the immune system as seen in primary reactions in the testis, may involve some other mechanism.

The resident cell types of the interstitial tissue are of interest in this regard. The Leydig cells are the steroid secreting cells of the testis and have been suspected as possible sources of the suppression as steroids are known to have strong immuno-suppressive effects. However, it is unlikely that the Leydig cells produce high enough quantities of steriods (Maddocks and Setchell, 1988a; Selawry and Whittington 1988) to create suppressive conditions in the local environment.

The macrophage plays a central role in mammalian immunity and is a highly active in the response ellicited during graft rejection. A number of mammalian species possess a

resident population of testicular macrophages. eg. rat, guinea pig, man (Fawcett et al 1973). However recent work (Pollanen and Maddocks, 1988) has shown that a notable exception to this list is the ram. The most exciting aspect of this discovery is that while the other species that have been studied show some degree of immune privilege status in the testis, the ram, which lacks a resident testicular macrophage population does not grant immune privilege to intra-testicular grafts (Maddocks and Setchell, 1988c).

The fact that the presence of the macrophage, an immuno-active antigen presenting cell, seems to correlate with the immune suppression in the testis is particularly intriguing and it is the reverse of what one would perhaps expect. However, several instances of non-specific immunoregulation by macrophages and their products have been cited by Nelson (1976). It has been shown that where target cells are killed solely or primarily by cytolytic effector T cells (as in the case of many tissue allografts) the presence of excess macrophages and/or activated macrophages can inhibit cytolysis (Kiessling et al 1974; Vasudevan et al,1974), through the release of macrophage-derived suppressive factors.

Thus it may be possible that the presence of the resident macrophages in the testis is somehow involved in the creation of the immune privilege status of the rat testis either directly through the production of biologically-active immuno-modulatory factors or indirectly by affecting or altering antigen processing or presentation.

1.3.2 Sources of Immune Suppression in the Testis

As discussed earlier, the testis appears to be somewhat unusual in that its immune privilege status is incomplete. That the afferent arm of the immune system is selectively blocked and only activated when the host has been pre-sensitised to the foreign tissue suggests that a number of factors in the testis must be acting in the organ to prevent the primary immune response. The presence of potent immuno-suppressive factors in the testicular interstitium have already been discussed but there also exists within the testis a

number of cells and molecules which must be considered for their immuno-suppressive qualities.

The immunology of the Leydig cell is still largely unexplored, yet the presence of some immunologic properties and its close relationship with the macrophage, have implicated it as a possible source of local immune suppression. Leydig cells have the capacity to bind lymphocytes spontaneously and non-specifically supress lymphocyte proliferation in vitro (Born and Wekerle, 1981; Rivenson et al, 1981) which may suggest that Leydig cells have some immune function related to the macrophage.

Alternately the high local levels of testosterone have been suggested as the cause of immune suppression, as androgens have been widely observed as having immunosuppressive properties when in high enough concentration (Roubinian et al, 1977; Clemens et al, 1979). However as previously mentioned, it is unlikely that the concentrations of androgen reach high enough levels to create a suppressive environment on their own. The Leydig cells also have the ability to synthesise prostaglandins (Ellis et al, 1975; Haour et al,1979; Molcho et al, 1984) which are known to have immunosuppressive properties. Although it is not known if these substances do create immune suppression in the testis, it is important to realise that prostaglandins can suppress macrophage accessory cell function and this may be a primary mechanism for immune supression at this site.

It is also important to note that the interstitial fluid is probably a conduit for all cellular secretions in the testis. The composition of this fluid is very important as it comes into contact with all the interstitial cells, and the foreign tissue grafts (Maddocks and Setchell, 1988b). Moreover, the hormones and immunosuppressive factors present in it will have a direct effect on target cells. It is therefore of interest to determine the contribution of these factors to the immune suppression observed in the interstitial space.

The origins of the immunosupressive factors are still under investigation, but at least one group, protectins, are thought to be secreted by the Sertoli cells inside the blood-testis barrier, where they act to supress the risk of an immune response (Pollanen, 1990). Sertoli cells have been shown to suppress lymphocyte proliferation in vitro (Pollanen et al, 1990) and are also known to secrete the cytokine interleukin (IL)-1α (Gerad et al, 1991) and express mRNA for transforming growth factor (TGF) ß (Skinner and Moses, 1989), as well as a range of other large molecular weight factors. In contrast to IL-1 which initiates lymphocyte activation, TGFß a cytokine which has been shown to inhibit lymphocyte proliferation (Kherl et al, 1985) and is known to have a suppressive action on the immune response (reviewed by Bock and Marsh, 1991) has also been suggested as a factor produced by the Sertoli cells.

The presence of high local hormone levels, or of factors originating from the seminiferous tubules being the sole cause of this situation seems to be annulled by the comparison of the rat and ram testis. As shown by Pollanen and Maddocks (1988), and Maddocks and Setchell in (1988c), the ram has no testicular macrophage population and surprisingly shows no immune privilege status. It therefore seems likely that the testicular macrophage is involved in the process of establishing immune privilege either by itself, or more likely through its associations with the Leydig cell or with the local suppressive factors.

In organs such as the testis, a model for the provision of immune privilege might be constructed from a composite of interactions of the local environment. In this instance the immune privilege is likely to be the result of a unique local microenvironment which contains locally produced factors that can suppress and regulate accesory cell function. It therefore seems likely that the testicular macrophage is involved in the process of establishing immune privilege either by itself, or more likely through its associations with the Leydig cell or with the local supressive factors.

1.4 Immunology

1.4.1 The Immune System

Within vertebrates a system of immunity has evolved known as the lymphoreticular system. This term encompasses the lymphatic and vascular channels of the body as well as the tissues, organs, and cells. Within this system are the lymphocytes and macrophages, the cells which form the immune system and immune response.

In the absence of antigen, lymphocytes are small, resting cells, essentially inactive and in a low secretory phase. Lymphocyte activation is dependant on two critical and distinct events, both requiring antigen recognition. The first involves the trapping of antigen by acessory cells such as dendritic cells and macrophages, and the second is the presentation of antigen which in turn stimulates proliferation and differentiation of B and T cells (Unanue and Allen, 1987)(Fig. 1.3.).

The B lymphocytes which constitute the humoral immune response produce circulating antibodies primarily to combat viruses, bacteria and the toxins they produce (Jeliner and Lipsky, 1987). The T-lymphocytes form the other arm of the immune system; cell-mediated immunity. Their role in the immune system is far more diverse, ranging from the destruction of infected cells to the regulation of the immune response. T-lymphocytes are divided functionally into cytotoxic, suppressor and helper subsets, each with a distinct role in providing cell mediated immunity (Paul, 1989). The T cells are also the principal agents in the rejection of tissue transplants.

Alien cells (grafted or infected) display a unique protein arrangement (antigen) on their membrane surface which is identified as foreign or "non-self" by the host body's immune system. When foreign antigen is introduced to the host, lymphocytes specifically targetted to that antigen are stimulated to proliferate and differentiate in order to remove the source of antigen (Roit, 1988).

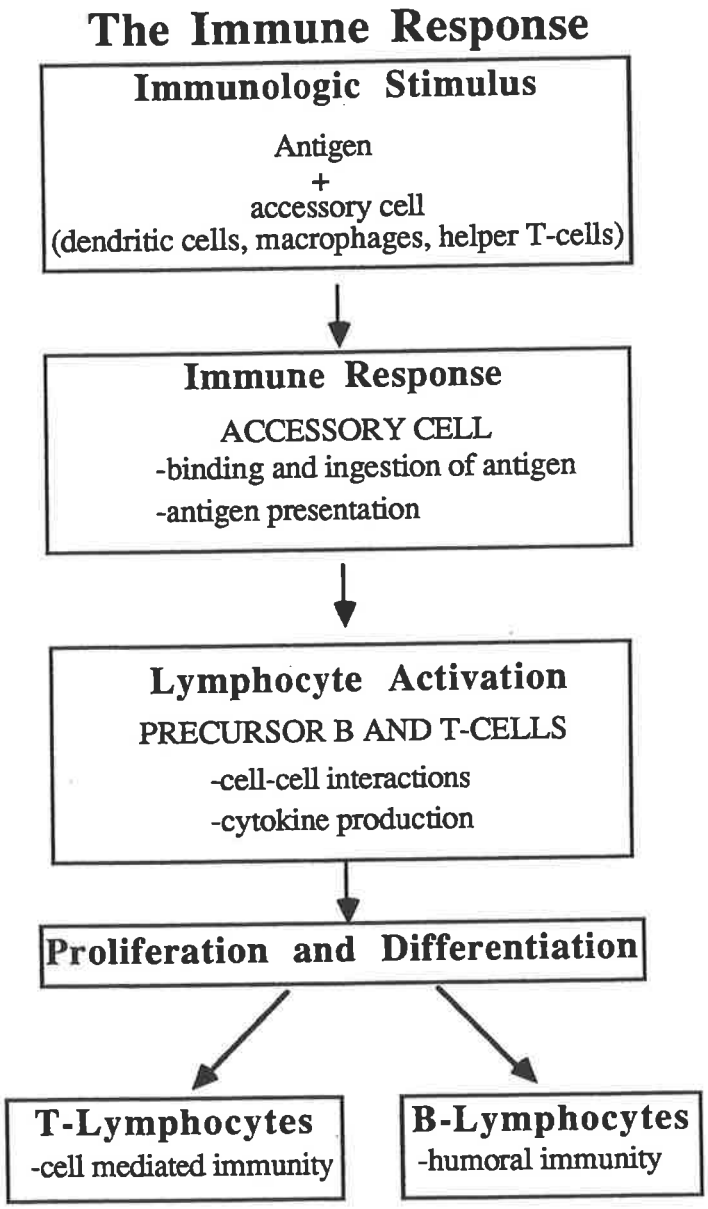

Figure 1.3 The Immune Response

A schematic diagram of the steps involved in the generation of an immune response (adapted from Roitt, 1988).

The mechanisms by which T-cells co-operate to produce a cell mediated immune response are related to the genes responsible for the expression of surface antigen on the cell. The cell surface antigens are known as histocompatibility antigens and the major group of genes that determines the expression of these antigens is the major histocompatibility complex (MHC)(Shearer and Schmitt-Verhurst, 1977).

Major histocompatibility complex class I antigens are expressed by most nucleated cells and represent a provision for the recognition of self, thereby enabling identification of foreign cells and tissues. MHC class II antigens, act as receptors for the presentation of antigen to helper T cells and are mainly associated with the surface of dendritic cells and macrophages (Brodsky and Guagliardi, 1991). The role of these cells, termed antigen presenting or accessory cells, is to present antigen to helper T-cells and activate them immunologically.

Before the discovery of lymphoid dendritic cells it was thought that the macrophage was the primary component involved in initiating T-lymphocyte activation. However while these cells display similar capabilities as accessory cells, recent observations suggest lymphoid dendritic cells are more potent than the unstimulated macrophages in activating resting lymphocytes during the initial phases of the immune response (Inaba and Steinman, 1984; Steinman, 1991). Although macrophages and dendritic cells inhabit many of the same tissues the expression of Fc receptors, non-specific esterase activity and cell linage markers such as F4/80 are exclusive to macrophages and may be used to distinguish them from dendritic cells (Buckley et al, 1987; Mettay et al, 1991).

The activated helper T-cells subsequently stimulate quiescent macrophages to activate and alter their phenotype. Expression of MHC II is rapidly upregulated thereby increasing the cells antigen presenting capacity, as is the expression of Fc receptor which is necessary for immuno-phagocytosis. The activated macrophage also displays cytocidal and anti-microbial activities not seen in the quiescent cells.

The activated macrophage typically secretes factors such as the cytokines interleukin (IL)-1ß, IL-6 and tumor necrosis factor (TNF) α in order to stimulate rapid proliferation of lymphocytes (Dinarello, 1989). It also secretes a wide array of other factors such as the cytotoxic oxygen intermediates and arachadonic acid metabolites all of which can modulate positively and negatively the proliferation of T and B cells depending on the state of the immune response. These macrophage factors in particular are also important for down-regulating the immune response after the antigen has been removed or destroyed.

1.4.2 The Macrophage

The macrophage is a large ubiquitous mononuclear cell type, first characterised by Metchnikoff in 1882 for its ability to phagocytise particulate material (cited in Karnovsky, 1981). A similar function was noted by Kupffer (1894) (cited in Vernon-Roberts, 1972) for some cells in the liver and so it was assumed that the role of the macrophage was primarily to scavenge cellular debri. This view was held for a considerable period until it was realised that the macrophage was a ubiquitous cell type that existed in most tissues.

Macrophages are members of the mononuclear phagocyte system, defined as a specific group of cells which are derived from the stem cells of the bone marrow. The bone marrow precursors are induced by a variety of colony-stimulating factors to differentiate from a myelotic precursor into monoblasts, promonocytes, monocytes in the bone marrow, blood bourne monocytes and finally macrophages (van Furth and Cohn, 1968; Johnston 1988). Blood bourne monocytes in the circulation either remain mobile as free monocytes or migrate into tissue to become resident macrophages and take on specific functions eg. Kupffer cells in the liver (Nathan et al 1980; Johnston 1988). However because of the large numbers of roles macrophages provide around the body the morphology and function of the cells not surprisingly varies according to location and state of differentiation (Vernon-Roberts 1972).

In free form, macrophages occur scattered diffusely throughout the mammalian body, existing outside the major lymphatic organs. They are found in connective tissues, serosal sacs, inflammatory exudates and in small amounts in blood. In general, free macrophages are considered homogeneous in morphology and function, a good example of this being the peritoneal macrophage.

Fixed macrophages occur in many of the major organs (Vernon-Roberts 1972) and are those which become tissue bound upon maturation at sites such as the liver, lungs, spleen and kidney. The developing macrophages are recruited to the organ via delivery as peripheral blood monocytes and subsequently remain there for life (Gordon et al,1986; Fawcett, 1986). As the specific tasks performed by fixed macrophages at other sites have been well documented, eg. the endocytosis of toxins in the liver and the filtration of blood in the spleen and kidney (Vernon-Roberts, 1972; Schreiner et al, 1981), there is now increasing interest in those found resident in other organs such as the testis.

1.4.3 Immunobiology of the macrophage

In forming the first line of defence the macrophage must display four important attributes, the basis of which forms a criteria for immuno-competance (Fig. 1.4). The presence of these attributes is critical in determining the macrophages ability to initiate and regulate an immune response. Firstly the macrophage must display phagocytic activity in order to operate as the frontline cell of the immune system. In this capacity the macrophage acts primarily as a scavenger cell by removing dead cells as well as endogenous and exogenous debri from both lymphatic and vascular circulation. While this function is mainly non-specific, the capacity to recognise and initiate immuno-phagocytosis via the Fc receptor and components of the complement receptor, C3 is vital in dealing with invading micro-organisms. Macrophages possess no less than three types of Fc receptors for the IgG immunoglobin (Unkeless et al 1977; Diamond and Yelton 1981) and at least one for IgE (Anderson and Spielberg 1981), as well as C3 (complement) receptors (Griffen et al, 1975; Ehlenberger and Nussenzweig 1977).

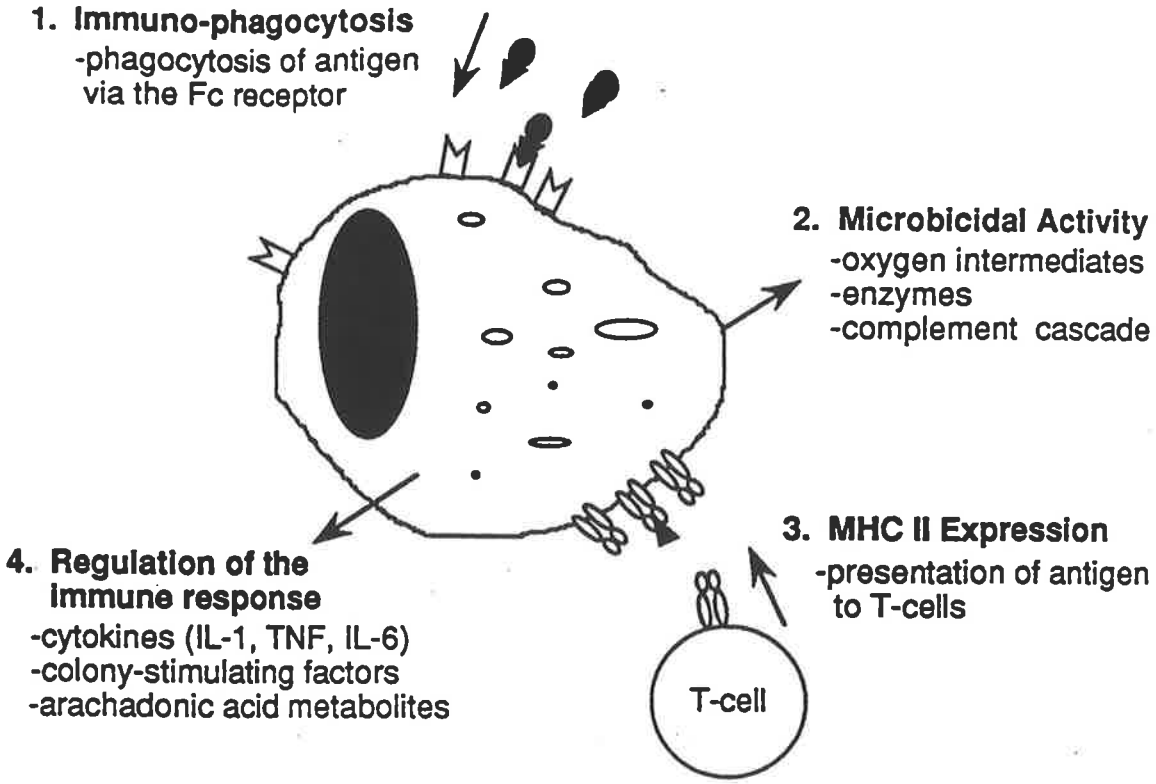

Figure 1.4 Macrophage Immuno-competence.

In order to be regarded as immuno-competent, the macrophage must display four attributes; Fc surface receptors (1), microbicidal activity (2), MHCII immune complexes (3) and the ability to regulate an immune response via secreted factors (4).

Hence it has ample machinery to deal with immuno-mediated phagocytosis, making it particularly effective against invading microorganisms and other parasitic infections.

As the bodies primary defence mechanism against microorganisms the subsequent destruction of these invaders by the macrophage forms the second attribute. Immuno-phagocytosis by the macrophage activates the cell to undergo a respiratory burst facilitating the release of reactive oxygen and nitrogen intermediates such as peroxides and nitric oxide (Murray and Cohn, 1980; Stuehr and Marletta, 1987), enzymes such as plasminogen activator, collagenase and lysosyme which breakdown bacterial cell walls, complexed antigen or other harmful structures (Takemura and Werb, 1984). It also mobilises the macrophages vast secretory arsenal initiating the production of cytokines, leukotrienes and prostaglandins and so creates the inflammatory response.

The third attribute is MHC II expression by the macrophage. These gylcoprotein complexes regulate the immune response genes that control the T-lymphocyte recognition of antigen, allowing processing and presentation of foreign antigen to lymphocytes in order to activate the humoral and cellular arms of the immune system. By these means the macrophage can communicate the nature of the antigen to the cellular arm of the immune system (T lymphocytes) and instigate an immune response (Rosenthal and Shevach 1973; Shevach and Rosenthal 1973; Pierce et al., 1976).

It is essential at this point to note that not all resident macrophages possess a MHC II complexes within the tissue they inhabit. Macrophage populations vary in their expression of the class II gene (Cowing et al, 1978) and often sub-populations of MHC II postive and MHC II negative macrophages are found in the one tissue. It may be that MHC II negative macrophages represent suppressor populations within tissues (Cowing et al, 1978), capable of inhibiting T-cell cytolysis (Kiessling et al 1974; Vasudevan et al,1974). However under normal circumstances T cells exposed to antigen release gamma-interferon (IFNγ) which in turn upregulates MHC II expression and enhances the

immuno-regulatory properties of these macrophages (Wong et al, 1983; Tweardy et al, 1986).

Fourthly, the macrophage must then be able to initiate and regulate the ensuing proliferation of lymphocytes. The macrophage manages this task by synthesising and releasing a number of immuno-active peptides, enzymes and low molecular weight factors such as cytokines, colony stimulating factors, prostagladins and proteases (see Table 1.1) (Takemura and Werb, 1984). These factors control the proliferation of specific T lymphocyte subpopulations as well as the actions of other leukocytes.

The great diversity in macrophage immunocompetance and function seems directly dependant on the site of residence. Macrophages are well known to possess several properties in both specific and non-specific immunity (to be discussed later); but the ability to display these characteristics when resident in an organ seems to be heavily regulated. As already discussed, the presence of MHC II on various macrophage populations is known to vary greatly depending the organ in which they reside. Peritoneal and alveolar macrophages for instance have only approximately 15% of the population expressing MHC II while macrophages resident in the spleen, thymus and liver have between 50-75% expressing the MHC II antigen (Cowing et al, 1978; Humphrey and Grennan, 1981; Lasser 1983).

Despite the low proportion of MHC II positive peritoneal macrophages in the resting state, it is well known that after activation with factors such as lipopolysaccharide LPS and IFNγ, macrophages fron the peritoneum and spleen are able to present antigen and stimulate lymphocyte proliferation in vitro adequately. In contrast, macrophages from the lung (alveolar) and liver (Kuppfer cells) do not induce an immune response and appear unable to coalesce with the lymphocytes in the initiation of the immune response (Unanue, 1984)

In these instances however, the inability to stimulate lymphocyte proliferation may be beneficial. As there are many peripheral blood monocytes in these organs with high vascular flow it could be that the resident macrophages in the tissue are more effective at filtering blood-bourne debri without initiating an immune response which would be deleterious to the function of these vital organs (Vernon-Roberts, 1972; Hume and Gordon, 1983).

The macrophages which do not express MHC II still retain many of the ordinary macrophage characteristics. They are still capable of secreting highly potent and active molecules into their surroundings often targeting cells in the immediate area. The type and purpose of the secretions is governed by the immediate organ and may involve any combination of factors from the macrophages enormous secretory arsenal.

Resident tissue macrophages may be refractory to immunologic stimulus and as a result be unable to release the cytotoxic molecules involved in the inflammatory response (Lepay et al, 1985a,b). Furthermore, as a source of factors such as prostaglandins, particularly PGE_2 and some colony stimulating factors which have been shown to suppress the immune response, the macrophages may act locally to prevent immune reactions in tissues where such an event would be deleterious to the organ. These macrophage phenotypes may be created by developmental events during differentiation and maturation of the cells.

Evidence supporting this model is provided from a number of studies which suggest that exposure to certain cytokines and colony-stimulating factors during the development of the monocyte will alter its functional and immunological properties. While interleukin-3 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) have a broad specificity and produce mature cells of multiple linage (Sieff et al, 1985), cytokines such as Interleukin 4 will specifically produce mast cells (Mosmann et al, 1986). Likewise the action of GM-CSF and Colony Stimulating Factor-1(CSF-1) on bone marrow derived

monocytes at different developmental stages produces macrophages with different immunological capacities (Nicola 1989). For example, CSF-1-derived macrophages respond to lipopolysaccharide (LPS) whereas GM-CSF-derived macrophages require IFNγ in addition to LPS treatment in order to attain cytotoxic status (Rutherford and Schook 1992a,b).

These differences in function serve to demonstrate that the resident macrophage is ultimately a product of its environment. While a macrophage may retain the capacity for a number of basic immunological functions it is also likely to be partake in specific functions for the organ concerned. It would therefore seem likely that macrophages resident in the testis may contibute some testis-specific role in addition to acting as regulators of the local immune response.

1.4.4 Macrophages in the cell-mediated immune response

It is only in the presence of cell-mediated immune responses that the macrophages become "activated" and although these cells are less secretory than those stimulated non-specifically during inflammatory reactions they possess a number of features not normally seen in resident or inflammatory cells. The induction of microbicidal and cytocidal activities as well as the display of the four attributes associated with immuno-competancy (see 1.4.2) are all vital components of cell-mediated immunity.

The importance of macrophages for this response has been recognised for years (MacKaness, 1964), as has their role in expressing MHCII complex and the presentation of antigen to helper T-cells. Macrophages are recruited to the site of infection by activated T-cells producing IL-2, GM-CSF and interferon gamma (Unanue and Allen, 1987). These stimulae in turn activate the macrophages, upregulating MHCII expression (Willman et al, 1989) and the production of the proinflammatory factors IL-1, IL-6 and TNFα (Unanue and Allen, 1987). Macrophage production of IL-1 is central to the immune response, stimulating the IL-2 production in T-cells which drives

lymphoproliferation (Lowenthal et al, 1986). This part of the response serves to recruit and activate additional macrophages and T-cells to the site of infection. Both TNFα and IL-6 have similar roles in regulating cell proliferation during the induction of cellular immunity (Hackett et al, 1988; Hirano et al, 1990).

The pleiotropic nature of these cytokines allows the macrophage to enhance proliferation in both arms of the immune system. The development of the humoral immune response requires the secretion of IL-1 and IL-6 by macrophages, as well as expression of MHCII and processed antigen on the macrophage surface (Dinarello, 1989).

1.4.5 Suppressor Macrophages and their Supressive Mechanisms.

Immune suppression is generally refered to as the downregulation of the immune response. Although it may be used to describe the "switching off" of the immune response once an antigen has been successfully removed from the host, it is more commonly used when referring to the absence of an immune response in the presence of antigen. While the cell-mediated arm of the immune system employs the secreted factors of suppressor T-cells and leukocytes to halt a complete immune response, the nature of immunological non-response is still largely unexplored.

Immune suppression and the cells which mediate the downregulation of the immune response have been shown to play a role in autoimmunity, infectious disease, tumors, and various other responses. This review will deal specifically with concepts which relate to macrophage induced suppression and how it may relate to the immune privilege afforded the testis.

Suppressor monocytes and macrophages are part of the cellular immune network which regulates the immune response. Currently there appears to exist a distinct macrophage suppressor phentope which when stimulated to increase numbers may give rise to a number of testicular malfunctions (Khansari et al, 1985).

It was observed as long ago as 1966 that under certain conditions, macrophages may become immuno-suppressive (Parkhouse and Dutton, 1966) and in some cases the same stimulus which initiates macrophage activation will also induce immunosuppression. This macrophage immuno-suppression may be induced after activation by microbial or fungal infection or more interestingly, it can be a normal function for macrophage populations.

It has been known for some time that macrophages isolated from the lung or peritoneum can inhibit immune responses *in vitro* (Waldman and Gottleib, 1973; Goodwin, 1977). It also seems likely that the macrophage populations from tumors or the lung may be intrinsically immuno-suppressive (Kirchner et al, 1974; Pelus and Bockman, 1979; Mbawuike and Herscowitz, 1988). The studies which subsequently investigated these reports found that prostaglandin and respiratory burst metabolites (reviewed by Allison, 1978) were the primary cause of the supressed immune response.

Prostaglandins negatively regulate many of the cytokines and colony stimulating factors associated with the immune response (Pelus et al, 1979; Kunkel et al, 1986a,b) and the associated T-cell responses (Goodwin et al, 1978; Goodwin and Webb, 1980; Gualde et al, 1985). Recently it has also been shown that prostaglandin E_2 acts on T-cells to block the expression of both IL-2 and its receptor, thus explaining its inhibition of lymphocyte proliferation in vitro.

Nitric Oxide has only recently been implicated in macrophage mediated suppression (Mills et al, 1991). It has been shown that production of this factor by macrophages can inhibit lymphocyte responses to concanavalin A in vitro (Albina et al, 1991).

While some large molecular weight (between 30 000 and 80 000MW), protein suppressor factors have been documented (Greene et al, 1981; Krakauer, 1985a; Fujiwara et al, 1987) few have been identified outside of the known macrophage-derived

cytokine family. Cytokines such as gamma interferon, interleukin-4, interleukin-10, GM-CSF and TGFß have all been cited as having immuno -suppressive effects and all but interleukin-10 is produced by the macrophage (Rapolee and Werb, 1988). Although GM-CSF alone has been shown to be immuno-suppressive in tumour bearing hosts (Walker et al, 1992), it more often than not exerts suppresion via the production of arachadonic acid metabolites, particularly PGE_2 (Morrisey and Ireland, 1991). TGFß is thought to exert its effects in a similar manner (Brock and Marsh, 1991).

The action of gamma-interferon in this respects is somewhat paradoxical. In most cases its downregulates prostaglandin production and stimulates the production of IL-1 (Boraschi et al, 1984), however it is also a powerful inducer of nitric oxide synthase and its immuno-suppressive effects are thought to mediated through this pathway perhaps by interaction with interleukin-4.

It is clear that while macrophages appear to have a number of suppressive mechanisms, the two main factors which mediate immune suppression are prostaglandin and nitric oxide. Even the suppressive effects of cytokines appear to be mediated in this manner. Therefore it is possible that production of these factors in a local environment could concievably reduce the prospect of an immune reaction at that site.

1.4.6 The macrophage as an effector cell.

It is apparent that macrophage immunobiology is in fact central to most aspects of the immune response. The macrophage operates in both afferent and efferent responses, influencing and interacting with the lymphocytes for the induction of an immune response. While their role in cell mediated immunity is highly defined, their role as cells resident in a tissue or as responders to non-specific stimulation is less defined. As potent effector cells they have the capacity to release an enormous range of secretory products enabling them to exert a wide range of actions upon cells in local tissues (Table 1.1).

Table 1.1 Macrophage Secretory Products.

Product	Function
Enzymes / Enzyme Inhibitors	
plasminogen activators	inflammatory response, tissue repair
collagenase I, II, III, VI, V	inflammatory response
complement components	inflammatory response
coagulation factors	coagulation, tissue repair
acid hydrolases	inflammatory response
lysosyme	anti-microbial activity
α2-macroglobulin	regulation of plasma enzyme activies
plasminogen activator inhibitor	regulation of enzyme activity
Growth Factors and Cytokines	
IL-1α , IL-1ß, IL-1 receptor antagonist	immunoregulatory
TNFα	immunoregulatory
IL-6	immunoregulatory
TGF-α, TGF-ß1, TGFß2	wound healing, inflammation
Interferon α and ß	antiviral activity
GM-CSF, G-CSF, C-CSF	proliferation and and differentiation of-haemopoietic precursors
Leukemia inhibitory factor (LIF)	immunoregulatory
Fibronectin	opsonic, adhesive
PGDF-A, PGDF-B	wound healing, inflammation
Low Molecular Weight Substances	
Radical oxygen intermediates	microbicidal, tumoricidal
Prostaglandins	inflammatory, immunoregulation
Leukotrienes	inflammatory, immunoregulation
Platelet activating factor	inflammatory, activates platelets
Nitric Oxide	microbicidal, tumoricidal

Compiled from Takemura and Werb (1984) and Rapolee and Werb (1988)

The classic and more familiar role of the macrophage is in the efferent arm of the immune response, as an effector cell involved in inflammatory reactions. The presence of an inflammatory stimulus, causes macrophages to develop increased secretory activities, and in this capacity the macrophage fulfils a number of general functions, such as removal of dead cells and tissue debris during wound healing as well as playing a significant role in tissue remodelling (Nathan, 1987; Knighton and Fiegel, 1989).

Although the inflammatory response is non-specific and involves a number of leukocyte cell types, macrophage production of the pro-inflammatory cytokines IL-1, TNFα and IL-6 is vital for the induction of cell proliferation and the tissue remodelling that occurs in the aftermath. TNFα and IL-1 have several effects during the inflammatory response. The first is to stimulate and induce self-production, the production of IL-6 and a host of CSF's as well as a number of chemotactic and adhesion factors in the recruited macrophages. This has the effect of inducing the respiratory oxidative burst in the recruited cells as well as the release of enzyme for digesting the extracellular matrix, complement components, and coagulation factors (Takemura and Werb, 1984). In conjunction with this, the polymorphonuclear leukocytes, neutrophils, endothelial cells and fibroblasts are all co-ordinated by the macrophage in aspects of chemotaxis, proliferation and cytoxicity necessary for destroying microbial invaders.

However, recent studies have suggested that the role of macrophages and inflammation need not be limited to disease control mechanisms. A plethora of reviews now describe macrophages resident in the female reproductive tract as using these same factors to modulate physiological function. For example, inflammation-like reactions modulated by macrophages and controlled by cytokines have been observed in the female reproductive tract as mediating various facets of oestrus, ovulation and changes in uterine epithelial structure congruent with ovulation (Adashi, 1990; Hunt, 1990). Studies of cytokine production and regulation in the female uterus have implicated GM-CSF as a possible paracrine regulator of uterine remodelling in this tissue (Robertson et al, 1992).

1.5 Macrophage Secreted Factors

1.5.1 Macrophage derived cytokines and colony-stimulating factors.

Cytokines and colony -stimulating factors (CSF's) belong to a large group of molecules loosely termed growth factors. The number of known growth factors and growth factor families has grown continually in recent years as research into this field has increased. In contrast to other cell types, the macrophage remains unique in that it has the capacity to produce so many of these factors, in all manner of combinations in response to a wide range of stimulii.

Cytokines and CSF's are factors responsible for mediating the survival, growth, differentiation and proliferation of cells in the haemapoietic system (Dinarello, 1989; Horiguchi et al, 1987; Becker et al, 1987;Hoang et al, 1989; Rappolee and Werb, 1992) and have a number of characteristics. They are generally secreted, synergise and act on nearby cells in a paracrine or autocrine fashion within a highly localised area. While they are not stored within the cell, they are readily inducible and as a consequence of their potency, their production is under tight regulation at all levels. Finally cytokines act on the target cell through a transmembrane receptor.

Resting macrophages usually produce no or low levels of cytokines. Increased cytokine production normally requires stimulation or activation of the macrophage to effector status. The common inducers of macrophage activation are endotoxin or lipopolysaccharide (LPS), IFNγ and interactions with macrophage Fc and complement receptors during immunophagocytosis.

LPS is a single moiety from the bacterial cell wall which stimulates macrophage activation both *in vivo* and *in vitro*. A non-specific stimulant of macrophages, it evokes a rapid response generating the production of the inflammatory molecules. IFNγ has a more specific action on macrophages. Released by T-cells, it is a potent inducer of MHCII expression on the macrophage as well as stimulating effector status. When used in

combination with LPS macrophages may be primed and triggered to full cytotoxic status (Kleinerman et al, 1984; Adams and Hamilton, 1984; Schreiber and Celada, 1985). Immuno-phagocytosis via the Fc receptor has also been shown to induce cytokine production by the macrophage. Opsonisation of antibody has been shown to stimulate the release of TNFα (Debets et al, 1990; Szabo et al, 1990), IL-6 (Ling et al, 1990) and possibly IL-1(Chou et al, 1985; Arend et al, 1985).

Macrophage-derived cytokines are generally recognised for their role in initiating inflammation or the induction of an immune response, hence the cytokines involved are termed pro-inflammatory. The interleukins are the best known subset of the cytokine family, of which there are at least 14 characterised and published. The interleukins are best known for their role in initiation of the immune response. In particular IL-1α and ß and IL-2 are recognised for their essential role in T-cell stimulation during cell-mediated immunity. Although there are a number of pro-inflammatory cytokines, the main contigent of this group are IL-1α and ß, TNFα and IL-6. These three cytokines are all structurally distinct but have considerable overlap in function, such is the pleiotrophy of their action.

IL-1 (originally lymphocyte activating factor) and IL-2 are pivotal mediators in the activation of antigen-presenting T-cell populations. Briefly, the mechanism by which T lymphocytes proliferate is largely dependant on the release of IL-1 from a cell of monocyte/macrophage linage (Unanue, 1987; Dinarello, 1989) and its subsequent stimulation of both IL-2 production and receptor expression by the lymphocyte.

Lymphocyte activation with IL-1 results in a two fold response, stimulating T-cells to produce IL-2 and express IL-2 receptors. The T-cell derived IL-2 then acts in an autocrine fashion upon these cells and initiates the cellular proliferative phase of the immune response.

IL-1 exists in two forms, a free secreted molecule (IL-1ß) with more hormonal style effects, and a membrane expressed moiety of similar size (IL-1α) which appears to be involved in cell-cell interactions. While both are produced in large quantities in response to LPS (Dinarello, 1989) and react with the same receptor, activated macrophages produce 10 fold more IL-1ß than IL-1α in response to foreign antigen or tissue damage (March et al, 1985). These two forms of IL-1 display almost complete overlap in function (March et al, 1985; Rupp et al, 1986), and the release of either iso-form manifests itself in increased cell proliferation and differentiation, and what is also termed as the inflammatory response.

It is important to note that the role of IL-2 is distinct from that of IL-1. While IL-1 initiates the reaction, IL-2 is primarily responsible for T-cell proliferation and maturation. The stimulated lymphocytes express high affinity IL-2 receptors that amplify and mediate the proliferative phase (Robb, 1984). Recent studies have shown that B lymphocytes and macrophages may also express receptors for IL-2 (Herrmann et al, 1989), suggesting that they are sensitive to this cytokine.

Both these interleukins have additional roles outside of lympho-proliferation in what is commonly referred to as the inflammatory response, a response often associated with a number of symptoms such as fever, prostaglandin and collagenase synthesis, fibroblast proliferation, enzyme inhibiton and cartlidge and bone resorbtion (Dinarello 1989). This response usually involves other closely related cytokines such as TNFα and IL-6.

As well as its ability to kill tumour cells in vitro (Ruff and Gifford 1981), many TNFα mediated effects are remarkably similar to that of IL-1, although it is required in a much higher concentration (Ranges et al 1988). The relationship between the action of these cytokines is so close that it is likely that they augument each others immunologic action. Furthermore, the biologic effects of IL-1 and TNFα are often indistinguishable because the two display synergism when added together in vitro.

Despite the current conjecture as to the ability of TNFα to mimic the lympho-stimulatory effects of IL-1 on lymphocytes (Ranges et al 1988; Plaetnik et al 1987), there are many other similarities between the two. Both factors display anti-viral activity and can initiate fever through prostaglandin synthesis (Dinarello, 1989). In addition these factors can induce synthesis of colony stimulating factors, act as co-factors for stem cell activation and stimulate many cell types not directly involved in immunity. They may also be major factors directly responsible for arthritic diseases (Krakauer et al 1985; Bertolini etal 1986) as well as shock and related syndromes (Tracey et al 1986).

IL-6, formerly known as Interferon B2, B-cell-stimulating factor-2 and hybridoma growth factor, shares characteristics with IL-1 and TNFα in that it is an endogenous pyrogen and an inducer of the macrophage acute-phase response. Like IL-1 and TNFα, it too participates in the inflammatory response and can induce self-production and the production of other acute phase cytokines. IL-6 is more similar to IL-1 than TNFα in that it appears capable of activating lympho-proliferation in the presence of a mitogen through the IL-2 pathway. However, IL-6 does not induce TNFα or IL-1 auto-production as do these cytokines on themselves and infact it may suppress autocrine induced production of these factors (Dinarello, 1989).

The differentiation and subsequent induction of bone marrow derived macrophages into monocytes and macrophages is tightly regulated by several cytokines. IL-3 is instrumental in the intitial differentiation of the progenitor stem cells but their specific transformation and functional capabilities are decided by exposure to the cytokines released from peripheral lymphocytes (Arai et al, 1990).

Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and Colony Stimulating Factors (CSF's) are released constitiuitvely by many tissues (Le et al, 1988). These factors act on monocytes as they first enter a tissue in order to determine the macrophages final functional status. Thus the organ or tissue to which the blood borne monocyte

migrates has the capacity to direct and create the phenotype of the macrophage through the factors it uses to mature the monocyte. GM-CSF therefore is highly important in the production of the pluripotent stem cells supporting the growth and differentiation of a variety of cell lines.

This brief list of cytokines is by no means extensive in its number or description of these immunomodulatory molecules and it would be beyond the capacity of this review to cover this field in real depth. An intricate knowledge of the cytokines is not necessary for the purpose of this thesis. The intention is simply to demonstrate the variety of capabilities and interactions of these factors. That the power of cytokines are not only limited to cells of the immune system, and that their effects are not always related to disease control opens a whole new range of possibilties in cellular communication and local tissue regulation.

1.5.2 Regulation of macrophage-derived factors

As discussed previously the production of cytokines and CSF's is highly regulated due to their extreme potency. As a cell unequalled for cytokine synthesis, the macrophage also employs specific mechanisms for downregulating this production. Cytokines such as IL-1(α and ß), GM-CSF and TNFα are unstable due to a repeat motif at the trancription level which allows a half-life of around one hour in the cytosol (Shaw and Kamen,1986; Brawerman, 1989). Similarly other growth factors may be degraded by specific peptidases in the extracellular environment, ensuring that the effects of these factors do not remain after the inductive stimulii have abated.

Another mechanism employed by the macrophage is to buffer or inhibit the efficacy of such factors. Both TNFα and GM-CSF have forms of soluble receptor which can bind the respective cytokines thus preventing interaction with cell-bound receptors. The production of a competitive inhibitor, IL-1 receptor antagonist, with the same affinity for

the IL-1 receptor as the iso-forms of IL-* 1 has also been observed (Carter et al, 1990; Hannum et al, 1990)*. Delayed production of the IL-1 receptor antagonist occurs after the initial induction of IL-1, but the antagonist is stable for longer thereby sequestering the IL-1 moeity after the initial response .

During inflammation or activation, the production of such a wide range of macrophage factors is mainly regulated by negative feedback mechanisms (Soberman and Karnofski, 1981). For example, IL-1 production stimulated by TNFα, in turn inhibits the autoinduction and production of TNFα (Epstein et al, 1990). However, while cytokines regulate themselves to some extent, the production of arachidonic acid metabolites such as prostaglandins have the strongest effects.

On stimulation of the target cell, IL-1 also induces PGE_2 production. PGE_2 in turn works negatively to reduce the production of the pro-inflammatory cytokines (Kunkel et al, 1986; Daniel et al, 1987; Dinarello, 1989). Furthermore, prostaglandins and PGE_2 in particular have long been sited as potent suppressors of immune function due to their ability to suppress lymphocyte proliferation (Offner and Clausen, 1974; Goodwin et al, 1978; Stobo et al, 1979).

The methods of macrophage regulation may have special implications in the context of resident macrophages where the local environment may well favour the production of one factor over another. For example, in the immune system as free macrophages, there is a higher probability that cells will be activated or primed for an immune response than those in the tissue. Tissue bound macrophages are often found in an immunologically quiescent state, secreting small amounts of substances relevant to the physiology of the organ. For example, in the kidney macrophages may secrete prostaglandin to regulate vasodilation (Hume and Gordon, 1983).

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*A soluble receptor for IL-1 has been found since the completion of this review which only includes literature up to and including December 1992.

Furthermore, macrophages from organs such as the liver, kidney and lung appear to have the ability to filter and remove antigen present in the blood (Vernon-Roberts, 1972; Schreiner, 1981; Nakstad et al, 1989), without initiating a deleterious immune response. It therefore appears that the activity of the (resident) macrophage in a tissue may relate directly to the immune function of the organ.

In this sense, an interesting comparison may be made with the immunobiology of other tissue bound macrophages, such as those present in the spleen. The spleen has a high percentage of B lymphocytes present and thus may be considered as an organ with imune function. Accordingly, the splenic macrophages are capable of both ingesting antigen and initiating a full range of lymphocyte responses.

1.6 Macrophages in the female reproductive tract

Macrophages have been found throughout the female reproductive tract (Adashi, 1990; Hunt, 1990) and evidence suggests they are involved in the paracrine regulation of these tissues. Macrophages from the mouse ovary have been shown to have a specific role in steroidogenesis by increasing granulosa cell progesterone production (Kirsch et al, 1981). Subsequent studies have demonstrated similar results in humans (Halme et al, 1985) and in pigs (Hughes et al, 1990).

Macrophages have been observed in myometrial and endometrial tissues of the uterus and change their size and distribution according to the stage of oestrous (Hunt et al, 1985). Although the function of uterine macrophages is yet to be fully determined, they are highly phagocytic (Tachi et al, 1981; Redline and Lu, 1988) and are postulated to play a role in preventing tumor-like trophoblast cells from invading maternal tissues (Hunt, 1990; Hunt and Pollard, 1992).

Although the exact mechanisms responsible for the paracrine action of macrophages in the female reproductive tract are not fully understood, it is well established that several macrophage derived products can influence these tissues. The iso-forms of IL-1 and TNFα have been found in the ovary and uterus (Khan et al, 1988; Bagavandoss et al, 1988, Hunt and Pollard, 1992) and factors such as GM-CSF and IL-6 have been shown to be secreted by macrophages in the uterus (Robertson et al, 1992). Uterine macrophages are also known to produce high levels of prostaglandin E_2 (Matthews and Searl, 1987), a factor well known for its anti-proliferative effects on lymphocytes.

The presence of resident macrophages in the female reproductive tract and importance of macrophage-derived factors in female reproductive function demonstrates a role for the macrophage as a paracrine regulator within the reproductive organs. It would therefore seem likely that the testicular macrophage may act similarly to regulate various events in the testes.

1.7 The Testicular Macrophage

The testicular macrophages comprise of up to 15%-20% of the interstitial tissue in rodents and humans and exhibit the general appearance of other tissue bound macrophages. They have been classified morphologically by ultrastructural and cytochemical methods (Carr et al 1973; Miller et al 1983) as having a single indented nucleus, paranuclear golgi bodies, rough endoplasmic reticulum, coated vesicles, and numerous heterogenous lysosomal vacuoles. The macrophage surface is highly invaginated and numerous filopodia and lamellopodia project from the plasma membrane, holding it in place in the interstitial space.

But despite their categorisation as macrophages on the basis of morphology and exogenous secretions, very little is known of their function. Previous studies have described the close association between macrophages and Leydig cells (Connell and

Christensen 1975; Wing and Lin, 1977) and there appears to be some interaction between them. For example, the association between the two cell types in the form of physical contact: the endocytosis of the Leydig cell cytoplasm (Miller et al, 1983, Hutson 1992), and the similarities in morphology (Bergh 1985, Hutson, 1992), suggest a functional coupling may exist in addition to the other roles of the macrophage. Functional changes induced in Leydig cells by means of gonadotropin withdrawal, cryptorchidism, or seasonal dysfunction, may lead also to alterations in the morphology of the testicular macrophage (Berg, 1985; Berg, 1987; Gondos et al, 1980; Hutson 1992; Wing and Lin, 1977).

Other factors which also support an interaction between the cells, are the common properties shared by macrophages and steroid producing cells in various organs around the body. Steroid activity by macrophages in other tissues is widely reported and has been in many cases implicated in the biotransformation of steroids (Vernon-Roberts, 1969; Kirsch et al, 1981; Milewich et al, 1982; Milewich et al, 1983; Lofthus et al, 1984). It may well be that the testicular macrophage in some way aids the Leydig cell, (which is the major steroid producing cell in the testis), in the production of testosterone and/or its metabolites.

Other recent studies have acknowledged the possibility that the testicular macrophage is responsive to pituitary hormones and through this medium, may regulate the function of the Leydig cell. An indication of this is the apparent ability of the testicular macrophages to bind Follicle Stimulating Hormone (FSH) (Orth and Christensen, 1977). Further studies on this topic (Yee and Hutson, 1983; Yee and Hutson, 1985a,b), have revealed that while the testicular macrophage is responsive to FSH, the peritoneal macrophage possesses no such surface receptor. These studies tend to support the concept that the testicular macrophage, as common for most resident macrophages, has adapted a functional specialisation for the testis.

This observation is of great interest in terms of testicular physiology. It is well known that the secretory products from macrophages modulate the activity of various cell types (Page et al, 1978). Conversely, it has been emphasised that various hormones are quite capable of altering macrophage secretions (Gemsa et al,1975). Since cell to cell communication is vital to most aspects of testicular function (Cameron and Markwald, 1981; Hutson and Stocco, 1981; Sharpe et al, 1981; Setchell, 1983; Sharpe 1984), the testicular macrophage may assume a role in the function of the testis via FSH regulated secretory products that alter Leydig cell steroidogenesis. This hypothesis is supported by a very similar situation which appears to exist in the ovary (Kirsch et al, 1981) whereby ovarian macrophages have been shown to influence the secretion of steroids by luteal cells.

It is worth at this point, while discussing the possible functions of the testicular macrophage, to draw comparisons between the resident macrophages of the testis and those of the kidney. Both of these organs originate from the same embryonic structure. The kidney has a high population of macrophages in the medulla where it is thought that they may be active in the control of circulation and ultrafiltration (Hume and Gordon, 1983). It is conceivable then, that the testicular macrophage may also perform some sort of filtration role in the testis. The macrophages in the medulla of the kidney can release prostaglandins which may provide a mechanism for changing local circulation within this organ.

The testes have a unique system of microcirculation, the capillary permeability of which is known to be altered in response to gonadotropin (Setchell and Sharpe, 1981; Sharpe, 1983), and thought to be mediated via the Leydig cell (Setchell and Rommerts, 1985; Head et al, 1983; Maddocks et al, 1987). Considering macrophages in general can produce a variety of vaso-active factors it is reasonable to assume that the testicular macrophage may interact with the Leydig cell to regulate bloodflow in the testis. Similarly, other cells in the testicular interstitium such as the fibroblast and myoid cell

populations appear to be involved in support of and in the contractility of the seminiferous tubules as well as the blood and lymph vessels (Setchell and Brooks, 1988).

It would therefore be reasonable to expect that the testicular macrophage assumes several roles in testicular function, and that these range from simple phagocytic functions, to regional immunobiology, and as the functional complement of the Leydig cell. It also seems likely that in performing these tasks, the testicular macrophage may at least in part be responsible for granting the immune privilege afforded the testis.

1.7.1 Possible functional roles for the testicular macrophage

While it has been evident for some time that there exists a paracrine and autocrine network for local regulation of testicular function, the majority of these studies have centred on the Sertoli and Leydig cells. Intitial studies by Yee and Hutson (1983, 1985a,b) suggested the testicular macrophage secreted a factor(s) capable of stimulating testosterone production by Leydig cells. The production of this macrophage-derived factor appeared time and dose responsive and inducible by FSH. Moreover Leydig cells optimally stimulated with LH, significantly increased testosterone production in the presence of macrophage-conditioned medium.

In contrast to the initial findings, subsequent studies have shown that testicular macrophage-conditioned medium inhibits testosterone production by the Leydig cell (Lombard-Vignon et al, 1991; Mayerhofer et al, 1992). Furthermore, neither of these studies observed the production of testosterone-inducing factors by the testicular macrophage factors in response to FSH. However, it was observed that when stimulated with LPS, the testicular macrophages increased Leydig cell testosterone production (Lombard-Vignon et al, 1991).

The identification of cytokines as local messengers in the ovary and reproductive tract of the female (Adashi, 1990; Hunt 1990) has generated speculation that the testicular macrophage may be a potential source of cytokines and paracrine regulation within the testis. Studies of macrophage-derived cytokines, IL-1 and TNFα added exogenously to Leydig cell culture have also shown effect on testosterone production yet once again the results are contrasting. Whereas Warren et al, (1990) reported that recombinant TNFα enhanced both basal and LH stimulated testosterone production, Mauduit et al (1991) reported the opposite and Calkins et al (1990) reported no effect of exogenous TNFα on testosterone production.

Studies using recombinant IL-1 in Leydig cell culture reveal a highly intricate relationship between dosage and α and ß forms of IL-1. When added in low concentration to Leydig cell culture IL-1 has been shown to enhance testosterone production but in high concentrations it has been observed to suppress it (Verhoeven et al, 1988; Calkins et al, 1988; Moore and Moger, 1991). The two forms of IL-1(α and ß) do not appear to have identical effects despite the fact their action is thought to be mediated through the same receptor. IL-1α appears a more potent inhibitor of testosterone production than IL-1ß (Calkins et al,1990) and this situation appears to be supported by the observation that receptors in the testicular interstitium of the mouse have a higher affinity for IL-1α than IL-1ß (Tako et al, 1990).

Constituitive TNFα production by the testicular macrophage has been recently reported (Hutson, 1992) as has the presence of mRNA for IL-1 in mouse testicular macrophages (Hales et al, 1992), but the use of recombinant IL-1 and TNFα in the reported studies and the contrasting observations make it difficult to assess the role of the testicular macrophage and these cytokines in regulating testosterone production *in vivo*.

Identifying the origin of these cytokines and their action on Leydig cells *in vivo* may also be difficult, especially where there may be more than one source. It has been recently

observed that IL-1 is produced by Sertoli cells within the seminiferous tubule (Gerad et al, 1991) and IL-1ß induces IL-1α mRNA in the Leydig cell in the testicular interstitium (Wang et al, 1991).

However, more relevant studies of the role of the testicular macrophage in Leydig cell steroid production have been recently completed. Testicular macrophages were removed exclusively from the testicular interstitum in vivo by injecting the testis with liposomes containing dichloromethylene diphosphonate (Cl2MDP). It was observed that the macrophage depleted testes secreted less testosterone than those with macrophages (Maddocks et al, 1992; Berg et al, 1992). It would appear from these studies that the testicular macrophage may indeed be involved in regulating testosterone production as a functional complement to the Leydig cell.

1.8 The purpose of this study

The presence of a resident macrophage population in the testis has only recently been speculated as contributing to the immune privilege status of the interstitum and only in the last decade has the close association between the testicular macrophage and Leydig cell been described (Bergh 1985, Bergh, 1987). Also during this time macrophages in general have been implicated as cells unparalleled in their secretory and effector capabilites (Rapolee and Werb, 1992). The macrophage is now recognised as a cell important to the intitiation and regulation of the immune response through its ability to secrete cytokines and colony-stimulating factors. Furthermore, the macrophage and these factors involved in the processes of immunology are now understood to play a pivotal role in female reproductive physiology.

Although traditionally viewed as a component of the immune system, the macrophage is now more widely recognised as a cell with a specific physiological role in many tissues. The presence of the macrophage in a site of immune privilege, the factors it secretes and

its close physical association with the Leydig cell may belie its role in testicular function. Therefore the aim of the present study was to characterise the immunobiology of the testicular macrophage with respect to roles it may play in the immunology and the physiology of the testis.

CHAPTER 2: Materials and Methods.

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2.1 Reagents

Bovine serum albumin (BSA), collagenase type V (250U/mg), 3-3'-diaminobenzidine (DAB), 3-[4,5 dimethylthiazol-2yl]-2,5 diphenyl tetrazolium bromide (MTT), sulfanilamide, napthylethylene diamine dihydrochloride, *S. enteritidis* lipopolysaccharide (LPS), Concanavalin A, *C. ensiformis* , α-napthyl acetate, Nitro BT, n,n-dimethylformamide, nicotinamide adenine dinucleotide ß, fast red TR salt, methyl green stain, trypan blue, ß-mercaptoethanol and indomethacin were all purchased from the Sigma Chemical Co, St Louis U.S.A.

Ficoll-Paque for the *in vitro* isolation of lymphocytes was purchased from Pharmacia LKB, Sweden. Dialysis tubing (<14000MW cutoff)was purchased from Selby-Anax, Australia. Amicon and Centricon microconcentrators were purchased from W.R. Grace & Co, Beverly, MA. U.S.A.

Haemolysin; sheep erythrocyte antiserum IgG, heat-inactivated fetal calf serum (HI-FCS), Heparin and penicillin/streptomycin mixture, glutamine (200mM) and Monomed-A (serum replacement) was purchased from CSL Parkville, Victoria, Australia. Phosphate buffered saline, RPMI 1640, Hams F12 and Hanks Balanced Salt Solution (HBSS) were purchased from Cytosystems, Castle Hill NSW, Australia.

Taq DNA polymerase, 25mM MgCl and pUC 19 DNA (restricted with HpaII) were purchased from Bresatec, Thebarton, South Australia.

Nutridoma-NS 100x, Recombinant cytokines IL-1α, IL-1ß, IL-6and TNFα and IFNγ, DNase I (from bovine pancreas) and RNase inhibitor (from human placenta) were purchased from Boehringer Mannheim, (Mannheim, Germany). Recombinant murine GM-CSF was kindly provided by N. Nicola (Walter and Elisa Hall Institute, Melbourne, Australia).

Superscript RNase H reverse transcriptase was purchased from Gibco, BRL. dNTP Ultra pure dNTP set was purchased from Pharmacia Biotech. Guanidine Thiocyanate was purchased from Fluka Biochemica, Buchs, Switzerland.

Mouse anti-rat CD11b, ED1, ED2, ED3, ED5, and W3/25 monoclonal antibodies (mAb) were purchased from Serotec Ltd, Oxford, England. The embedding mixture for cryostat sections was purchased from Jung, Lecia instruments, GMBH, Nussloch Germany.

The anhydrous Magnesium Chloride, Sodium Chloride, Sodium Citrate, Glucose and the Haemotoxylin and Eosin stains were prepared from concentrates marketed by Gurr Laboratories, BDH chemicals Ltd, Poole, England. The coatasil, used to siliconise glassware was bought from Ajax chemicals, Sydney, Australia.

Polyclonal rabbit anti-human antibodies for cytokines TNFα (αTNFα) and IL-1 (αIL-1)were purchased from Genzyme, Cambridge MA. A goat polyclonal antibody to murine GM-CSF (goat α GM-CSF; Miyajima et al, 1986) and a rat monoclonal antibody to murine IL-6 (αIL-6) (Shabo and Sachs 1988) were kindly provided by J. Schreurs (DNAX, Palo Alto, CA) and L. Sachs (Weismann Institute of Science, Rehevot, Israel) respectively.

Tritiated PGE_2 (specific activity 7.4TBq/mM), $PGF_{2\alpha}$ (specific activity 6.81TBq/mM), Testosterone (specific activity 3.74 TBq/mmol), [3H] thymidine (specific activity 1.04TBq/mM), Biodegradable Counting Scintillant (BCS), streptavidin-horseradish

peroxidase (SAv-HRPO) and biotinylated sheep anti-mouse immunoglobulin antibody (biotin-SHAM) were purchased from Amersham Australia.

2.2 Animals

Rats were of the albino Wistar strain or DA rat (where specified) bred in the Adelaide University central animal house. All rats used were adult males, sexually mature, and weighing no less than 300g.

2.3 Cell Isolation

2.3.1 Peritoneal Macrophages

Peritoneal macrophages were isolated by peritoneal lavage as described by Stuart *et al* [17]. The peritoneal cavity of the rat was flushed with phosphate buffered saline (PBS) (20ml) and recovered cells were collected by centrifugation at 250g for 10 minutes then resuspended in sterile RPMI-FCS for culture. The cells were then cultured for 20 minutes, after which non-adherent cells were removed by rinsing the cultures dishes vigorously with PBS. Macrophages isolated in this manner were approximately 90% pure as judged by non-specific esterase activity and reactivity with the macrophage-specific mAb CD11b.

2.3.2 Isolation of Testicular macrophages and Leydig cells

Testicular macrophages and Leydig cells were isolated from the interstitial tissue by enzymatic digestion of the testis followed by density gradient centrifugation and cell elutriation. Decapsulated testes were washed three times in sterile phosphate buffered saline. Six testes were incubated in 50 ml PBS containing collagenase (0.25mg/ml) in a shaking waterbath at 34°C for 10 minutes. The seminiferous tubules were then allowed to sediment and the supernatant carefully removed with a sterile 20ml syringe. The suspension of

interstitial cells was then carefully layered over 15ml of Ficoll-Paque and centrifuged (1200g) at 4°C for 20 minutes to pellet tubule remnants, germ cells, erythrocytes and cellular debri. A crude interstitial cell fraction containing the testicular macrophages and Leydig cells was collected from the supernatant-Ficoll interface, washed twice by centrifugation in HBSS at 250g for 5min, and resuspended in 10ml HBSS (1% BSA) ready for elutriation

For cell elutriation, a Beckman J2-21 centrifuge and Beckman JE-6B elutriation rotor (Beckman Instruments, Palo Alto, CA) were used with the rotor speed set at a constant 2000 ± 40rpm. Sterile HBSS containing 1% BSA was pumped through the rotor under postive pressure by a Desaga pump (Heidleberg,Germany). The testicular cell preparation (10ml) was injected into the elutriation mixing chamber, and after an initial wash of 150ml with the pump set at a flow rate of 15ml/min, separate fractions of cells (150ml each in volume) were eluted.

2.3.3 Elutriation and adherence purification of testicular macrophages

Eluted fractions collected at the pump flow rates of 19ml/min, 28ml/min and 31ml/min provided the highest yields of testicular macrophages as described by Dirami *et al* 1991. Cells in these fractions were washed once by centrifugation (250g for 5min) and resuspended in RPMI-M at a concentration of 10^6 cells/ml. The cells were then further purified in culture by exploiting the adherence of macrophages to plastic and glass substrates. After culturing the cells for 20 minutes, cultures were washed vigorously with PBS five times in order to remove non-adherent cells. This provided cultures of highly purified testicular macrophages (95%) as judged by immunohistochemical and biochemical markers.

2.3.4 Elutriation and Percoll purification of Leydig cells

Leydig cells were collected after the removal of testicular macrophages by elutriation. With the pump still going, the rotor was stopped and remaining cells were collected. The Leydig

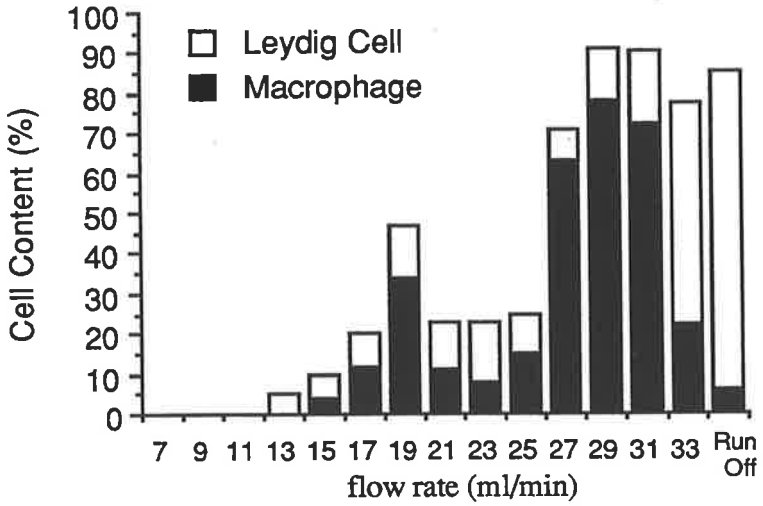
cells were then further purified using the protocol described by Klinefelter (1988). The crude Leydig cell suspension was then washed once in HBBS and resuspended in 14ml of HBBS with sodium bicarbonate (0.35g/L) and BSA (0.25%). The cell suspension was then thoroughly mixed with 21ml of iso-osmotic Percoll (11:1; v/v of percoll with 10x HBSS, Ca^{++} and Mg^{++} free) to make a 60% Percoll solution. A further 60% Percoll solution was prepared that contained density marker beads for 1.062g/ml and 1.075g/ml but no cells.

The two solutions were centrifuged in a fixed angle rotor (Beckman JA 20) at 20 000g for 60 minutes at 4°C. After centrifugation the different cell types became partitioned due to differing bouyant desities and Leydig cells were found in the fraction between the densities of 1.062g/ml and 1.075g/ml. The purified Leydig cells were then washed in HBBS (250g for 10 minutes) to remove the Percoll and resuspended in RPMI ready for culture. Leydig cells isolated in this manner were stained with ß-hydroxysterioddehydrogenase and judged greater than 95% pure.

2.3.5 Isolation of Lymphocytes

Peripheral blood lymphocytes were obtained from rats as described by Weir (1986). Blood was collected by means of cardiac puncture and then diluted 1:1 with saline. Aliquots (20ml) of the resulting suspension were pippetted onto 10ml of Ficoll in 50ml conical centrifuge tubes. These were then centrifuged at 1200g for 20 minutes at 4°C. The centrifugation produces an interphase layer of blood plasma in the Ficoll gradient. These cells in this layer were collected by sterile pippette and made up to 50 ml in RPMI 1640. The cells were then washed twice for 10 minutes at 400g and the resultant pellet was then resuspended in RPMI 1640 media containing 10% HI FCS to 10^7 cells/ml.

Figure 2.1 Elutriation Flow Rates for Testicular macrophages and Leydig Cells. The percentage of macrophages and Leydig cells within each elutriated fraction as determined by staining with non-specific esterase for macrophages and ß-hydroxysteroid dehydrogenase (ßHSD) for Leydig cells.


Leydig Cell
Macrophage
Cell Content (%)
100
90
80
70
60
50
40
30
20
10
0
7 9 11 13 15 17 19 21 23 25 27 29 31 33 Run Off
flow rate (ml/min)

2.4 Cell Culture

2.4.1 Media

Media used included RPMI-1640 or HamsF12 supplemented with 10% FCS, 5 x 10^{-5} M ß-mercaptoethanol and antibiotics (RPMI-FCS and HamsF12-FCS respectively); RPMI supplemented with 0.5% FCS, 1% Nutridoma-NS, 5 x 10^{-5} M b-mercaptoethanol and antibiotics (RPMI-NS) and RPMI-1640 supplemented with 2 mM glutamine, 4% Monomed A, 2% FCS and antibiotics (RPMI-M), as specified.

2.4.2 Cell Counts/Cell Viability

Once isolated, the various cell populations were counted to allow adjustments to be made for culturing cells at the desired concentrations. A small sample from the cell suspension was taken and diluted 1:1 in 0.2% trypan blue immediately before the cell count. The number of cells in suspension , was estimated by standard procedure in a haemocytometer counting chamber. Viable cell numbers were counted (dead cells stain blue), as a proportion of the total cell population per millilitre of medium. Cells were cultured at densities specified in the experimantal section and the purity of these cultures was determined by both biochemical and immuno-histochemical procedures.

The purity of the testicular and peritoneal macrophage population was ascertained by staining for nonspecific esterase (NSE) and macrophage-specific antibodies CD11b, ED1, ED2, ED3 and W3/25. The purity of Leydig cell cultures (and their presence in macrophage cultures) was determined by ßHydroxysteroiddehydrogenase activity, a marker specific for the Leydig cell.

2.4.3 Culture of Macrophages

Both testicular and peritoneal macrophages were cultured in RPMI-M on glass coverslips at 34°C (normal testis temperature) and 37°C respectively in an atmosphere of 95% air and 5%

CO_2. After an intial 20 minute attachment period, non-adherent cells were removed by five vigourous washings in PBS. LPS (10mg/ml), indomethacin (10mg/ml) or interferon gamma (IFNγ, 200U/ml) were added alone or in combination to some cultures. After 48 hours culture, the culture medium was collected, centrifuged at 11000g for 5min and the supernatant stored at -80°C for bioassay.

2.5 Identification of Macrophages

2.5.1 Haemotoxylin and Eosin

Cell cultures were removed from the incubator and rinsed thoroughly in PBS. Following the wash, the monolayer was fixed in a solution of methanol and acetone (1:1), and then rinsed again. The haemotoxylin solution was then added in excess to the slide and left to stain for approximately one minute. The slide was then rinsed and stained briefly with eosin before being rinsed again. Coverslips were then "wet-mounted" onto microscope slides using nailpolish applied around the margins, prior to viewing.

2.5.2 Stain for Non-Specific Esterase Activity

A stock solution containing 1g α-napthyl acetate 1g, 50ml acetone, and 20ml distilled water was prepared as described by Miller and Morahan, (1981). A working substrate was then made up fresh on each occassion in the following manner:

Stock solution of α-napthyl acetate	2.0ml
0.1mol/L phosphate buffer (pH 7.3)	15ml
distilled water	15ml
fast red TR salt	15mg

This working solution was then filtered through a Milliex GS 0.22um filter prior to use. It was then added to the harvested coverslips which were then allowed to incubate for 30-40min. The coverslips were then rinsed in tap water and counterstained with 1% methyl

green for 2 minutes. The coverslips were then rinsed again and mounted on a glass slide for viewing (as in 2.5.1).

2.5.3 Stain for ß-Hydroxysteriod Dehydrogenase (ßHSD)Activity

The stain for ßHSD activity was formulated as described by Lojda et al, (1981). A stock solution of aqueous incubation media was prepared from the following:

0.1M phosphate buffer (pH7.2-7.4)	10ml
0.1%-0.4% Nitro BT dissolved in	0.5ml
N,N-dimethylformamide and add	9.5ml
of distilled water)	10ml
0.05% sodium cyanide (pH 7.2)	4ml
047% anhydrous Magnesium Chloride	4ml
distilled water	8ml
Total	36ml

and stored in small portions in deep freeze.

A substrate stock solution containing 11ß-hydroxyandrostane (6mg) dissolved in N,N-dimethylformamide (10ml) was also prepared and stored in deep freeze.

For the actual staining an incubation medium was prepared with the following components:

Stock solution	2ml
NAD^+ or $NADP^+$	2-4mg
2mM substrate solution	0.2 mg

mixed, filtered and adjusted to pH 7.2-7.6. Coverslips carrying cultured cells were then incubated in the staining solution at 37ºC for 30 minutes. Following incubation, the monolayers were fixed in methanol and acetone (1:1), and mounted on a glass slide for

viewing (see 2.5.1).

2.5.4 Immuno-histochemical evaluation of macrophage specific markers

Testicular tissue for immuno-histochemical examination was prepared by freezing freshly removed testes in an embedding mixture using liquid nitrogen cooled isopentane. Frozen sections (6μm)were then cut transversely from frozen tissue blocks and placed onto glass slides. Isolated testicular macrophages and peritoneal macrophages were prepared for immuno-cytochemistry by culturing the cells on glass coverslips.

The sections and monolayers were fixed in cold ethanol (4°C) for 15 minutes and then rehydrated in sucessive dilutions of 100%, 70% and 50% ethanol also at 4°C. The samples were then washed three times in PBS at room temperature before incubation in a 1:400 dilution of either CD11b, ED1, ED2, ED3, W3/25 or ED5 ascites in PBS with 1% BSA and 10% normal rat serum (PBS-NRS) for 1 hour at room temperature.

After three more washes in PBS the samples were incubated with biotin-SHAM diluted 1:50 in PBS-NRS for 1 hour at room temperature. The coverslips were then rinsed three times in PBS and incubated with SAv-HRPO diluted 1:50 in PBS-NRS for 30 minutes at room temperature. After washing again with PBS, the coverslips were incubated with DAB (5 mg/ ml in 0.05 M Tris-HCl pH 7.2) / 0.02% hydrogen peroxide for 15 minutes and counterstained in haematoxylin. Positive cells stained brown using this procedure. Coverslips were then mounted in Depex and the antibody-positive cells were quantified.

2.5.5 Erythrocyte phagocytosis by macrophages

The testicular macrophages were isolated and cultured in complete media at a density of $2.4x10^6$ cells/cm on the coverslip at both 32°C and 37°C. At times of 24, 48 and 72 hours after the initial culture, the macrophages were then incubated with immunoglobin- coated sheep erythrocytes.

The freshly obtained sheep erythrocytes were diluted in a 1:1 ratio with Alseviers solution, which allows for their storage and use for between 3 and 14 days. Blood/Alseviers solution (1ml) was then added to Hanks M199 (9ml) and washed three times by centrifugation at 250x g for 5minutes. The resultant pellet was then resuspended to 5% solution in Hanks M199 (1ml).

At this point a 1% solution of rabbit anti-sheep red blood cell IgG (anti E-IgG) (400ul) was prepared in Hanks M199 and added to 400ul of the 5% erythrocyte solution. After incubation at 37°C for 15min the solution was washed three times in M199 by centrifugation and the pellet resuspended in Dulbeccos MEM (4ml).

Coverslips were then washed to remove any non-adherent cells and 10^9 anti-E-IgG coated erythrocytes (0.5ml of blood solution) were added to the macrophage cultures and allowed to incubate for one hour. The cultures were then washed, stained and viewed to determine the extent of red blood cell rosetting around macrophages. To quantify the ingestion rather than the peripheral binding of erythrocytes to macrophages, the non-ingested red blood cells in the culture were lysed by washing the coverslip in a solution of Hanks M199 and water (1:4) before fixing.

2.5.6 Quantification of Erythrocyte Binding and Ingestion

Quantification of binding and ingestion was followed as described by Bianco and Pytowski, (1981). The degree of binding of opsinized erythrocytes to macrophages (rosetting) was expressed as the percentage of macrophages forming rosettes. A rosette was defined as a macrophage binding 3 or more target erythrocytes.

By lysing the free erythrocytes before fixing and staining the monolayer, only blood cells ingested by the macrophages remained. Thus it was possible to count the number of

macrophages ingesting erythrocytes and compare these to the total number involved in binding (rosettes).

2.5.7 Preparation of Alseviers Solution

Glucose (24.6g), Sodium Citrate (9.6g) and Sodium Chloride (5.04g), were disolved in distilled water (1.2 L). The pH was then adjusted to 6.1 using saturated citric acid, and then sterilized by filtration. Sheep red blood cells were then added in a 1:1 ratio, and were subsequently used and stored in this manner for a time span of 3-14 days.

2.6 Radioimmunoassay.

2.6.1 Testosterone Assay

Buffer

Phosphate buffer solution (PBS) 0.01M pH7.5

Na_2HPO_4 (anhydrous)	1.42g
EDTA	0.34g
Na azide	1.00g
NaCl	8.18g

adjusted to pH 7.5 with diluted HCl or NaOH and made up to one litre using double distilled H_2O.

Gel Buffer

0.2% Gel PBS

Add 2.0g of gelatine to 1.0L of PBS

Dextran coated charcoal mixture

Charcoal Norit-A	625mg
dextran T_{70}	62.5mg

made up to 100ml using PBS.

2.6.1.2 Stock solutions and Assay Solutions

^{3}H-Testosterone stock solution

^{3}H-testosterone (250μCi) was dissolved in 5ml ethanol (AR grade) to make a stock solution.

3H-Testosterone Assay Solution

250μl of the above stock solution was placed into a conical flask and dried under air. Gel PBS (100ml) was added to to this to make an assay solution with an activity of 10 000cpm/200μl.

Testosterone Standards

The testosterone was diluted in ethanol (1μg/ml) and then used for making the stock solution. For each standard curve, 64μl of testosterone stock solution was added to 4.0ml of Gel PBS to produce a 1600pg/100μl top standard. This standard was then serially diluted to make 9 standards; 1600, 800, 400, 200, 100, 50, 25, 12.5 and 6.25pg/100μl.

Non-specific binding (NSB)

200μl of Testosterone stock solution (1μg/ml) was placed into a small conical flask and dried under air. Gel PBS (5ml) was then added to reconstitute the NSB solution.

Testosterone antiserum

The testosterone antiserum (lot 457) was raised in sheep against testosterone-3-carboxymethyloxime-BSA conjugate and was a gift from Dr. R.I. Cox (CSIRO, Prospect, NSW Australia). The cross reactivity of this antiserum is 98% with 5a-dihydrotestosterone,

47% with 4-andosten-3ß, 17ß-diol, 4.7% with androstenedione, 3.6% with 4-androsten-17ß, 19-diol-3-one and less than 1% with other steroids.

The antiserum stock was prepared by dissolving 10mg of antiserum (crystaline) into Gel buffer 10ml. This stock was aliquoted (500µl) and stored frozen at -20°C. The working solution of testosterone anitsera was made by adding Gel PBS (100µl) to the antiserum stock (500µl).

2.6.1.3 Assay protocol

Toluene:hexane (2:1) (1ml) was added to each of sample, NSB or standard (100µl) in disposable glass test-tubes (12x75mm) and extracted for 30 seconds on a vortex mixer. The aqueous phase was then frozen in a liquid nitrogen and ethanol bath, allowing the organic phase to be decanted into clean tubes. The extracted samples were then placed in a water bath (40°C) and dried under a stream of compressed air. 3H-testosterone assay solution (200µl) together with testosterone antiserum (200µl) was then added to the dried samples, NSB and standards and then incubated overnight at 4°C. Free and bound testosterone were separated with dextran coated charcoal (200µl) for 10 minutes before being centrifuged (1000g) for 20 minutes. The supernatant was then transferred to polyethelene scintillation vials ready for scintillation and counting.

2.6.1.4 Scintillation fluid and counting

Biodegradable counting scintillant (BCS) was used as a scintillation fluid in all testosterone assays.After adding the sample and scintillation fluid (1ml/vial), the polyethylene vials were shaken for 20 minutes on a mechanical shaker. The samples were then counted for ß emmissons in a LKB-Wallac, Rack Beta II liquid scintillation counter.

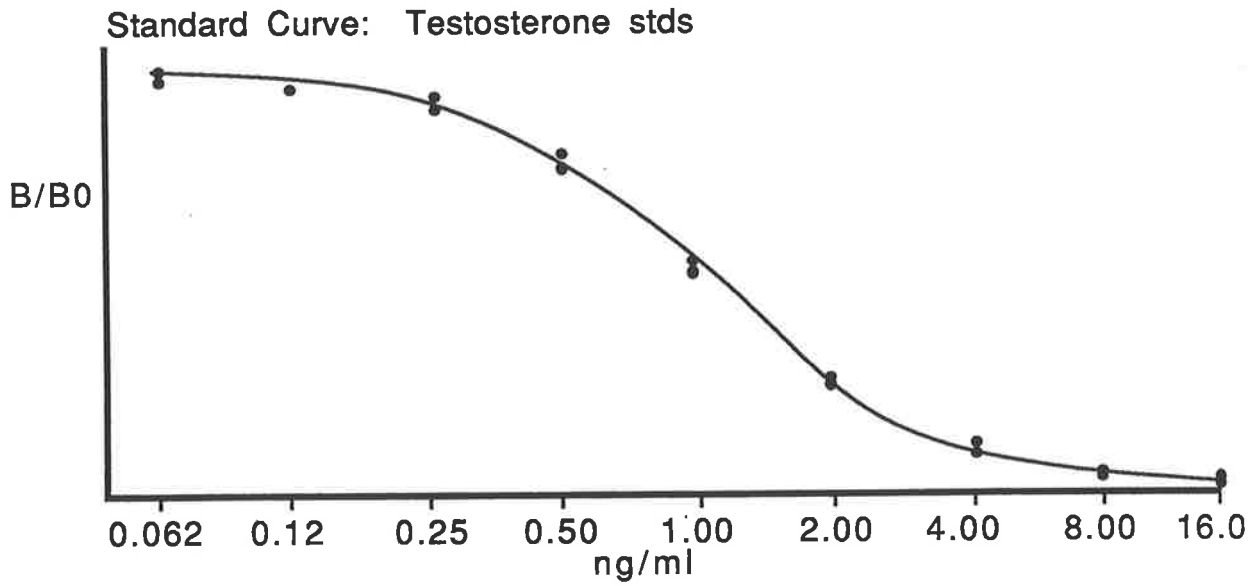


Figure 2.2 Testosterone Assay: Standard Curve

2.6.2 Prostaglandin Assays

2.6.2.1 Buffer and Solutions

Tris-EDTA-Gelatin Buffer pH 7.4

Tris	12.11g
EDTA	0.5g
Gelatin	1.0g

Made up to 1L in distilled H_2O

Acetate Buffer 1.5M pH5.1

Anhydrous Sodium Acetate 123g

Made up to 1L in distilled H_2O

Phosphate Buffer pH7.4

KH_2PO_4 (anhydrous)	0.14g
Na_2HPO_4(anhydrous)	0.426g
NaCl	8.776g
Gelatin	1.0g
NaN_3	1.0g

disolved in 1L distilled H_2O at 37°C

Dextran coated charcoal mixture

Charcoal Norit-A	500mg
dextran T_{70}	25mg

was added to 100ml phosphate buffer at 4°C.

2.6.2.2 Stock solutions and assay solutions

^{3}H-Prostaglandin stock solution

To ^{3}H-Prostaglandin E_2 (PGE_2)(100µCi) or ^{3}H-Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)(100µCi) methloxime hydrochloride (500µl) and acetate buffer (2ml) were added. After heating at 60°C for 30 minutes the prostaglandin was extracted in ether:ethylacetate 3:1 (10ml). The organic phase was washed in H_2O, recovered and evaporated under nitrogen. Ethanol (1ml) was then added to reconstitute the tracer.

^{3}H-Prostaglandin methyloxime assay solution

The prepared primary stocks of PGE_2 and $PGF_{2\alpha}$ (200µl) were then placed into a conical flask and dried under air. Tris-EDTA-gelatin buffer was then added to this to make an assay solution with an activity of 5000 cpm/100µl.

Prostaglandin standards

Both PGE_2 and $PGF_{2\alpha}$ primary standards were diluted in methanol (1mg/ml) and stored at -20°C.

For PGE_2 the primary standard (100µl) was then diluted in ethanol (10ml) to form standard B. Standard B (10µl)was further diluted by adding ethanol (10ml) to create a top standard of 1000pg/100µl. The top standard was then serially diluted in Tris-EDTA-gelatin buffer to make 8 standards; 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8pg/100µl.

For $PGF_{2\alpha}$ the primary standard (50µl) was then diluted in ethanol (10ml) to create standard B. Standard B (800µl) was then diluted in PBS (100ml) to give a concentration of 4624pg/100µl. This top standard was then serially diluted in Tris-EDTA-gelatin buffer to make 8 standards; 4624, 2312, 1156, 578, 289, 144, 72.2 and 36.1pg/100µl.

Non-specific binding

200µl of either PGE_2 or $PGF_{2\alpha}$ stock solution (1mg/ml) was placed into a small conical flask and dried under air. Tris-EDTA-gelatin buffer was then added to reconstitute the NSB solution.

Prostaglandin antiserum

PGE_2 antiserum was raised in angora cross-bred goats against PGE_2 bovine serum albumin conjugate and supplied by S. Deam (Department of Obstetrics and Gynaecology; University of Adelaide). The cross-reactivity for this antiserum is 100% with PGE_2, 53% with PGE_1, 31% with PGE_3, 1.5% with $PGF_{2\alpha}$ and less than 1% with all other prostglandins.

$PGF_{2\alpha}$ antiserum was raised in rabbits against $PGF_{2\alpha}$ bovine serum albumin conjugate (Department of Chemical Pathology, University of Natal, Durbin). The cross-reactivity of this antiserum is 100% $PGF_{2\alpha}$, 10%$PGF_{1\alpha}$ and less than 1% with all other prostaglandins

2.6.2.3 Assay Protocol

Samples were methyloximated as for the ^{3}H-prostaglandin stock solution. All standards, NSB's and samples (100µl) were diluted in 10mM phosphate buffer, ph 7.4, containing 0.1% gelatin and 0.1% NaN3 (200µl). Tritiated PGE_2 and $PGF_{2\alpha}$ tracer, and diluted antisera (PGE_2 1:1750, $PGF_{2\alpha}$ 1:250) were combined and incubated at 37°C for one hour before being stored at 4°C overnight.

Unbound radio-labelled prostaglandin was removed by the addition of 500µl of cold Dextran-Charcoal for 10 minutes at 4°C before centrifugation at 4000g for 10 minutes. After centrifugation the supernatant was decanted into scintillation vials to which BCS scintillant (1ml) was added.

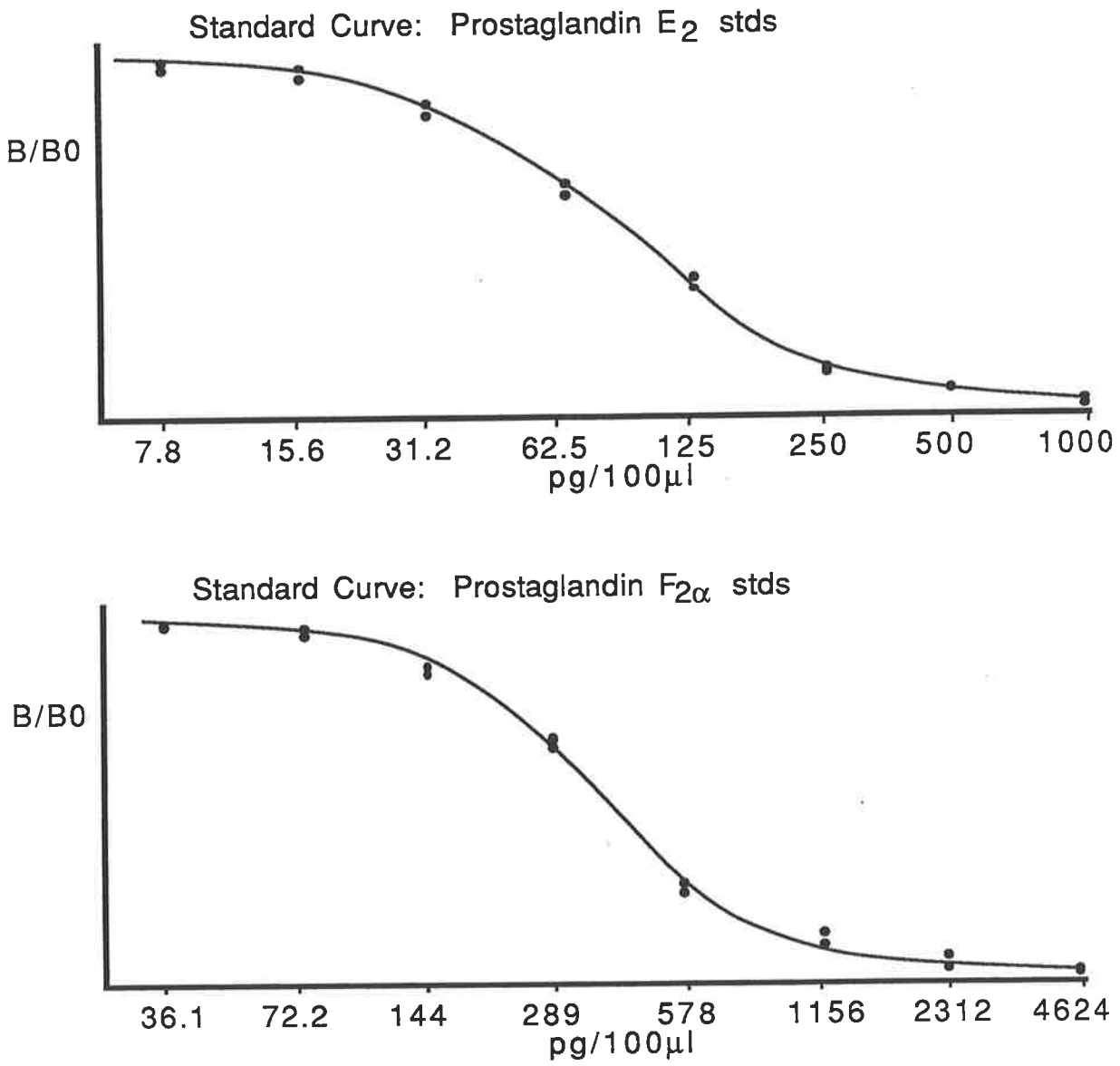


Figure 2.3 Standard curves for prostaglandin E_2 and $F_{2\alpha}$.

2.6.2.4 Scintillation fluid and counting

Biodegradable counting scintillant (BCS) was used as a scintillation fluid in all testosterone assays.After adding the sample and scintillation fluid (1ml/vial), the polyethylene vials were shaken for 20 minutes on a mechanical shaker. The samples were then counted for ß emmissons in a LKB-Wallac, Rack Beta II liquid scintillation counter.

NOTE

This chapter only addresses general protocols used throughout this thesis. Where specific techniques and methods have been established and their suitability and specificity then verified prior to further use within a specific section of this work, the procedures employed will be described in the appropriate chapter.

CHAPTER 3: Characterisation of the Testicular Macrophage by Macrophage Markers.

Chapter 3. The characterisation and heterogeneity of rat testicular macrophages.

3.1 Introduction

Circulating monocytes differentiate into macrophages once they migrate from the peripheral blood to the tissues (Van Furth, 1972). Once resident in a tissue they adopt a phenotype characteristic of the environment in which they reside and the function they must perform (Flotte et al, 1982; Haines et al, 1983). As a result macrophage populations from different tissues are heterogenous, and even within a single organ such as the spleen the macrophage population may differ depending on location (Buckley et al, 1987). The differences between these cells and the markers they express are important not only in the identification of these cells but also in determining their functional characteristics, particularly with respect to the cells immuno-biology.

One such way in which these markers relate to macrophage immunobiology is the presence of the Fc receptor on the macrophage. The Fc receptor is essential for immuno-phagocytosis of antibody coated antigen. It recognises the Fc fragment on the IgG antibody and so initiates opsonisation and the subsequent phagocytosis of the foreign particle by the macrophage. The presence of the Fc receptor may also be used to determine whether the cell is activated or at rest.

Sub populations of macrophages within a tissue may also be detected by observing the manner in which they express different surface and cytoplasmic antigen. For example, splenic macrophages have been shown to express different combinations of these antigenic determinants in relation to their location within the spleen (Dijkstra et al, 1985).

In this study, several macrophage specific monoclonal antibodies and an opsinisation/ingestion test were used to characterise and examine the heterogentiety of macrophages isolated from the rat testis. This allowed characterisation of the isolated macrophages in culture, providing both identification and a method of measuring purity in cell culture. The expression of these markers by testicular macrophages in vitro was then verified in vivo by immunocytochemical screening of cryostat testicular tissue sections.

To test Fc receptor expression, testicular macrophages were compared with peritoneal macrophages (in resting and activated states) for their ability to bind and ingest IgG-coated sheep red blood cells through receptor-mediated phagocytosis. This allowed a direct comparison between the cell types and a subsequent assesment of the immuno-phagocytic function of the testicular macrophage.

3.2 Materials and Method

Macrophages were isolated from enzyme-digested testes (see 2.3.2), using a novel three step protocol to attain highly purified testicular macrophage preparations and peritoneal macrophages from the peritoneal cavity were isolated as in 2.3.1. Cryostat sections of testis, spleen, kidney and lymphatic tissue were prepared for immuno-cytochemistry as outlined in Chapter 2 (see 2.5.4.)

Whole testicular interstitial cell suspensions recovered from Ficoll density gradients were further fractionated by cell elutriation. The flow rates employed correspond to those used successfully by Dirami *et al* (1990), (see 2.3.3), to obtain semi-pure testicular macrophages. In a final step to reduce contaminating cells, macrophages were positively selected from whole or elutriated fractions on the basis of their rapid adhesion to glass coverslips. After

purification the peritoneal and testicular macrophages were cultured (10^6 cells/coverslip) at 37°C and 34°C respectively for 24 hours in a humidified atmosphere of 5% CO_2.

The macrophage content of preparations were determined by measuring the proportion of cells positive (stained brown) for the macrophage markers CD11b, W3/25 or for macrophage specific monoclonal antibodies ED1, ED2, ED3. Macrophages were also identified by the histochemical stain for non-specific esterase activity, and contaminating Leydig cells were identified by their βHSD enzyme activity. The presence of lymphoid dendritic cells within the testis and cell cultures was screened for with the monoclonal antibody ED5.

The antibody CD11b was chosen for its ability to recognise the Mac 1 receptor, a complement receptor common to most macrophage species, while W3/25 is specific for the CD4 receptor on rat macrophages, and a sub-population of (helper/inducer) T cells. ED1, ED2 and ED3 are monoclonal antibodies which recognise antigens exclusive to rat tissue macrophages. ED1 recognises a cytoplasmic anitgen specific to both tissue bound and free macrophages. The ED 2 antibody is specific for a membrane antigen found on resident tissue macrophages while the ED3 antibody is specific for lymphoid tissue macrophages. The ratio of these three macrophage specific antibodies may be used to discriminate between distinct subpopulations of macrophages within organs (Dijkstra et al, 1985). ED5 is a monoclonal antibody which recognises denritic cells present in B-cell follicles of the spleen and lymph nodes (Jeurissen and Dijkstra, 1986).

Erthyrocyte rosetting and ingestion by the peritoneal and testicular macrophages were carried out as described in Chapter 2. The presence and expression of the Fc receptor on these cells was used to determine the immuno-competance of the testicular macrophage and the cells ability to participate in immuno-phagocytosis and antigen-antibody complexing. Only

instances where macrophages bound three or more erythrocytes were counted as rosettes (see 2.5.5).

The Students t-test was used to compare expression of macrophage markers in the crude testicular fraction with the elutriated fractions in Table 3.1. In Tables 3.2&3.3, the t-test was used to compare non-stimulated and LPS treated macrophages from the same isolation procedure. A two way ANOVA was used to determine differences in Fc expression between macrophages from different isolations.

3.3 Results

3.3.1 Macrophage Expression of Immunocytochemical Markers.

Cryostat sections of testicular tissue were positive for the monoclonal antibodies CD11b, W3/25, ED1, ED2 and ED3 (Plates 3.1, 3.2, 3.3 & 3.4). Coverslip cultures of crude macrophage preparations obtained after density gradient fractionation and glass adherence were found to be comprised of 82±2.1%, 86±1.8%, 78±1.7%, 85±1.9%, 22±3.1% and 87±1.2%, CD11b, W3/25, ED1, ED2, ED3 and NSE-positive cells respectively (Plate 3.6, 3.7 &3.8; Table 3.1). Staining for ED5 was not observed in any of the testicular cell populations, or on any cryostat sections of testicular tissue, however it was shown to be present in the white pulp of the spleen (Plate 3.2).

The expression of ED1, ED2 and ED3 by the isolated testicular macrophages was uniform with the exception of fraction 1 (19ml/min; Plate 3.7). The cells eluted at a lower flow rate in fraction 1 are smaller in size than those eluted in fractions 2 and 3. Cells in Fraction 1 also express higher proportions of ED1 and ED3 relative to ED2 than observed in Fractions 2 and 3. In fraction one, ED1 expression was equal ED2 expression, although ED2 expression was significantly higher in the whole macrophage preparation and fractions 2 and 3. The

Table 3.1

Expression of macrophage and Leydig cell specific markers by elutriated fractions of isolated testicular macrophages

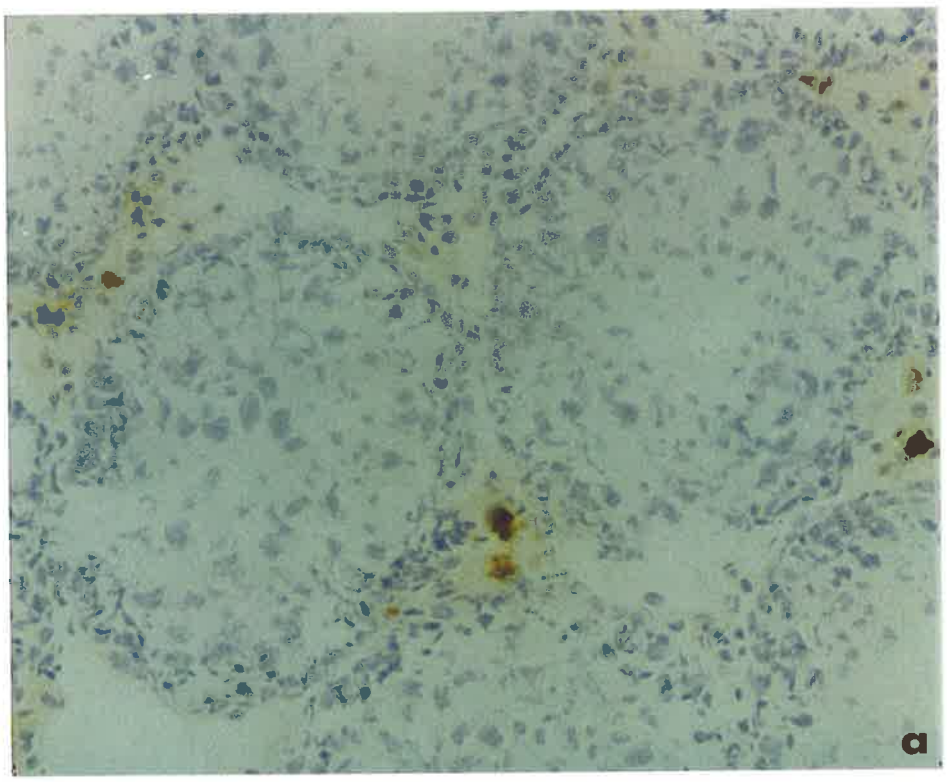
	% Cell Expression (mean ± SEM)						
	CD11b	W3/25	ED 1	ED 2	ED 3	NSE	ß-HSD
TM whole fraction	82±2.1	86±1.8	78±1.7	85±1.9	12±3.1	87±1.2	8.0±0.8
	Elutriated Fractions and Flow Rates						
Fraction 1 (19ml/min)	66±2.0*	64±2.8*	67±2.3*	62±3.6*	12±1.5	48±3.6*	2.5±0.5*
Fraction 2 (28ml/min)	94±1.1*	95±1.1*	87±1.7*	95±1.3*	11±3.7	93±1.1*	3.6±0.7*
Fraction 3 (31ml/min)	83±2.3	84±2.0	78±1.9	83±2.5	12±3.0	84±4.0	9.9±1.0

n=6 macrophage isolations. * = $P<0.001$ when compared to TM whole fraction.

Plate 3.1

Cryostat sections of adult rat testis (a) and spleen (b) stained with monoclonal antibody ED2 (brown colour) and counterstained with haemotoxylin. Note that the testicular macrophage stains strongly for ED2 (representative of resident macrophages) and staining in the spleen is restricted to the red pulp and the boundary of the follicle (white pulp) but not inside the follicle itself.

x250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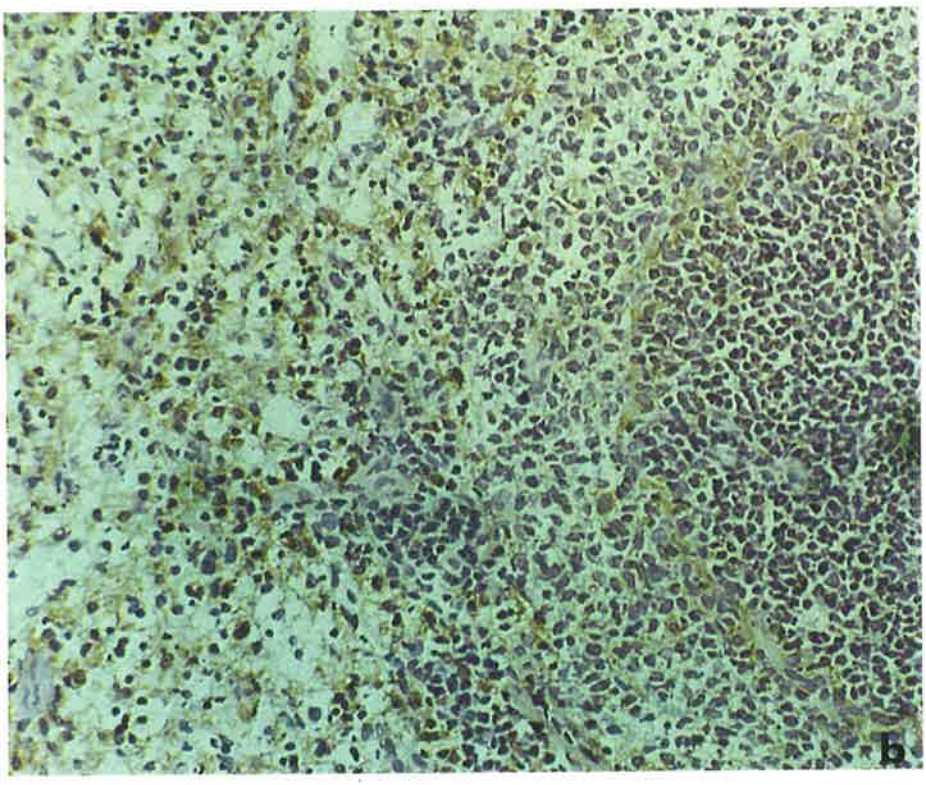
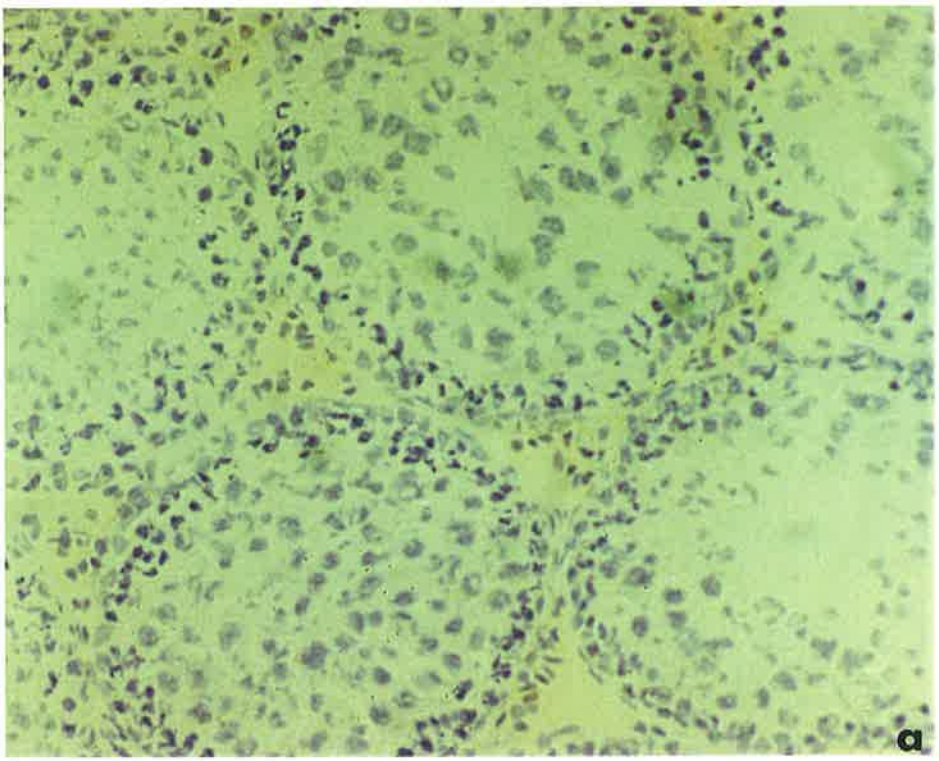

b

Plate 3.2

Cryostat sections of adult rat testis (a) and spleen (b) stained with monoclonal antibody ED5 (brown colour), specific for lymphoid dendritic cells. While negative for cells in the testis, in the spleen ED5 was specific for dendritic cells within the white pulp.

x250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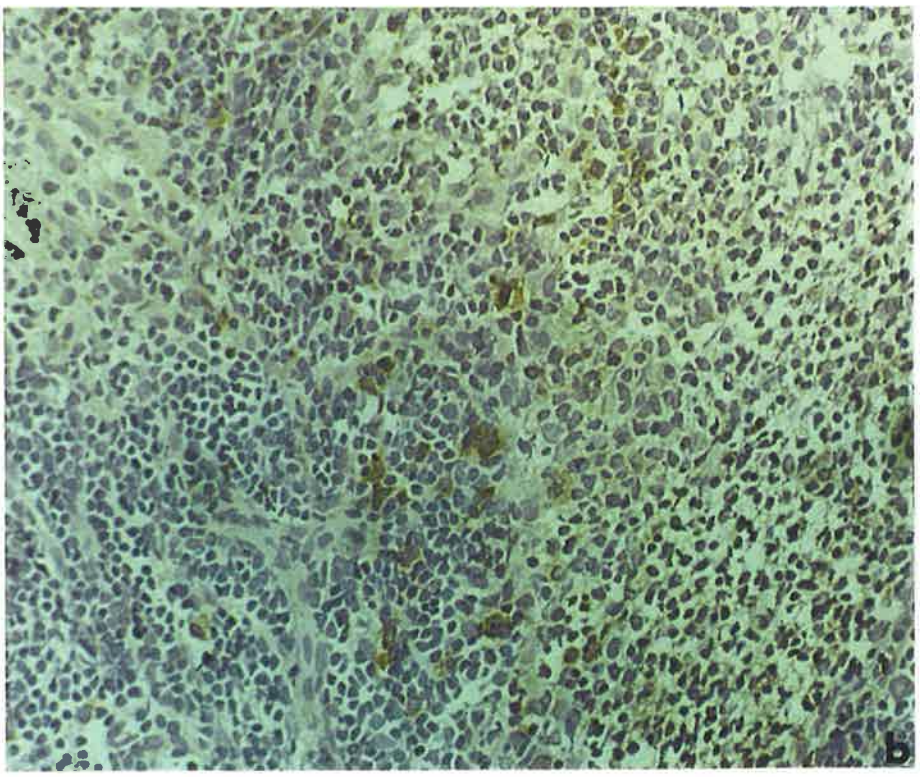
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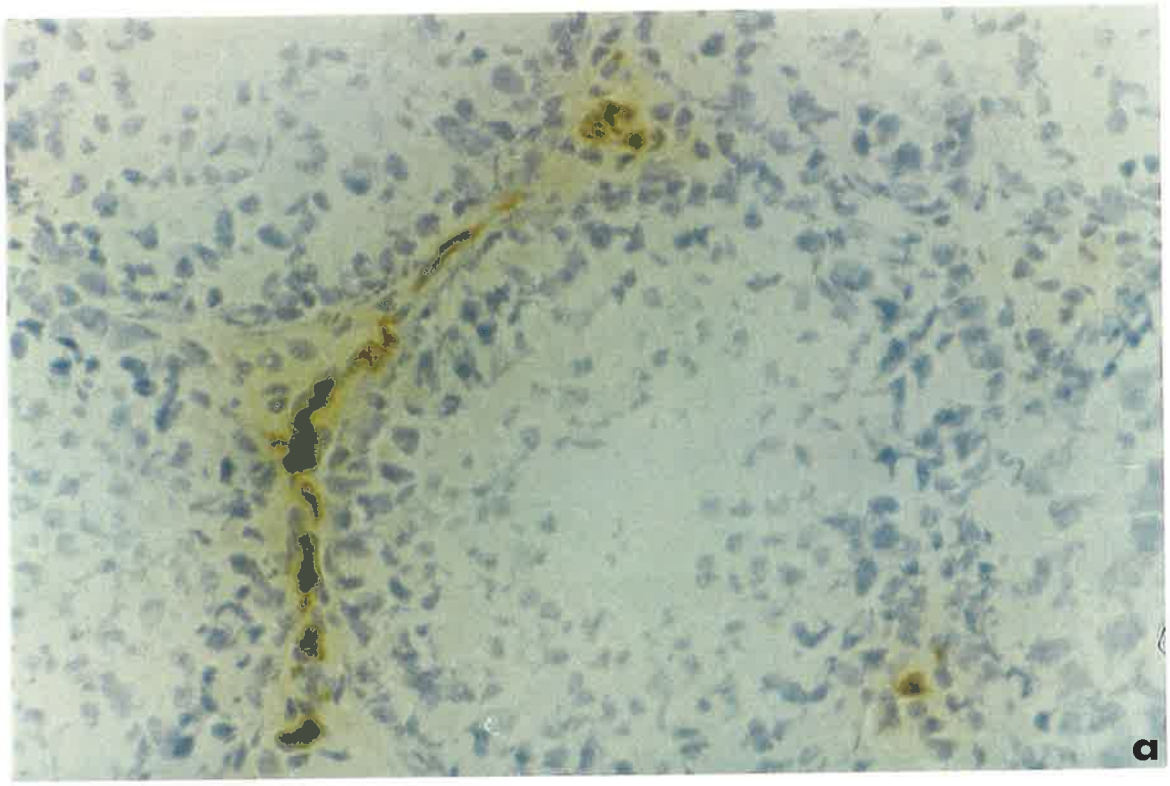
Plate 3.3

Cryostat sections of adult rat testis stained for ED1 (a) and W3/25 (b); both stained brown. ED1 is specific for cells of the monocyte-macrophage lineage, while W3/25 is recognises the CD4 receptor on rat macrophages and a sub-population of helper T-cells. x250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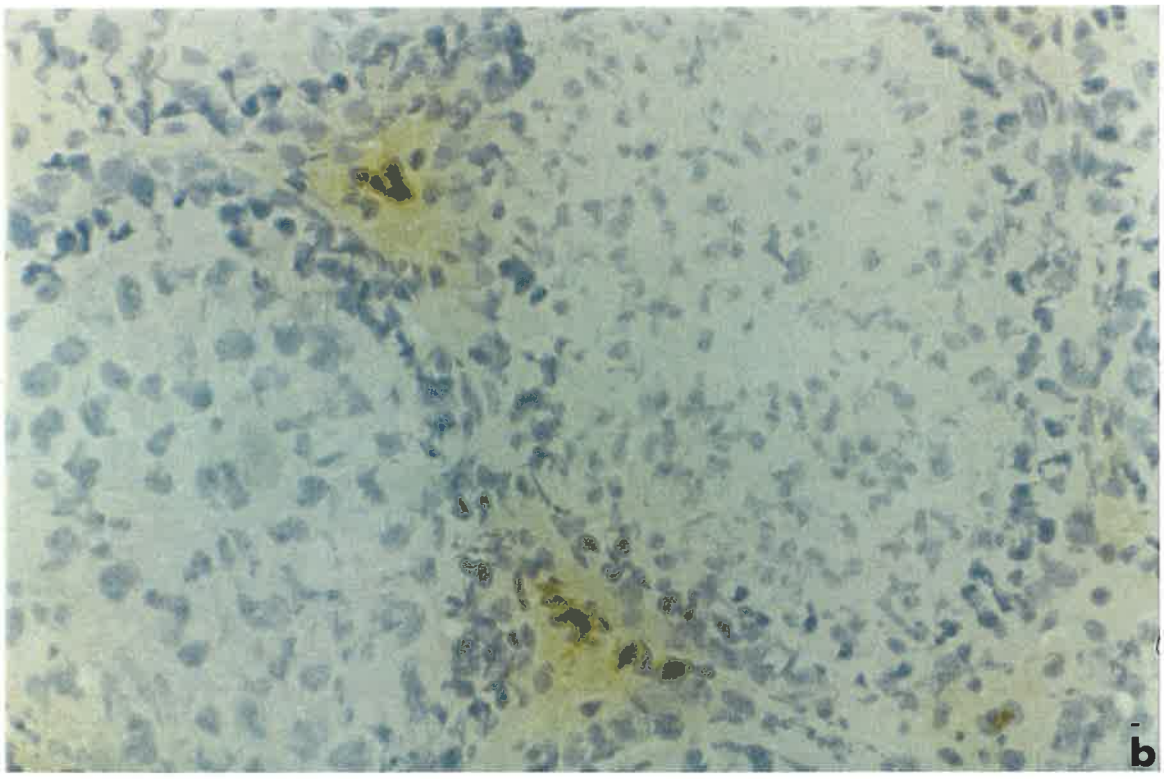
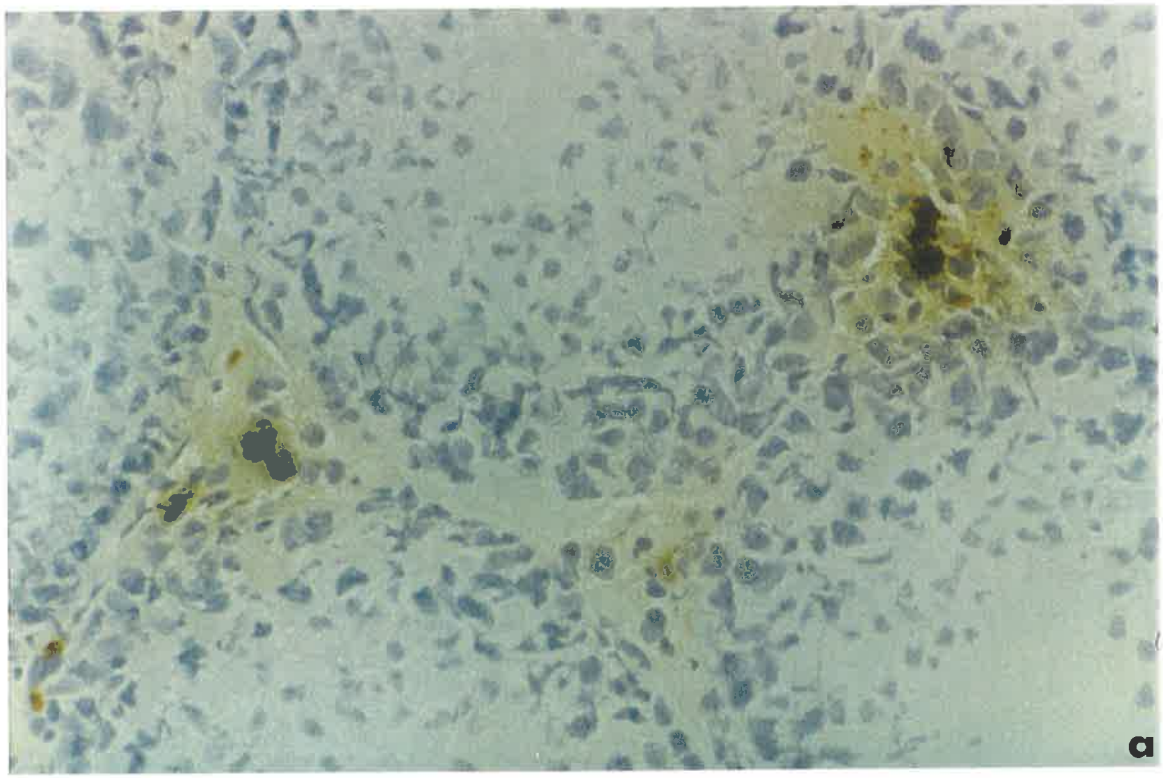

b

Plate 3.4

Cryostat sections of adult rat testis stained for ED2 (a) x 250 and CD11b (b); both stained brown. ED2 is specific for resident macrophage populations while CD11b is stains for the Mac 1receptor, a complement receptor common to most macrophage species.

x 125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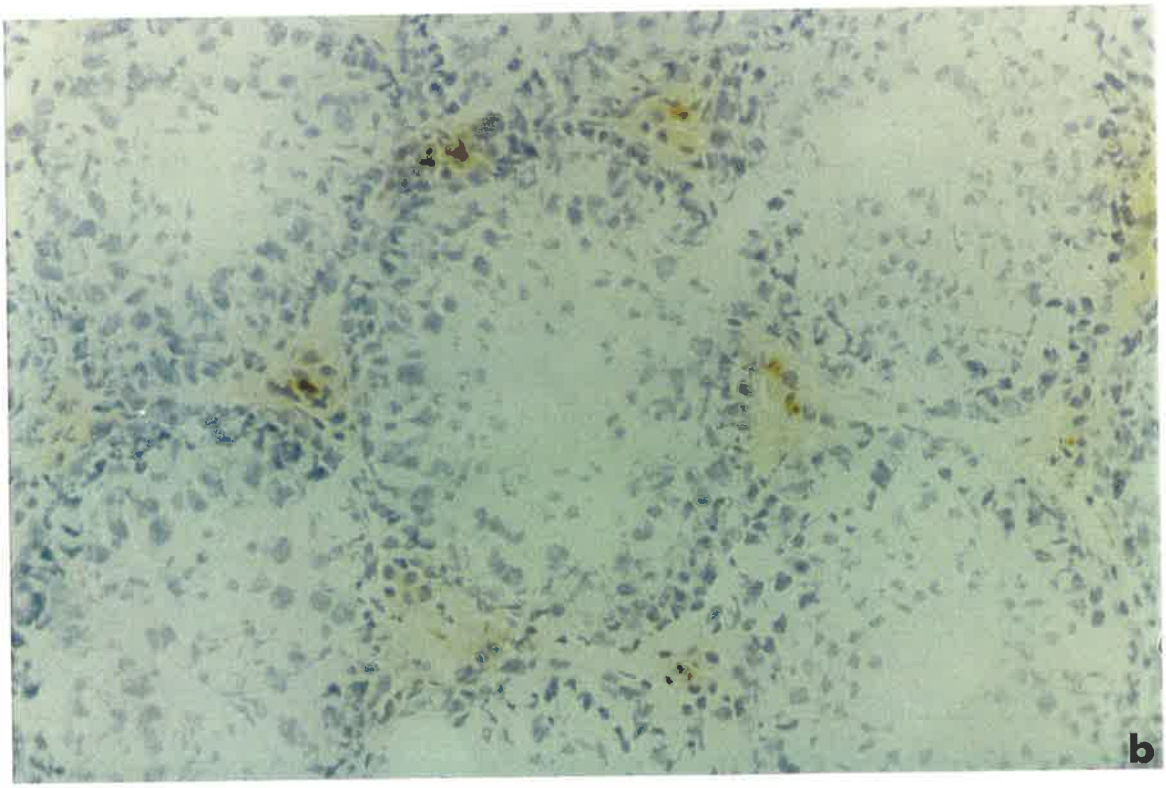
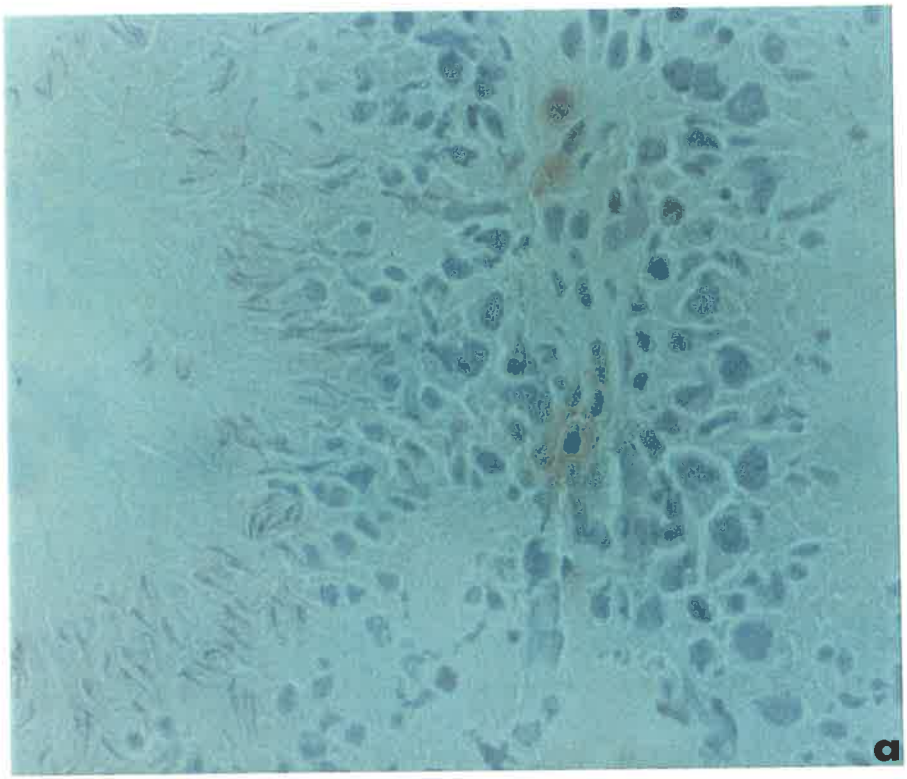

b

Plate 3.5

Cryostat sections of adult rat testis (a) and kidney (b) stained with the monoclonal antibody ED3 (brown colour). ED3 is specific for lymphoid tissue macrophages. Compare the weak staining of ED3 in the testis with the strong staining by mesangial cells in the kidney.

x 320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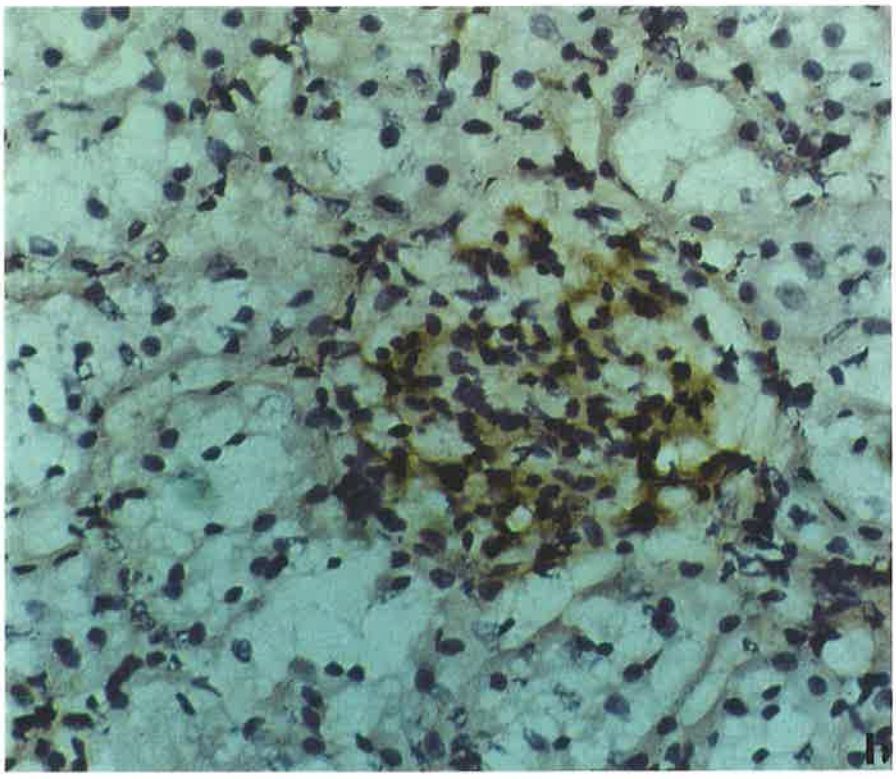
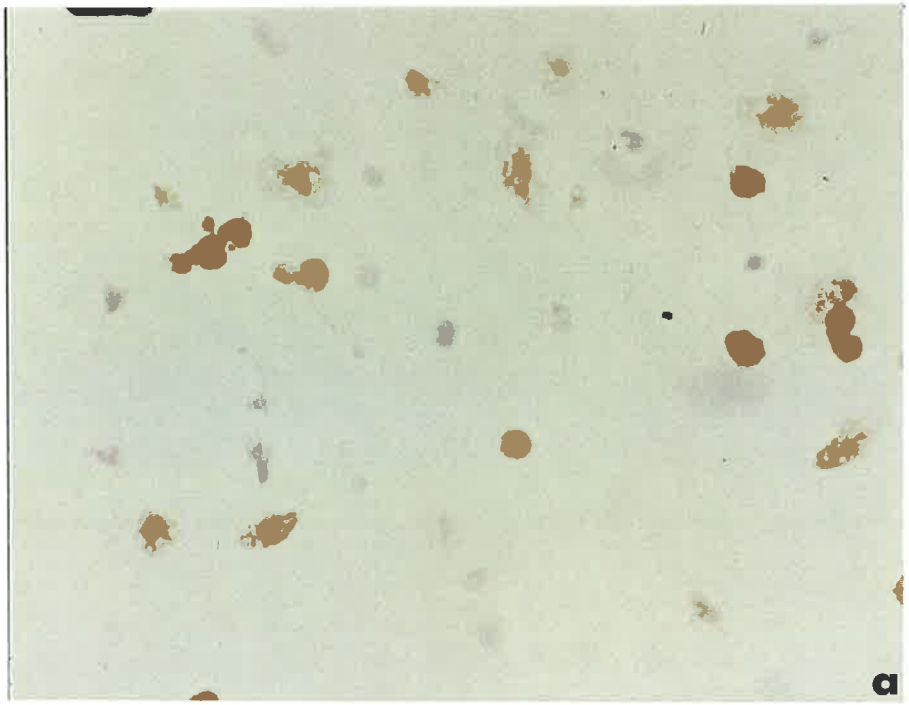

b

Plate 3.6

Macrophage cultures isolated using only ficoll and glass adherence, and stained for non-specific esterase activity (stained brown; a) or for the presence of ßHSD activity by contaminating Leydig cells (stained purple; b). Note the high level of Leydig cell contamination in these cultures due to the absence of elutriation during the purification procedure (see also Table 3.1).

x125

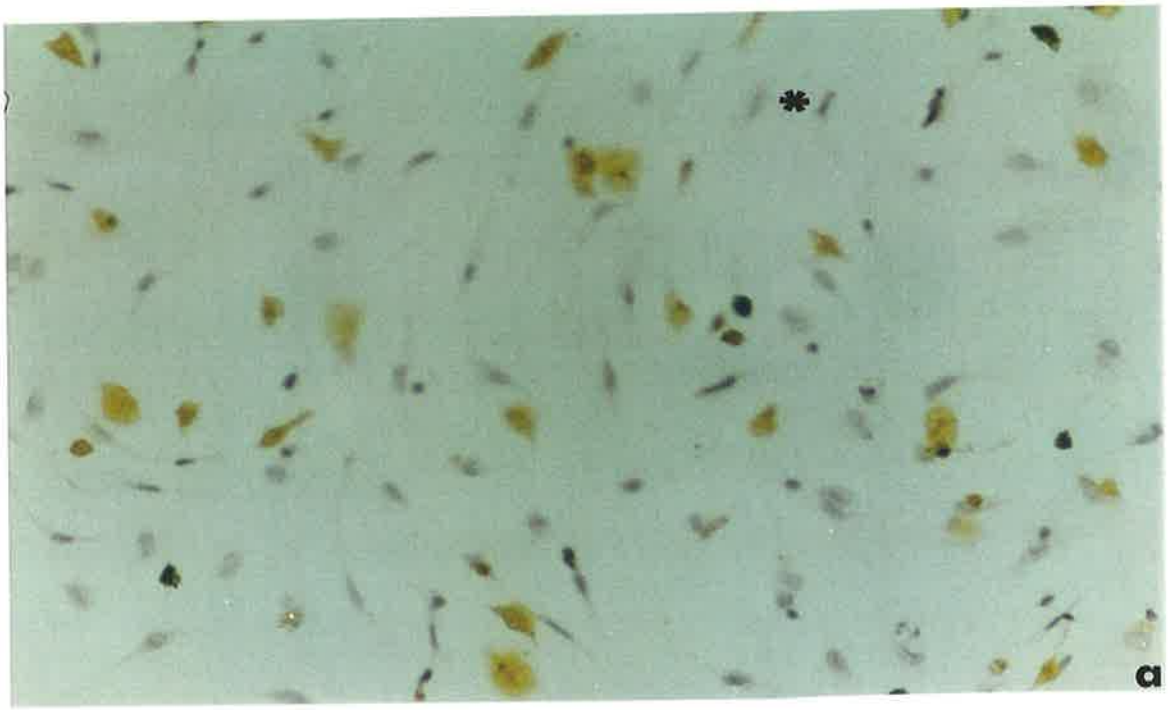

a


b

Plate 3.7

Cultures of testicular macrophages purified by elutriation prior to glass adherence and stained with the monoclonal antibody ED2 (brown colour) and counterstained with Haemotoxylin. Macrophage cultures from fraction one (flow rate 19 ml/min) were contaminated with a considerable number of fibroblast-like cells (stained purple; asterix) while cultures from fractions 2 (flow rate 28ml/min) and fraction 3 (flow rate 31 ml/min) were more highly purified.

x 250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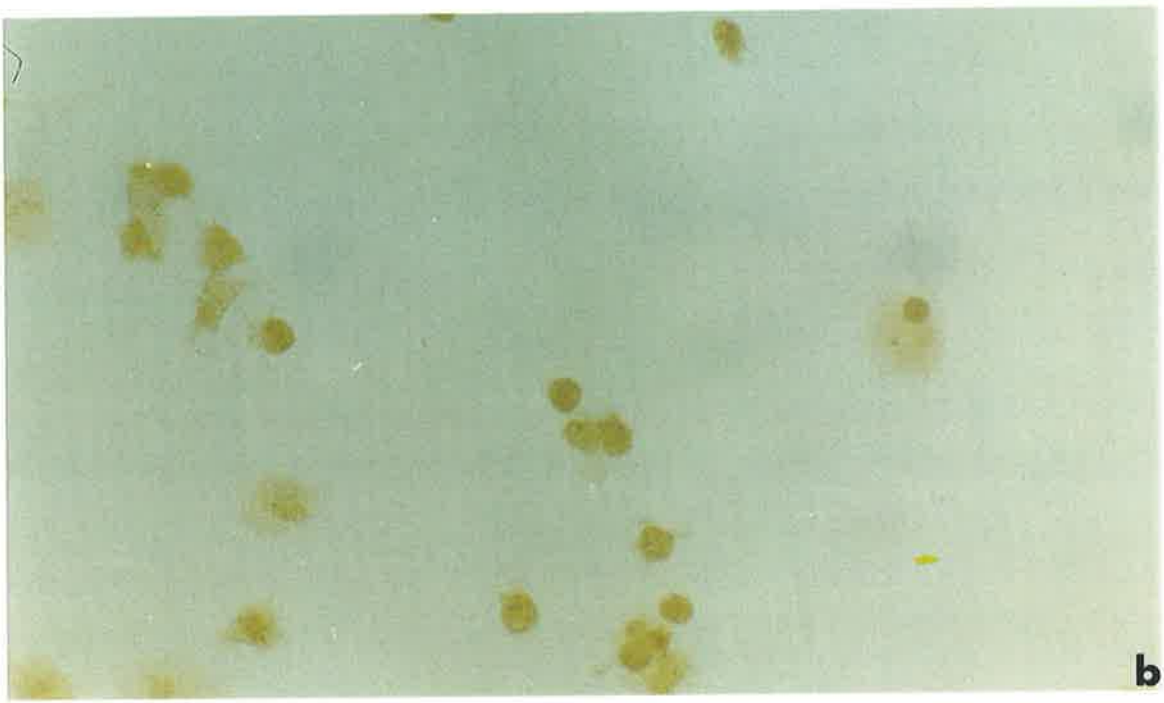
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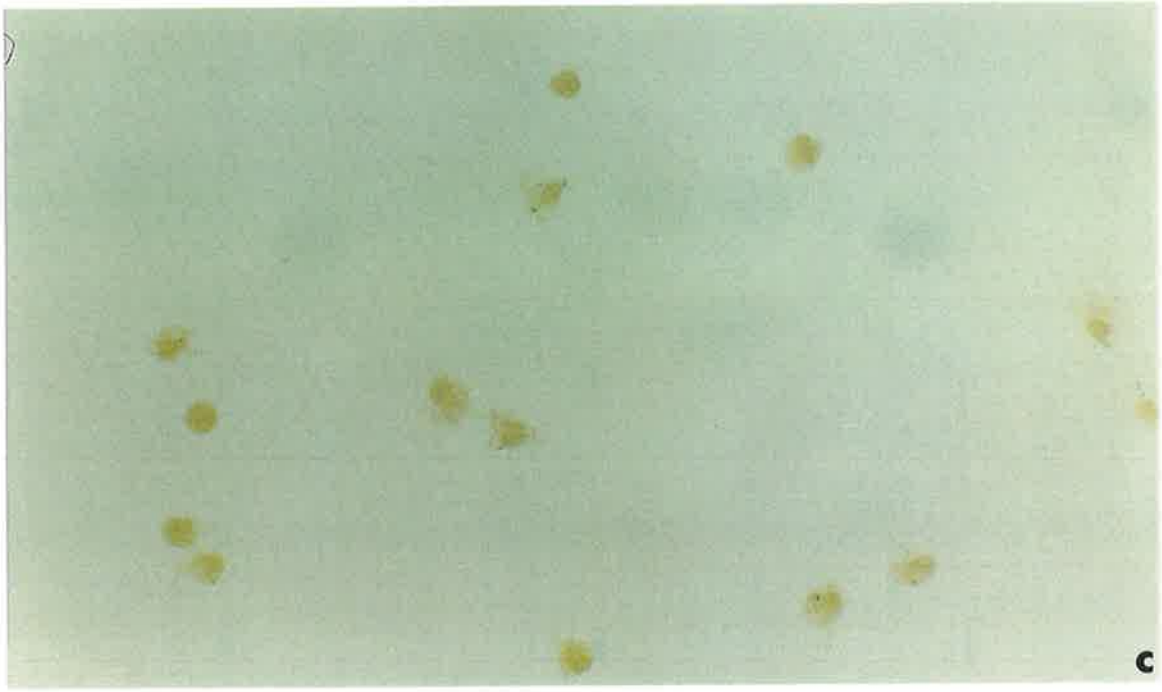
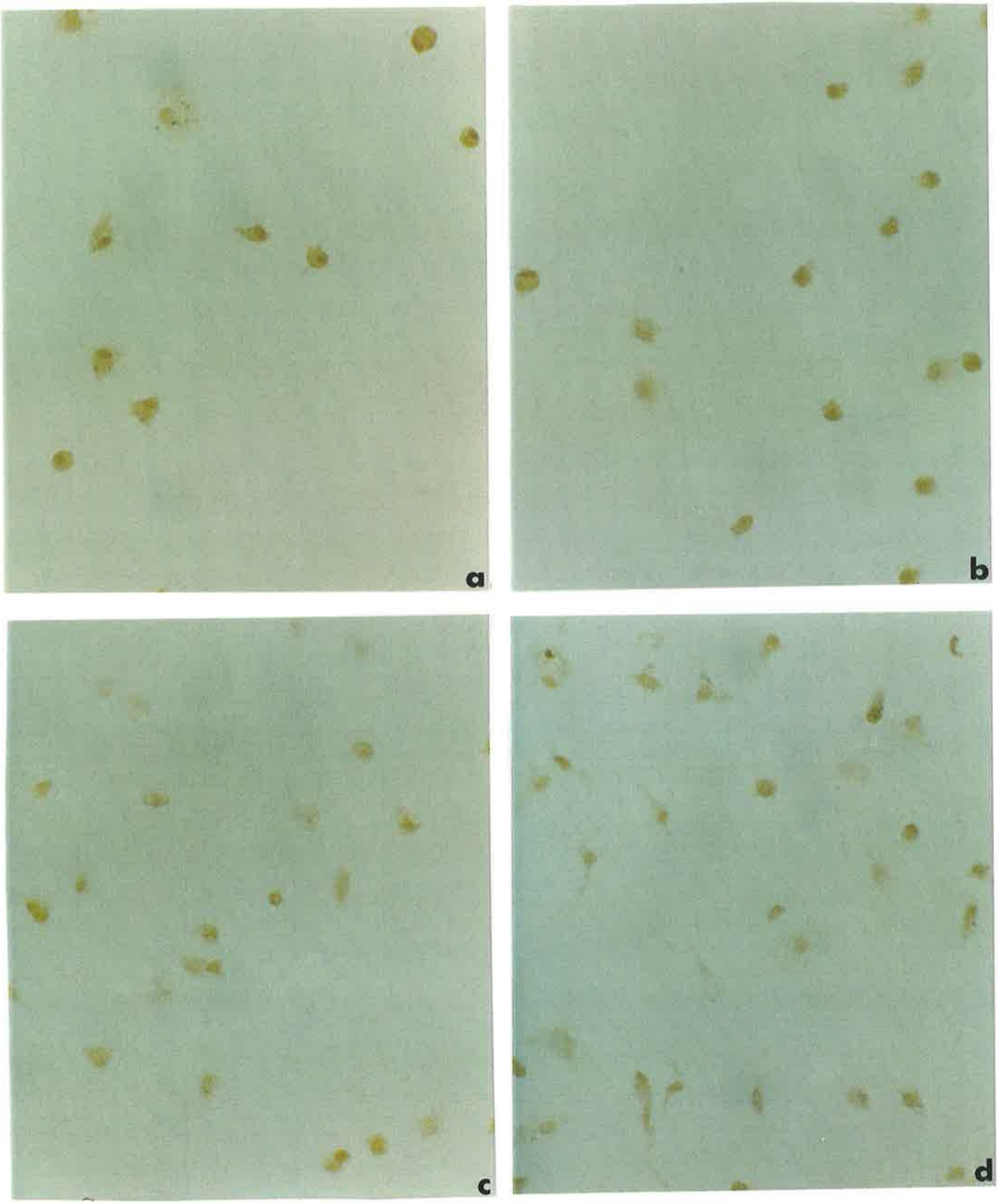

c

Plate 3.8

Cultures of testicular macrophages elutriated in fraction 2 (flow rate 28/ml/min) and stained with monoclonal antibodies for ED1(a), W3/25(b), CD11b (c) and ED3 (d).

x 125


a
b
c
d

highest expression of ED3 was also observed in fraction 1. However, ED3 expression was present on a comparitively small number of testicular macrophages, and stained weakly in comparison to ED1, ED2 and the other monoclonal antibodies used. ED3 while present in testicular tissue was more readily present on macrophages from the kidney (plate 3.5)

When whole macrophage preparations were subjected to elutriation prior to glass adherence macrophages were found to be significantly enriched ($P<0.001$) over cultures relying solely on glass adherence. Cultures of cells elutriated in fraction 2 (plate 3.8; cells collected at a flow rate of 28ml/min) had 94±1.1% of cells expressing CD11b, 95±1.1% expressing W3/25, 87±1.7% expressing ED1, 95±1.3% expressing ED2, 11±3.7% expressing ED3 and 93±1.1% expressing NSE activity (Plate 3.6).

The Leydig cell content in fraction 2 was just 3.6±0.7% compared with 9.9±1.0% in the whole macrophage preparation (sig $P<0.001$). Although the Leydig cell content of fraction 1 (2.5±0.5%) was even less than that of fraction 2 (3.6±0.7%), fraction 1 was found to be contaminated with large numbers of cells, judged on the basis of their morphology in culture to be predominantly fibroblast cells, and hence the expression of the macrophage markers was significantly less than that in the whole macrophage preparation (Plate 3.7). Fraction 3 (31 ml/min) was found to have a similar macrophage and Leydig cell content to the whole macrophage preparation.

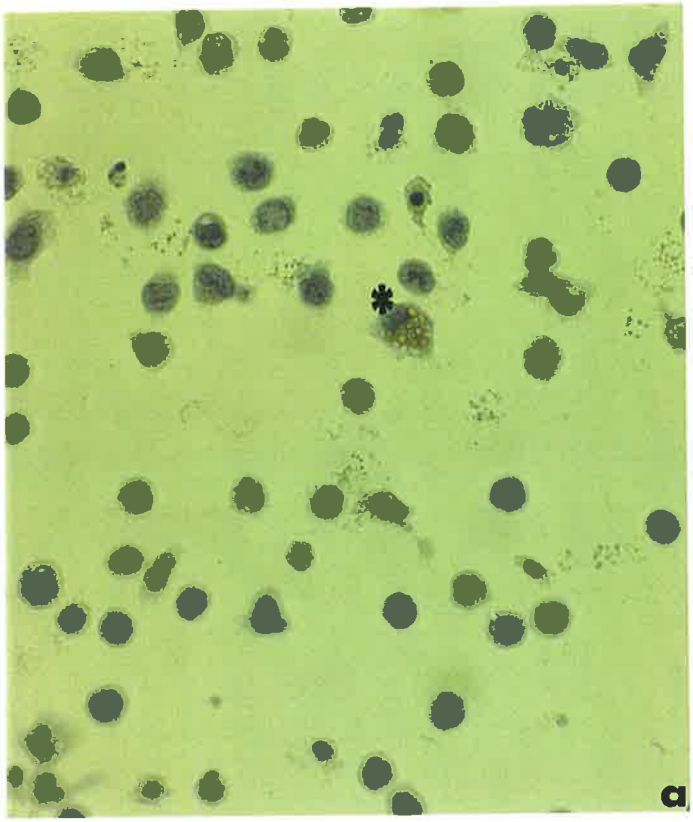
3.3.2 Fc receptor expression by testicular macrophages

The majority of peritoneal macrophages exist in a resting state which are readily activated by the non-specific stimulant LPS. This makes them ideal as a guide in determining the functional state of the testicular macrophage both in culture and *in vivo*. Freshly isolated peritoneal macrophages exhibited very little phagocytic activity at all (Fig 3.9; Tables 3.2&3.3), with the expression of Fc receptor (16±0.7% rosetting, 15±1.0%ingesting)

Plate 3.9

Quiescent peritoneal macrophage populations display low Fc receptor expression (a), although a proportion of the population is always active (asterix). After 24 hours, LPS-activated peritoneal macrophages (b) increased Fc receptor expression and bound and ingested immunoglobin coated red blood cells at levels comparable to the testicular macrophage (c & d). Note the avidly phagocytic nature of the testicular macrophage and that the levels of binding were unchanged from 0 hours (c) to 24 hours (d).

x 125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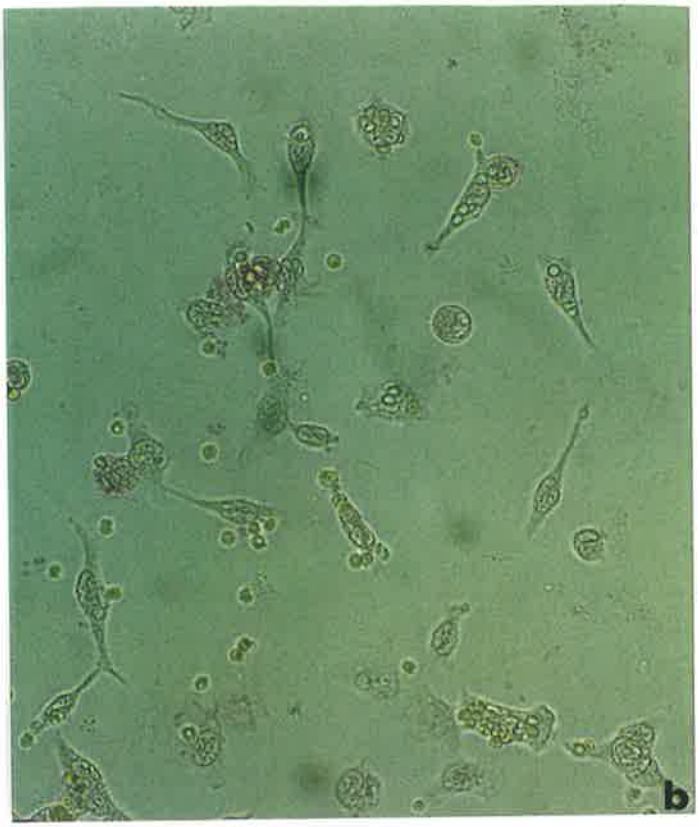
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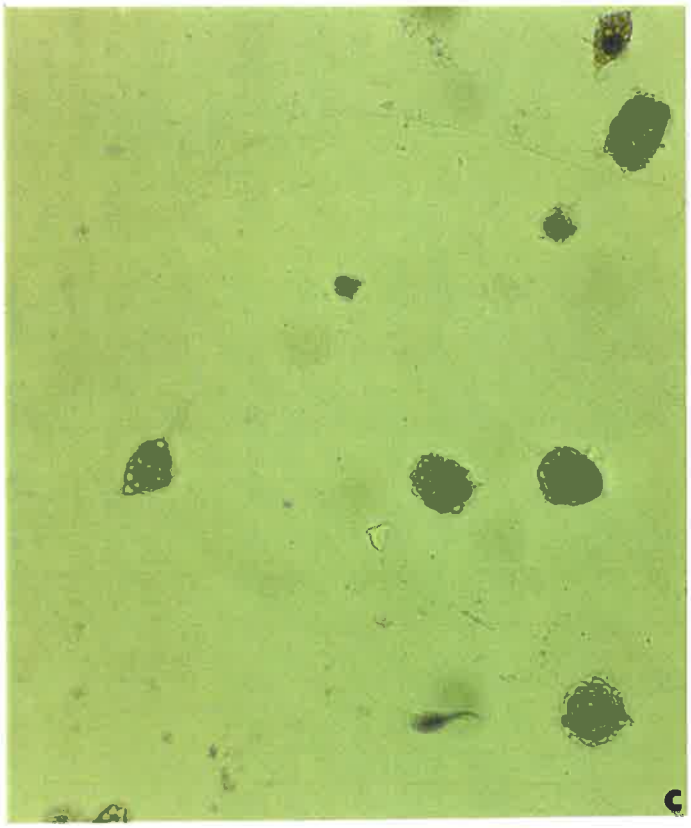

c


d

Table 3.2. Rosetting of IgG Coated Erythrocytes by Isolated Macrophages

Time (hours)	0	12	24	36	48
	%macrophage binding	%macrophage binding	%macrophage binding	%macrophage binding	%macrophage binding
Peritoneal Macrophages †	16±0.7	15±1.7	13±0.9	14±0.8	13.2±0.9
Peritoneal Macrophages +LPS †	15±1.0	35±3.0*	49±3.0*	75±2.4*	81±2.9*
Testicular Macrophages (whole fraction)	86±2.0	85±2.0	87±3.0	83±2.4	84±2.2
Testicular Macrophages (whole fraction) +LPS	84±2.9	86±1.6	83±2.7	79±2.2	81±3.8
Testicular Macrophages † F 1:Flow rate 19ml/min	45±2.2	41±2.6	45±1.7	39±2.0	40±2.2
Testicular Macrophages +LPS † F 1: Flow rate 19ml/min	46±2.0	39±1.6	44±2.1	40±1.9	40±0.8
Testicular Macrophages † F 2: Flow rate 28ml/min	91±2.4	94±2.9	90±2.0	94±2.5	95±2.2
Testicular Macrophages +LPS † F 2: Flow rate 28ml/min	94±2.6	91±1.9	92±1.0	93±1.5	93±2.7
Testicular Macrophages F 3: Flow rate 31ml/min	89±1.7	84±1.3	86±3.0	88±4.3	89±1.8
Testicular Macrophages +LPS F 3: Flow rate 31ml/min	85±2.2	87±3.0	84±2.4	82±2.1	90±1.3

*=P<0.001 when compared to non-stimulated cultures (students t-test)

†=P<0.001 when compared with expression in testicular crude fraction (2 way ANOVA)

Table 3.3 Ingestion of IgG Coated Erythrocytes by Isolated Macrophages

Time (hours)	0	12	24	36	48
	%macrophage ingestion	%macrophage ingestion	%macrophage ingestion	%macrophage ingestion	%macrophage ingestion
Peritoneal Macrophages †	5.6±0.4	6.7±0.8	3.9±0.4	4.7±0.5	5.4±0.4
Peritoneal Macrophages +LPS †	6.2±0.6	25±0.8*	48±1.6*	69±1.7*	75±3.0*
Testicular Macrophages (whole fraction)	70±2.1	70±2.0	74±3.0	70±2.3	77±2.8
Testicular Macrophages (whole fraction) +LPS	74±2.1	72±1.9	77±2.0	71±1.6	71±2.4
Testicular Macrophages † F 1:Flow rate 19ml/min	36±2.0	40±2.0	43±2.2	44±3.0	42±3.8
Testicular Macrophages +LPS † F 1: Flow rate 19ml/min	38±2.0	39±3.5	36±2.0	32±2.6	33±2.3
Testicular Macrophages † F 2: Flow rate 28ml/min	83±4.0	84±3.0	88±2.8	82±2.3	82±1.4
Testicular Macrophages +LPS † F 2: Flow rate 28ml/min	85±2.5	86±2.0	85±2.1	84±1.8	80±2.0
Testicular Macrophages F 3: Flow rate 31ml/min	74±2.4	71±2.6	70±3.2	77±3.9	74±2.0
Testicular Macrophages +LPS F 3: Flow rate 31ml/min	72±2.6	76±2.9	76±1.7	78±2.6	74±1.6

*=P<0.001 when compared to non-stimulated cultures (students t-test)

†=P<0.001 when compared with expression in testicular crude fraction (2 way ANOVA)

limited to a small active population or occuring purely by opportunistic means. The level of binding by the unstimulated peritoneal macrophages did not alter over 48 hours. However in the LPS activated cultures, there was a significant increase in the rosetting and ingestion of erythrocytes after 12 hours of culture (35±3.0% rosetting, 25±0.8% ingestion), which continued to increase during the 48 hours of culture (81±2.9% rosetting, 75±3.0% ingestion).

The increase in Fc receptor expression by the LPS stimulated peritoneal macrophages as they became activated highlighted the transition and differences between the functional states (activated, resting) that can be displayed by macrophages from the same tissue. Rosetting and ingestion of Fc receptor by freshly isolated whole preparations of testicular macrophages (86±2.0% and 70±2.1% respectively)was significantly in excess of the peritoneal populations and comparable to the binding observed in LPS stimulated peritoneal cultures after 48 hours (Figs 3.9; Tables 3.2&3.3). Futhermore, this level of Fc receptor expression was maintained without decline over 48 hours. Interestingly, the addition of LPS to testicular macrophage culture made no significant difference to Fc receptor expression over the course of the experiment.

Macrophage cultures derived from elutriated fractions expressed Fc receptor in a pattern that mirrored the purity of these cultures (Table 3.1.). Cells isolated at a flow rate of 19ml/min displayed significantly lower rosetting and ingestion (45±2.2% and 36±2.0% respectively) than either the crude isolate or the other two elutriated fractions. While cells isolated at a flow rate of 31ml/min demonstrated similar expression of the Fc receptor to the crude isolate, cells eluted at 28ml/min displayed significantly higher rosetting and ingestion again (91±2.4% and 83±4.0%).

3.4 Discussion

3.4.1 Macrophage Expression of Immunocytochemical Markers.

The results in Table 3.1, suggest that the macrophages resident in the testicular interstitium express many of the common macrophage surface markers. Futhermore, the detection of these immunohistochemical markers both *in vivo* and *in vitro* suggests the isolation procedure does not alter their expression. The uniform expression of these markers also suggests that macrophages resident in the testis consist of a large homologous population and possibly a smaller sub-population.

While the testicular macrophages were observed to stain for the common leukocyte markers, CD11b and W3/25, of particular interest was the expression of ED1, ED2 and ED3. These monoclonal antibodies recognise macrophage-specific markers, and have been used by Dijkstra et al (1985) to discern subpopulations of macrophages within rat tissues. The testicular macrophages expressed all three of these monoclonal markers although expression of ED3 appeared weaker than for the other two. This is in contrast to a study by Wang et al, (1994) which found no expression of ED3 within the testis.

Although the macrophages resident in the testis appear to be uniform in their expression of surface and cytoplasmic antigen, it seems there may be a minor population of macrophages expressing a different phenotype in fraction 1. The higher expression of both ED1 and ED3 relative to ED2 in fraction 1, may be the result of an immature population developing within the testis, or may be leukocytes migrating through the tissue.

It is somewhat unusual for macrophages to stain for all three of these antibodies with only a subpopulation of Kupffer cells (liver) and splenic macrophages from the red pulp exhibiting similar expression patterns (Dijkstra et al, 1985). It of interest to note that some macrophages from liver and spleen share certain functional charateristics with the testicular

population depending on their location in the tissue. The most striking of these is the phagocytic nature of these cells which both have high expression of Fc receptor (Wardle, 1987; Buckley et al, 1987). This probably reflects the function of these cells in clearing particulate and soluble substances in the liver, and phagocytosing erythrocytes in the red pulp of the spleen.

The absence of ED5 from the testicular cell populations rules out the presence of dendritic cells in the tissue or in culture. Macrophages from tissues very often have a dendritic appearance and discerning the identity of the two cells types are often compounded by dendritic cell expression of macrophage related antigen. Dendritic cells however, generally do not express Fc receptors, and hence this may be used as another method besides immunohistochemistry for discerning between macrophages and dendritic cell types.

3.4.2 Fc receptor expression by testicular macrophages

The comparison between the peritoneal and testicular macrophage populations provided interesting results in relation to the Fc receptor expression by the testicular cells. Peritoneal macrophages in a resting state displayed low metabolic activity and very low phagocytic ability. Once activated with LPS however, the normally quiescent peritoneal macrophages changed both their morphological and functional characteristics and became avidly phagocytic via receptor-mediated immuno-phagocytosis over 48 hours.

This was highlighted in the results by the low/binding and ingestion rates seen in the non-activated peritoneal macrophages in comparison to the 'LPS' activated population. Phagocytosis of the red blood cells by the quiescent macrophages appeared to be initiated mainly by non-specific binding, although a section of the population which always remain in an active state (Kaplin, 1977), bound some very large rosettes. The non-specific binding which occurs in the quiescent population has been observed previously (Griffen and

Silverstein, 1974) and can not be included as an example of immuno-phagocytosis. This is because it has been demonstrated that specific binding (immune-mediated) may only be accomplished by activated populations (Bianco et al, 1975). Thus only a minority of the binding observed in quiescent peritoneal populations are immune-mediated and this is due to a very small proportion of the population which always remains active (Kaplin, 1977). Conversely there is also the indication of a proportion of cells in activated populations which never become activated (Albrecht, 1980).

The responses of the peritoneal macrophage populations to receptor-mediated phagocytosis is of particular interest when used in comparison to gauge the response of testicular macrophages to immuno-induced phagocytosis. It is 12 hours before the LPS activated peritoneal macrophages begin to show a significant increase in rosetting and ingestion and 48 hours before expression is comparable to those of freshly isolated testicular macrophages. Although the effects of the isolation on these cells can not be ruled out, it is unlikely that the levels of Fc receptor expression displayed by the testicular macrophage are the result of this procedure. Testicular macrophages were avidly phagocytic immediately after the isolation procedure (Tables 3.2&3.3) and remained so for the duration of the experiment, in comparison to the peritoneal macrophages which required at least 12 hours to increase Fc receptor expression in response to LPS. That the testicular macrophage can display such Fc receptor expression immediately after isolation, whether LPS stimulated or not, suggests that these cells exist in the testis in an avidly phagocytic state.

Whereas the peritoneal macrophages must first be activated by the presence of antigen before they may perform their role in immune function, this does not appear to be the case for the testicular macrophage. It seems the testicular macrophage already exists in an activated state expressing a high proportion of Fc receptors and therefore most likely in a high secretory state (Takemura and Werb, 1984). Immuno-phagocytosis is also observed during the

inflammatory response and macrophages expressing Fc receptors usually also release factors such as the pro-inflammatory cytokines and prostaglandins. Miller et al, (1983), indicated that the testicular macrophage may be involved in Leydig cell degradation, and as such is an essential component in Leydig cell turnover in the testis. In this case, the highly phagocytic nature and secretive abilities of the cell would be fully implemented.

The testicular macrophage's ability to bind and ingest antigen is perhaps of more interest, as it suggests a role for these cells in the immune privilege of the testis. That the testicular macrophage *in vivo* may be as capable of binding antigen as the activated peritoneal macrophages requires further consideration. The reason for the 'activated ' state of the testicular macrophages is unknown, although it is likely to be induced by a local factor, perhaps of interstitial origin, such as the Leydig cell, or the interstitial fluid.

The activated peritoneal macrophage is known to be able to present the ingested antigen to lymphocytes and in so doing initiate an immune response. The testicular macrophages have been shown here to be capable of completing the first step, involving receptor-mediated immuno-phagocytosis, but it is unknown if they can complete the second; the presentation of the antigen. The inability to present antigen, or to initiate the production of factors associated with the proliferation of lymphocytes may be involved in the immune privilege seen in the testis. This may be due to the fact that while the testicular macrophage can bind and ingest antigen through specific immunological means, it may not be able to initiate a T cell response. Such a situation would significantly restrict the contact of T cells with antigen, and could result in a suppression of the immune response.

It is also possible that such a situation may give rise to immune tolerance, a state in which the host immune system is subtly led into the position of tolerating, or accepting as normal, the presence of foreign antigen. This possibility was discussed by Lafferty et al (1983), where

the induction of tolerance for tissue grafts is likely to develop as the result of a slow leakage of free antigen into the immune system of the recipient. Conversley in the ram where there are no resident macrophages in the testis (Pollanen and Maddocks, 1988), and no immune privilege status (Maddocks and Setchell, 1988), it is possible that antigen in the testis is readily taken up by circulating immuno-active phagocytic cells (perhaps the small acid phosphatase positive cells), which then present it to lymphocytes and initiate a normal rejection response.

CHAPTER 4: The Immunosuppressive Activity of the Testicular Macrophage.

Chapter 4. The immunosuppressive of activity rat testicular macrophages.

4.1 Introduction

The presence of putative peptide immuno-suppressive factors in the interstitium has been suggested as one of the primary mechanisms for assisting graft survival in the testis (Pollanen et al, 1988). However, such a hypothesis does not account entirely for all the immune interactions observed in the testis. Autoimmune responses against testicular autoantigens may contribute to infertility, but are easily induced experimentally (Tung and Lu, 1991). Furthermore Ferguson and Scothorne (1977) were able to induce rejection of secondary intratesticular autographs when the hosts were pre-sensitised by primary skin grafts. This suggests that the afferent arm of the immune response is somehow restricted in this environment, presumably as a means of maintaining a state of non-responsiveness against antigens usually present only in the testis (eg. germ cell autoantigens).

The testis differs markedly to other sites of immune privilege in the body in that it possesses a highly effective lymphatic and vascular network which allows the migration of T-cells through the tissue (Tilney, 1971; McCullough, 1975; Head et al, 1983a,b). It has been demonstrated already in Chapter 3, that rat testicular macrophages have express the Fc receptor and are avidly phagocytic. However, the initiation of an immune response within the testicular interstitum is likely to be dependent on the communication between the resident population of macrophages and the migratory lymphocytes.

To further understand the basis of the immune privilege afforded the testis, the present study investigated the ability of isolated rat testicular macrophages to initiate and regulate *in-vitro* the proliferation of isolated peripheral blood lymphocytes.

4.2 Methods

Adult male rats (>300g) of the inbred DA strain were euthenased by CO_2 asphyxiation. Testicular macrophages, peritoneal macrophages and peripheral blood lymphocytes were then isolated as described in Chapter 2.3.5 and prepared for culture alone or co-culture with lymphocytes.

4.2.1 Lymphocyte Proliferation Assays

Macrophage-Lymphocyte Co-culture

Viable peripheral blood lymphocytes ($5x10^5$ cells in 50µl), as determined by Trypan Blue exclusion, were then added to the 96 well culture plates with RPMI (100µl), and 10µg/ml Con A (50µl). Macrophages (10^4 cells in 50µl) or macrophage conditioned media (50µl) was then added to the co-cultures to create a ratio of 1:50 macrophages to lymphocytes, or an equivalent ratio of lymphocytes to macrophage conditioned medium. Control wells consisting of ConA-stimulated lymphocytes only were run with every assay.

Macrophage Conditioned Medium

The isolated macrophages as described above were cultured for 48 hours in 35mm Falcon culture dishes in RPMI 1640 (1ml containing 2mM glutamine and 10%FCS) with various combinations of Lipopolysaccharide (10µg/ml), indomethacin (10µg/ml) and interferon-gamma (200U/ml) after which the conditioned media was collected. Lipopolysaccharide is a non-specific stimulant of macrophages and gamma-interferon is a cytokine known to activate macrophages. Indomethacin inhibits the production of immuno-suppressive prostaglandins by blocking their synthesis via the cyclooxygenase pathway.

The conditioned media was then centrifuged at 11 000g for 5 minutes and stored as frozen aliquots. Prior to freezing, some of these aliquots were dialysed against PBS for 48 hours to exclude molecules of size less than 14000MW. The PBS was changed four times during this

time, and the conditioned medium increased in concentration by 10% during dialysis, as determined by weight. Dialysed and non-dialysed media were subsequently tested in the lymphocyte proliferation assay at the same time.

4.2.2 Fractionation of macrophage conditioned medium by centrifugation

Macrophage conditioned medium (2ml) was pipetted into Centricon-10 (10 000MW cutoff) and Centricon-30 (30 000MW cutoff) microseparators. Centricon-10 and Centricon-30 were centrifuged at 5000g for 30 minutes and 2000g for 10 minutes respectively and both the filtrate and retentate retained for addition to the lymphocyte proliferation assay. The concentration increase of the fractions by microcentrifugation as djudged by weight was as follows; <10000MW 2.5 fold, >10000MW 1.6 fold, <30000MW 3.3 fold, >30000MW 1.4 fold and 10000MW<>30000MW 4 fold.

4.2.3 [^{3}H] Thymidine Incorporation

The proliferation of lymphocytes was determined by measuring the cellular incorporation of ^{3}H thymidine. Lymphocytes were cultured either with macrophages or macrophage conditioned media for 48 hours after which, 1μCi [^{3}H] thymidine (specific activity of 5000mCi/mmole) was added to each culture well. Twelve hours later, cells were harvested onto glass fibre filter paper using a PhD cell harvester (Cambridge Technology Inc.) and the incorporation of thymidine was measured using a LKB 1215 liquid scintillation counter (LKB, Wallac, Finland).

4.2.4 Prostaglandin Assays

Samples of macrophage conditioned medium were assayed for prostaglandins E_2 and $F_{2\alpha}$ as outlined in Chapter 2 (section 2.6).

4.2.5 Statistical Analysis

The student's t-test was used to compare control and experimental groups. Values of $P>0.05$ were not considered significant

4.3 Results

4.3.1 Suppressor activity of the testicular macrophage

The proliferation of mitogen-stimulated lymphocytes was inhibited by both testicular macrophages and testicular macrophage-conditioned medium , (65±3% and 65±4% of the control response respectively; Fig. 4.1). This inhibition was not significantly altered when testicular macrophages were stimulated non-specifically with LPS. Peritoneal macrophages and the conditioned media from peritoneal macrophage cultures also significantly inhibited the mitogenic response of lymphocytes (82±5 % and 84±3 %), but in contrast to the testicular macrophages this inhibition did not occur when LPS was added to the macrophage cultures (Fig. 4.1).

4.3.2 Effect of Dialysis and Indomethacin

The negative effects of testicular macrophage-conditioned medium on lymphocyte proliferation in vitro were significantly reduced when the media was dialysed prior to addition to the lymphocyte cultures (Fig. 4.2). With the removal of small molecular weight compounds (<14000 MW) from the testicular macrophage conditioned medium the lymphocyte proliferation response was 93%±4 of the control, although this was still significantly lower ($p<0.05$) than the control value for ConA stimulated lymphocytes alone (100% PBL proliferation, Fig. 4.2). Dialysed medium from LPS treated testicular macrophages induced lymphocyte proliferation responses that were not significantly different to the control, ConA-stimulated PBL. That dialysis of the media could remove the previously inhibitory effects of LPS treated testicular macrophages suggests that the

Figure 4.1

Concanavalin A-induced peripheral blood lymphocyte proliferation in the presence of peritoneal (Pm) or testicular macrophages (Tm), in the presence of conditioned medium from cultures of peritoneal (Ps) or testicular (Ts) macrophages, alone or with the addition of LPS (+L). Results are expressed as a percentage of the PBL proliferation observed in the Con A stimulated control. Values are the mean ± SEM for 6 experiments. *= $P<0.05$ vs the control. There is no significant difference between any of the Tm or Ts values.

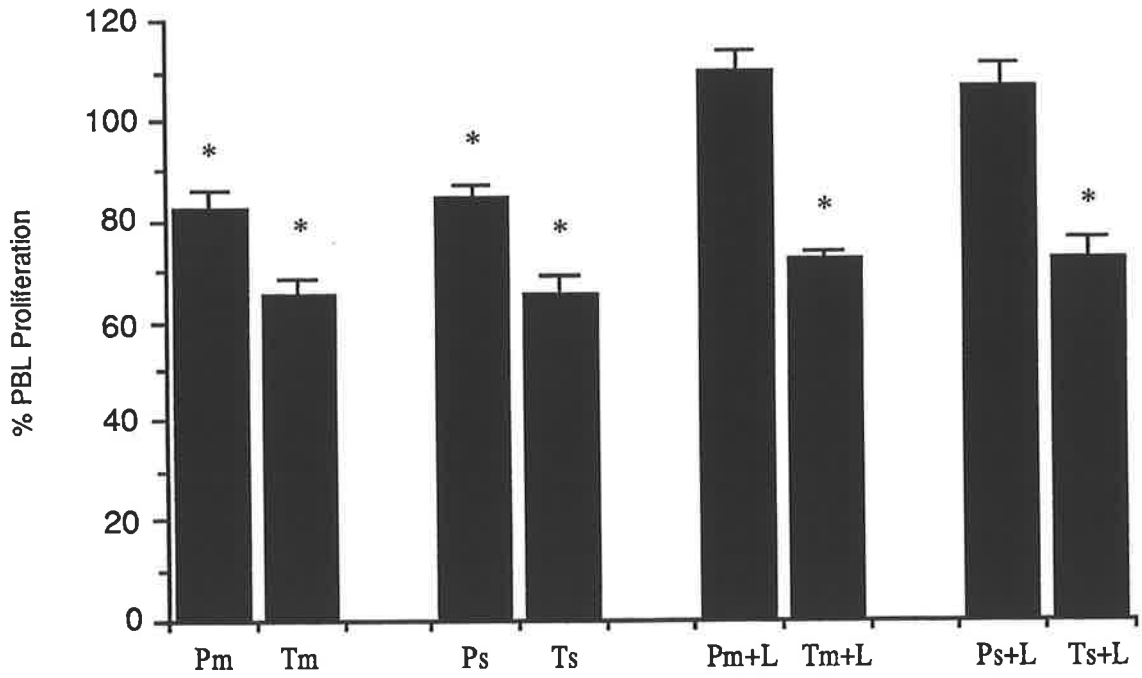

% PBL Proliferation
120
100
80
60
40
20
0
*
Pm
Tm
Ps
Ts
Pm+L
Tm+L
Ps+L
Ts+L

Figure 4.2

The percentage of peripheral blood lymphocyte proliferation observed relative to the Concanavalin A stimulated controls where (d) represents dialysed testicular macrophage-conditioned medium, (+L) is LPS stimulation of the macrophages (non dialysed), and (i) is the addition of indomethacin to macrophage cultures (non dialysed). Values are the mean ± SEM for 6 experiments. *= $P<0.05$ vs Con A stimulated PBL control (100%).

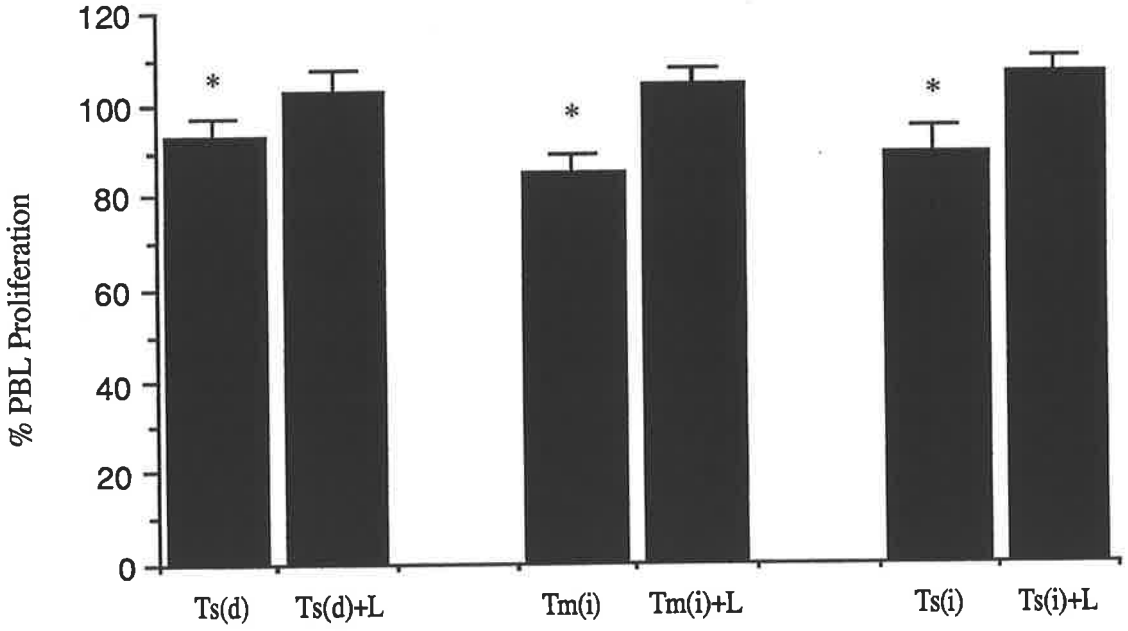
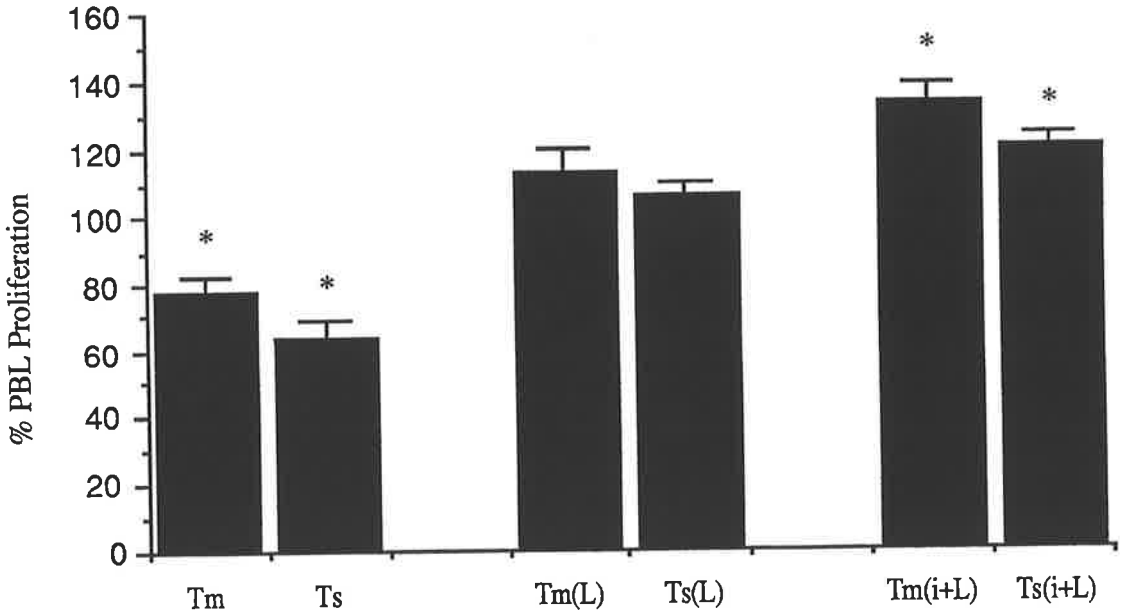

% PBL Proliferation
120
100
80
60
40
20
0
*
*
*
Ts(d)
Ts(d)+L
Tm(i)
Tm(i)+L
Ts(i)
Ts(i)+L

Figure 4.3

The proliferative responses observed following the addition of IFNγ primed testicular macrophages and macrophage-conditoned medium to concanavalin A stimulated peripheral blood lymphocyte culture. (L) denotes LPS stimulation of the macrophage and (i) denotes the addition of indomethacin to macrophage culture. Values are the mean ±SEM for 6 experiments. *= $P<0.05$ vs the control.


% PBL Proliferation
160
140
120
100
80
60
40
20
0
*
*
*
*
Tm
Ts
Tm(L)
Ts(L)
Tm(i+L)
Ts(i+L)

Lymphocyte proliferation in the presence of media from indomethacin treated cultures c macrophages was significantly improved over normal conditioned media and comparable to that of the dialysed medium (Fig. 4.2). Where the testicular macrophages were cultured wit LPS and indomethacin simultaneously, the proliferative response observed was equal to th control, suggesting that not only had the inhibitory factors been removed, but that LP stimulation had also contributed to the responses. Indomethacin clearly prevented th synthesis of prostaglandins as the prostaglandin concentrations measured in testicul macrophage conditioned medium were reduced to levels below the minimum sensitivity c the assay (Table 4.1).

4.3.3 Effect of IFNγ and LPS

inhibitory activity of the cell culture medium is largely due to low molecular weigl substances.

The addition of interferon gamma (IFNγ) to testicular macrophage cultures did not affect th inhibitory effects of these cells on PBL proliferation. When IFNγ and LPS were both adde to testicular macrophages in culture, the testicular macrophages and the testicul macrophage-conditioned media both induced PBL proliferation that was not significant different from the control (113%±6 and 106%±4 respectively) (Fig 4.3). Whe indomethacin was also added to these cultures the proliferation was significantly increas above that of the controls, (133%±6 for testicular macrophages and 119%±5 for testicul macrophage-conditioned medium; Fig 4.3).

4.3.4 Prostaglandin profile of TM

Testicular macrophage conditioned media was found to contain substantial concentrations PGE_2 (13.1±0.4 ng/ml) and $PGF_{2\alpha}$ (16.8±0.6 ng/ml) while conditioned medium from

Table 4.1 Prostaglandin Production

Treatment	PGE_2 (ng/ml)	$PGF_{2\alpha}$ (ng/ml)
Pm cultured alone	<1.0	<1.8
+ lipopolysaccharide	26.8±3.2	21.9±1.6
Tm cultured alone	13.1±0.4	16.8±0.6
+ lipopolysaccharide	29.4±2.6*	22.2±1.8*
+ indomethacin	<1.0	<1.8
+ lipopolysaccharide & indomethacin	<1.0	<1.8
+ dialysis	<1.0	<1.8
+ lipopolysaccharide (dialysed)	<1.0	<1.8
+ IFNγ	13.2±0.7	3.8±0.2*
+ IFNγ + lipopolysaccharide	25.3±1.8*	6.1±0.9*
+ IFNγ + lipopolysaccharide & indomethacin	<1.0	<1.8

Values ± SEM, n=3; * $p<0.05$ compared with Tm alone.

Figure 4.4

Concanavalin A-induced peripheral blood lymphocyte proliferation in the presence of fractionated testicular macrophages conditioned medium: factors<10000MW. Results are expressed as a percentage of the PBL proliferation observed in the Con A stimulated control. Values are the mean ± SEM for 6 experiments. *= $P<0.05$, **=$P<0.01$ and ***=$P<0.001$ vs the control. There is no significant difference between any of the Tm or Ts values.

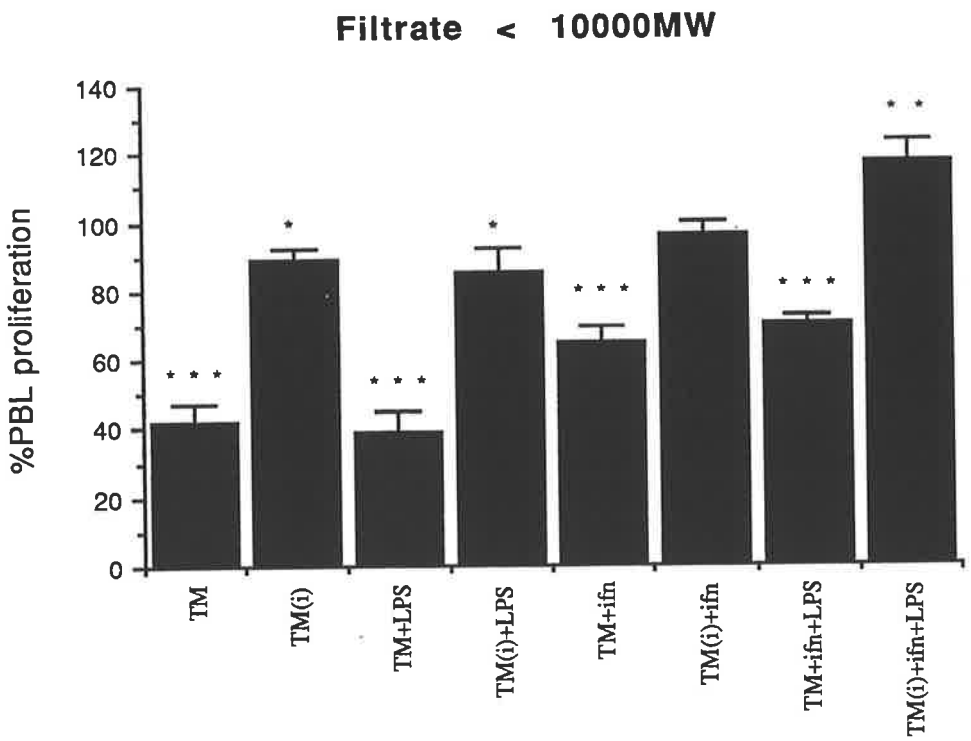
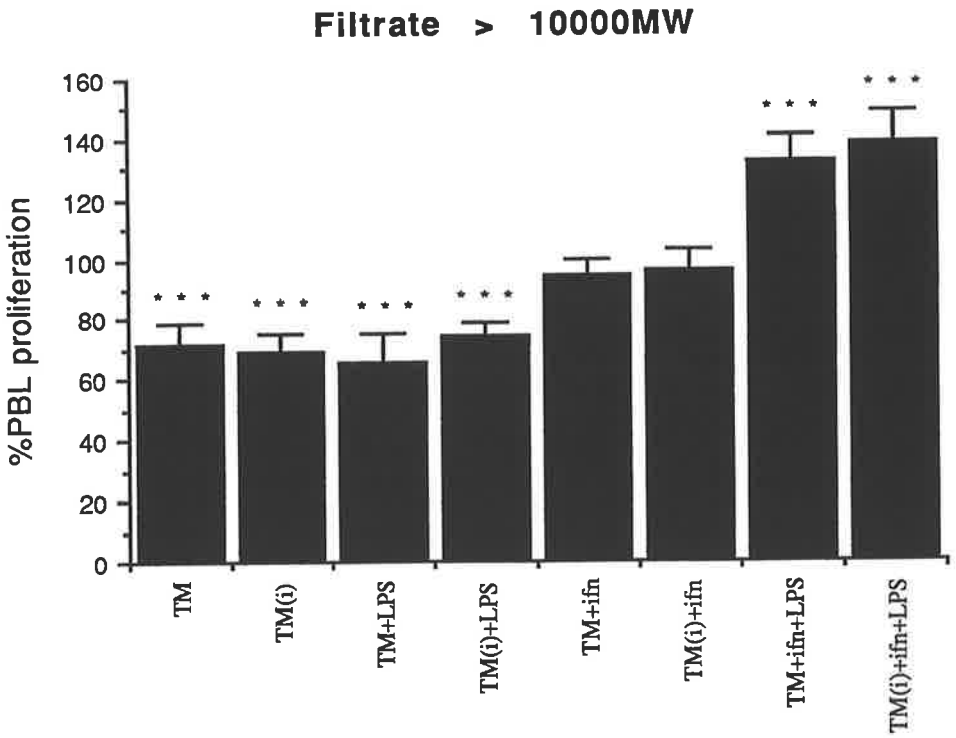

Filtrate < 10000MW
%PBL proliferation
140
120
100
80
60
40
20
0
TM
TM(i)
TM+LPS
TM(i)+LPS
TM+ifn
TM(i)+ifn
TM+ifn+LPS
TM(i)+ifn+LPS

Figure 4.5

Concanavalin A-induced peripheral blood lymphocyte proliferation in the presence of fractionated testicular macrophages conditioned medium: factors >10000MW. Results are expressed as a percentage of the PBL proliferation observed in the Con A stimulated control. Values are the mean ± SEM for 6 experiments. *= $P<0.05$, **=$P<0.01$ and ***=$P<0.001$ vs the control. There is no significant difference between any of the Tm or Ts values.


Filtrate > 10000MW
%PBL proliferation
160
140
120
100
80
60
40
20
0
TM
TM(i)
TM+LPS
TM(i)+LPS
TM+ifn
TM(i)+ifn
TM+ifn+LPS
TM(i)+ifn+LPS

unstimulated peritoneal macrophages did not contain detectable levels of these prostaglandins (Table 4.1). The inhibition of lymphocyte proliferation by the quiescent peritoneal macrophage (Fig 4.1) has been observed previously (Keller, 1975; Oehler et al, 1977), and is thought to be due to the presence of a proteinaceous factor. Following LPS stimulation of the testicular macrophages, these concentrations were increased significantly (PGE_2 29.4±2.6ng/ml and $PGF_{2\alpha}$ 22.2±1.8ng/ml). The addition of the prostaglandin inhibitor indomethacin to testicular macrophage cultures, reduced prostaglandin levels in the medium to below the sensitivity of the assay. The addition of IFNγ to testicular macrophage cultures reduced $PGF_{2\alpha}$ production to levels significantly lower than those expressed basally. There was no equivalent effect of IFNγ on TM production of PGE_2.

4.3.5 Characterisation of immuno-suppressive elements in macrophage conditioned medium.

Factors <10000MW

When conditioned media from testicular macrophage cultures with or without LPS stimulation was fractionated, the factors of less than 10000MW (Fig.4.4) when added to lymphocyte culture strongly inhibited lymphcoyte proliferation. Conditioned media from cells cultured with indomethacin, was largely free of this anti-proliferative effect although a small but significant inhibition remained sugggesting the presence of an inhibitor outside the cyclooxygenase pathway. IFNγ on its own, or in conjunction with LPS did not overcome the inhibitory effects of these low molecular weight secretions. However, conditioned medium from IFNγ stimulated testicular macrophages in culture supplemented with indomethacin displayed reduced lymphocyte inhibiton (96±4% of the control) and where LPS was used in conjunction with IFNγ, lymphocyte proliferation was actually increased (117±6%) compared to the control.

Figure 4.6

Concanavalin A-induced peripheral blood lymphocyte proliferation in the presence of fractionated testicular macrophages conditioned medium: factors <30000MW. Results are expressed as a percentage of the PBL proliferation observed in the Con A stimulated control. Values are the mean ± SEM for 6 experiments. *= $P<0.05$, **=$P<0.01$ and ***=$P<0.001$ vs the control. There is no significant difference between any of the Tm or Ts values.

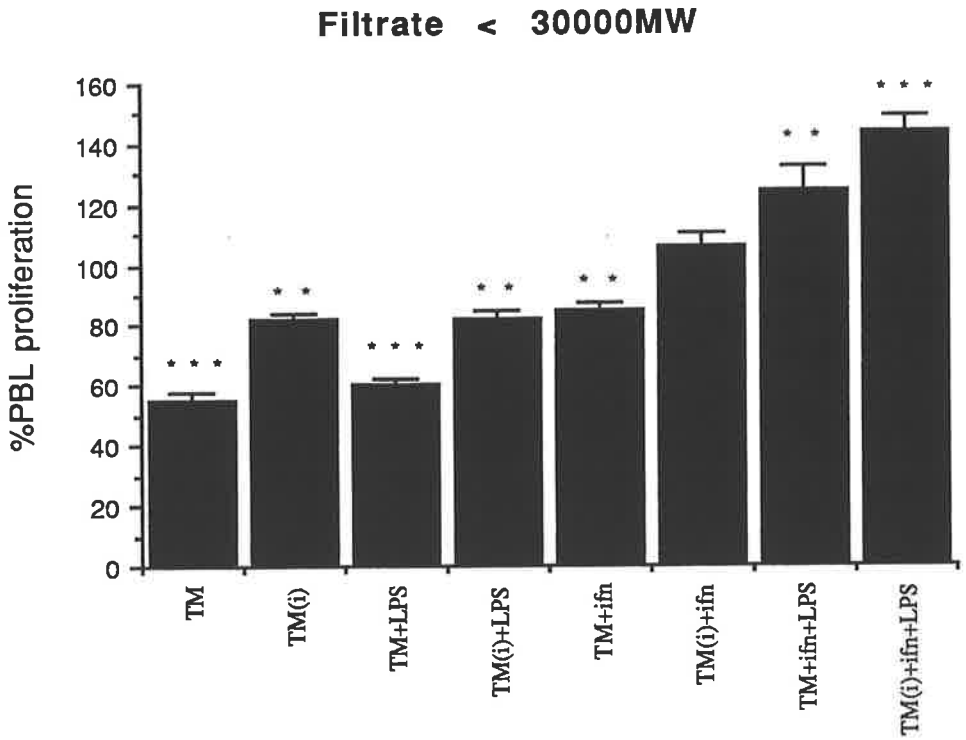

Filtrate < 30000MW
%PBL proliferation
160
140
120
100
80
60
40
20
0
TM
TM(i)
TM+LPS
TM(i)+LPS
TM+ifn
TM(i)+ifn
TM+ifn+LPS
TM(i)+ifn+LPS

Figure 4.7

Concanavalin A-induced peripheral blood lymphocyte proliferation in the presence of fractionated testicular macrophages conditioned medium: factors >30000MW. Results are expressed as a percentage of the PBL proliferation observed in the Con A stimulated control. Values are the mean ± SEM for 6 experiments. *= $P<0.05$, **=$P<0.01$ and ***=$P<0.001$ vs the control. There is no significant difference between any of the Tm or Ts values.

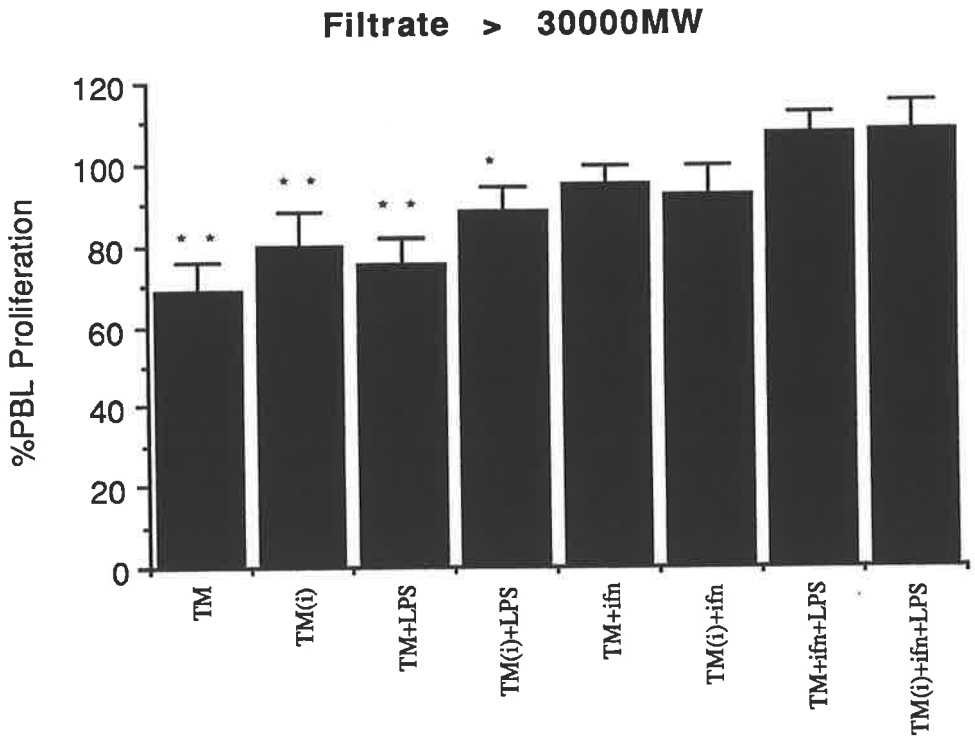
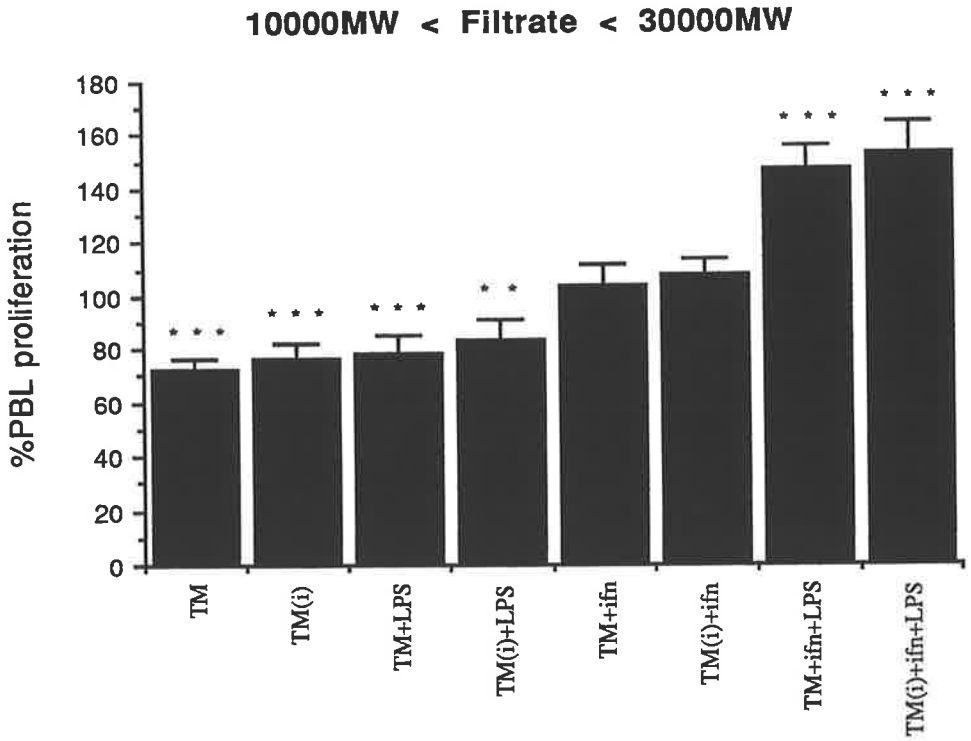


Figure 4.8

Concanavalin A-induced peripheral blood lymphocyte proliferation in the presence of fractionated testicular macrophages conditioned medium: 30000MW > factors >10000MW. Results are expressed as a percentage of the PBL proliferation observed in the Con A stimulated control. Values are the mean ± SEM for 6 experiments. *= $P<0.05$, **=$P<0.01$ and ***=$P<0.001$ vs the control. There is no significant difference between any of the Tm or Ts values.


10000MW < Filtrate < 30000MW
%PBL proliferation
180
160
140
120
100
80
60
40
20
0
TM
TM(i)
TM+LPS
TM(i)+LPS
TM+ifn
TM(i)+ifn
TM+ifn+LPS
TM(i)+ifn+LPS

Factors >10000MW

The fraction containing molecules of greater than 10000MW (Fig 4.5.) exerted a reduced inhibitiory effect on lympho-proliferation in comparison to factors less than 10000MW. A similar level of inhibition was observed in conditioned media from cultures without IFNγ. Cultures stimulated with IFNγ alone or supplemented with indomethacin displayed proliferation comparable to the control. Where LPS was added in conjunction with IFNγ, the proliferation observed was significantly greater than the control (142±9% and 148±12%) without or with indomethacin supplement, respectively.

Factors <30000MW

Isolated factors of size less than 30000MW from testicular macrophage conditioned medium (Fig.4.6) displayed a similar pattern of inhibition to non-fractionated macrophage medium (Figs 4.1-4.3). Significant inhibition of lymphocyte proliferation was seen in testicular macrophage conditioned medium, with or without LPS and/or indomethacin. While INFγ on its own did not reduce the inhibitory secretions of the testicular macrophage, in combination with indomethacin the proliferation observed was not significantly different to that of the control. IFNγ-LPS stimulated cultures again displayed enhanced lymphoproliferation (124±8%) and with indomethacin added to culture this figure increased to 143±6%.

Factors >30000MW

Conditioned media from cultures not IFNγ treated remained inhibitory to lymphocytes (Fig. 4.7). Fractions of greater than 30000MW from IFNγ treated cultures displayed lympho-proliferation not significantly different to that of the control.

10000MW<Factors <30000MW

In cultures not treated with IFNγ, the fractionated medium between 10000MW and 30000MW inhibited lympho-proliferation (Fig.4.8). Where IFNγ was added on its own or

with indomethacin to culture, these inhibitory effects were removed. Cultures primed and triggered with IFNγ and LPS, or with indomethacin in addition to this combination significantly increased proliferation (147±9% and 153±11%) compared with the control.

4.4 Discussion

4.4.1 Inhibition of Lymphocyte Proliferation

Prostaglandins of the E and F classes are well known to be associated both with aspects of reproductive function and with macrophages during pro-inflammatory immune responses. Their production by macrophages can be induced by a number of signals such as Fc receptor-mediated phagocytosis and immune complexes (Rouzer et al, 1982), stimulation by cytokines and LPS (Kunkel et al, 1986a,b;Bonney and Humes, 1984) and increased protein synthesis (Bonney et al, 1980). *In-vitro* studies have also established that prostaglandins of the E class are involved in the suppression of mitogenically induced lymphocyte proliferation (Offner and Clausen, 1974; Goodwin et al, 1978; Stobo et al, 1979).

In the present study, isolated testicular macrophages inhibited the proliferation of peripheral blood lymphocytes in-vitro via production of a factor(s) secreted into the culture medium. LPS stimulation of testicular macrophages, or the addition of conditioned media from LPS-stimulated testicular macrophage cultures to PBL failed to significantly increase lymphocyte proliferation when compared to testicular macrophages alone.

That most of this inhibition could be removed either by dialysing the conditioned media to remove low molecular weight molecules (<14000MW) or by culturing the testicular macrophages with indomethacin, a known inhibitor of prostaglandin production, suggests that prostaglandins may be responsible for the majority of the testicular macrophage induced-inhibition of lymphocyte proliferation in vitro. However, testicular macrophages and their

conditioned media from indomethacin treated cultures or dialysed culture medium still retained inhibitory characteristics (Fig.4.2). This may be due to the presence of a non-dialysable factor of greater than 14000MW, or to the inability of the TM to synthesise adequate lymphocyte stimulatory molecules such as the cytokines.

IFNγ is well known as an anti-viral agent and for its specific action on cells of the immune system (Ijzermans and Marquet, 1989) and by using a combination of LPS and IFNγ, macrophage populations can be induced to produce cytokines essential for the induction of lymphocyte proliferation *in vitro* (Adams and Hamilton, 1984; Schreiber and Celada, 1985). In the presence of IFNγ, LPS and indomethacin, testicular macrophages or their conditioned medium increased lymphocyte proliferation significantly above that of the control. Although it is unclear as to exactly how the combination of these agents mediated their effects, it appears likely this is due to IFNγ induced macrophage synthesis of cytokines such as IL-1 and TNFα (Adams and Hamilton, 1984). The additional presence of indomethacin in culture should prevent the down regulation of IL-1 and TNFα production by prostaglandins and restore synthesis of these cytokines (Kunkel et al, 1986b; Hart et al, 1988).

Our observations that the testicular macrophage produces significantly greater basal levels of prostaglandins than does the peritoneal macrophage, may explain the lower rates of PBL proliferation seen in the presence of testicular macrophage-conditioned medium. In normal circumstances prostaglandin production appears to regulate the immune response by down-regulating production of cytokines through a negative feedback mechanism. Basal secretion of prostaglandins by the testicular macrophage may be a mechanism employed in the testis to reduce acessory cell function by limiting cytokine production. This hypothesis is supported by other experiments in which we have found the production of certain cytokines by the testicular macrophage to be minimal, even after LPS stimulation (see Chapter 5).

The addition of LPS to cultures increased the production of PGE_2 and $PGF_{2\alpha}$ by both macrophage populations (Table 4.1). This observation corresponds with previous reports of LPS action on murine macrophages and human monocytes (Kurland and Bockman, 1978; Aderem et al, 1986). While the LPS-induced increase in PGE_2 and $PGF_{2\alpha}$ production suggests that the testicular macrophage is indeed stimulated by this endotoxin, there is no associated increase in PBL proliferation as there is for the peritoneal macrophages. This suggests that while LPS stimulates production of factors via the cyclo-oxygenase pathway, testicular macrophages are not responding by increasing cytokine production.

The addition of IFNγ to testicular macrophage cultures did not alter PGE_2 levels but did significantly reduce the levels of $PGF_{2\alpha}$ (Table 4.1). This led to a small but significant ($P<0.05$) reduction in the inhibition of lymphocyte proliferation (compare Tm in Figure 4.3 to Tm in Figure 4.1). When LPS and IFNγ were added simultaneously to testicular macrophage cultures, PGE_2 synthesis increased to levels equivalent to those in testicular macrophages stimulated by LPS alone while $PGF_{2\alpha}$ levels remained significantly reduced. These results strongly suggest that there may be differential regulation by IFNγ of these prostaglandins in the testicular macrophage and therefore the PGE_2 to $PGF_{2\alpha}$ ratios may be of significance.

The nature of the testicular environment is such that local physiological influences may account for the high concentrations of prostaglandins produced by the testicular macrophage in comparison to the peritoneal macrophage. Furthermore, the high basal levels observed suggest a possible role for testicular macrophage-derived prostaglandin in the testis.

One explanation may be that as a functional component of the testicular interstitum, the testicular macrophage produces prostaglandins for a specific physiological purpose. The testosterone producing Leydig cell could be one such target due to its close developmental

relationship (Berg, 1985; Berg 1987; Hutson 1990) and the intimate cellular association (Niemi et al, 1986; Hutson, 1992) with the testicular macrophage.

It has been inferred that $PGF_{2\alpha}$ and PGE_2 may regulate ovarian cell function by, respectively, decreasing and increasing transcription of steroidogenic enzymes in porcine granulosa cells (Li et al, 1993). Prostaglandins in the testicular interstitium may be performing similar regulatory tasks. That the source of these 'regulatory' prostaglandins in the testis is unlikely to be the Leydig cell (Abayasekara et al, 1990), gives greater significance to the possible role of the testicular macrophage in this respect.

The present study demonstrates that high basal levels of $PGF_{2\alpha}$ and PGE_2 produced by the testicular macrophage are likely to be a primary source of the inhibitory effect of testicular macrophages on peripheral blood lymphocyte proliferation in vitro. That the levels of these prostaglandins may be regulated independantly suggests that the testicular macrophage and its production of $PGF_{2\alpha}$ and PGE_2 may play a role in the normal physiology of the testis.

4.4.2 Characterisation of Macrophage conditioned media

The inhibitory effect of testicular macrophage conditioned medium on lymphocyte proliferation appears mainly to be due to factors less than 10000MW. Fractionation of the conditioned media suggests that the testicular macrophage may produce several inhibitory factors. While prostaglandin, and particularly PGE_2 are the main components of inhibition in testicular macrophage conditioned media, there appears to be other inhibitory factors of higher molecular weight present. The inhibition seen between 10000MW and 30000MW (Fig.4.8), and above 30000MW (Fig.4.7) although significant, is weaker than that of the prostaglandins.

Culturing the testicular macrophages with LPS does not seem to decrease the production of these inhibitory factors, or increase lymphocyte proliferation. That conditioned medium from cultures stimulated with LPS alone remains inhibitory highlights the refractoriness of testicular macrophages to LPS. This observation reinforces our findings on the non-responsiveness of testicular macrophages to LPS and the apparant lack of cytokine production in response to this stimulus (see Chapter 5).

That the production of these inhibitory factors may be down regulated in the presence of IFNγ suggests they may be under immunological regulation. Yet, despite removing the inhibition caused by the factors greater than 10000MW, IFNγ alone can not stimulate a significant increase in lympho-proliferation.

The proliferation in the lymphocyte cultures was significantly increased where IFNγ and LPS were used in combination to sucessfully prime and trigger cultures of testicular macrophages. When these two macrophage 'activators' are used together, macrophages in other tissues have been shown to become activated and release a host of immuno-active factors such as cytokines, colony stimulating factors and low molecular weight products (Adams and Hamilton, 1984; Schrieber and Celada, 1985)).

Therefore the immuno-activity in the 10000MW-30000MW fraction is of interest as it is within this MW range that cytokine activity is expected. The initial presence of an inhibitory factor within this MW range may suggest basal production by the testicular macrophage of macrophage derived immunosuppressive cytokines such as GM-CSF and TGFß (Kherl et al, 1985; Morrisey and Ireland, 1991; Walker et al, 1992; Pollanen et al, 1993). Although a role for these factors within the testis is yet to be determined, their production may belie a specific paracrine function while their suppressive action remains a secondary feature.

The nature of this fraction is altered if the macrophages have been activated with IFNγ and LPS, as is amply demonstrated by the significant increase in lymphoproliferation by the fraction between 10000MW and 30000MW (Fig 4.8). It is also worth noting at this point that after stimulation of the testicular macrophages with IFNγ and LPS, it was in this fraction which stimulated lymphocyte proliferation to levels significantly above the control. It is also within this molecular weight range the pro-inflammatory cytokines are found and the rapid production of cytokines such as IL-1, TNF and IL-6 by the activated macrophage (see Chapter 5) is central to the initiation of an immune response and vital for the proliferation of lymphocytes (Dinarello, 1989).

It is worth noting that no proliferative factors were observed in the greater than 30000 MW range, even after macrophage activation. Inhibitory factors of approximately 30000MW and larger (Fig 4.7) have long been associated with the suppressive proteins in the testis although the source of these factors has never been determined (Pollanen et al, 1990; Pollanen et al, 1994). The presence of such an inhibition in testicular macrophage conditioned medium suggests this factor may be a source of the high molecular weight inhibitory activity seen in the testis (Pollanen et al, 1988). Although its inhibitory effect is only relatively minor, and IFNγ downregulates its production, its presence requires further investigation.

The production of inhibitory factors by the testicular macrophage and their downregulation by IFNγ, is evidence of the testicular macrophage's effector status may be under immunologic control. Production of at least three separate inhibitors of lymphocyte proliferation suggests that the testicular macrophage may secrete factors which inhibit local immune responses and as a result contribute to the immune privilege afforded the testis.

CHAPTER 5: Cytokine Production by the Rat Testicular Macrophage.

Chapter 5. Cytokine secretion by macrophages in the rat testis

5.1 Introduction

Testicular macrophages exhibit many typical macrophage characteristics including Fc receptor-mediated phagocytosis (Miller et al, 1983), secretion of cytotoxic molecules (Wei et al, 1988), and MHC class II receptor expression (Pollanen and Maddocks, 1988). However, experiments in this thesis have found that the immunoaccessory function of testicular macrophages is deficient in comparison to that of macrophages resident in the peritoneal cavity. Testicular macrophages failed to support the proliferative response of lymphocytes stimulated *in vitro* with mitogen, and indeed were found to secrete proliferation-inhibiting factors including large amounts of prostaglandin (Chapter 4).

Macrophages resident in other peripheral tissues are remarkably versatile cells able to perform a variety of both immunological and specific physiological tasks primarily through their capacity to secrete a vast array of cytokines, enzymes and other biological mediators in a microenvironmental and differentiation state-specific manner. For example, the degree and type of immune response are known to be regulated by macrophage-derived cytokines both *in vitro* and *in vivo*. Interleukin 1 (IL-1) is a primary stimulator of Interleukin 2 (IL-2) synthesis in T-cells for the subsequent proliferation of lymphocytes (Lowenthal et al, 1986). Tumor Necrosis Factor alpha (TNFα) and Interleukin 6 (IL-6) (Hackett et al, 1988; Hirano et al, 1990) also have similar roles in regulating cell proliferation and differentiation during the induction of cellular immunity while Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) may be associated with the induction of suppressive responses (Fu et al, 1991; Tsuchiya et al, 1988).

In addition, there is a rapidly expanding appreciation of the critical roles for macrophages and their cytokine products in diverse tissue remodelling processes, with an abundance of studies now supporting roles for IL-1, TNFα, IL-6 and GM-CSF as paracrine regulators of female reproductive function (Adashi, 1990; Hunt, 1994; Robertson, 1994). In the testis, there is ample evidence that IL-1 and TNFα can alter Leydig cell function *in vitro* (Calkins et al, 1988; Warren et al, 1990), with Sertoli cells known to produce at least one cytokine (IL-1α) (Gerad et al, 1991). However, the potential for local macrophages to participate in cytokine-mediated cell communication processes in the testes remains largely unexplored.

In the present study we have isolated testicular macrophages by a novel cellular elutriation method and have examined their secretion of the cytokines IL-1, IL-6, TNFα and GM-CSF both basally and in response to activation by IFNγ and LPS. IFNγ is a lymphocyte-derived macrophage-activating cytokine and LPS a non-specific but potent activator of macrophages. IFNγ and LPS were chosen for their ability to stimulate maximal cytocidal activity in macrophages.

5.2 Methods

5.2.1 Cell isolation and culture

Peritoneal and testicular macrophages were isolated as described in chapter 2. Both testicular and peritoneal macrophages were cultured in RPMI-M on glass coverslips at 34°C (normal testis temperature) and 37°C respectively in an atmosphere of 95% air and 5% CO_2. After an intial 20 minute attachment period, non-adherent cells were removed by five vigourous washings in PBS. LPS (10mg/ml), indomethacin (10mg/ml) or interferon gamma (IFNγ, 200U/ml) were added alone or in combination to some cultures. After 48 hours culture, the culture medium was collected, centrifuged at 11000g for 5min and the supernatant stored at -80°C for bioassay.

5.2.2 Cytokine Bioassays

TNF α

TNFα was assayed in a cytotoxicity assay employing the TNFα sensitive murine fibroblast cell line L929 as described by Matthews and Neale (1987). Duplicate serial dilutions (1:2) of macrophaage-conditioned culture media were incubated with 20,000 L929 cells in 200 µl of HamsF12-FCS containing 4 mg/ ml cycloheximide for 3 days. Cell lysis was then measured by methyl violet uptake (0.5% in 20% methanol for 10 min at room temperature). The cells were then washed to remove excess dye and the remaining incorporated dye was dissolved in 50% acetic acid and quantitated by measuring absorbence at 570 nm using a multiwell ELISA plate reader.

IL-1

IL-1 was assayed in a cytotoxicity assay employing the IL-1 sensitive human melanoma cell line A375 as described by Nakei *et al,* (1988). Duplicate serial dilutions (1:2) of macrophage-conditioned culture media were incubated with 2000 A375 cells in 200 µl of RPMI-FCS for 4 days. Cell lysis was then measured as for the TNFα assay.

GM-CSF

GM-CSF was assayed using the GM-CSF-dependant murine myeloid cell line FD5/12, essentially as described by Kelso and Owens (1988). Duplicate serial 1:2 dilutions of macrophage culture supernatants were incubated with 2000 FD5/12 cells in 200 µl of RPMI-FCS. After 2 days, cultures were pulsed with 1 mCi of ^{3}H thymidine for 6 h and harvested onto glass-fibre filter paper using a PhD automated cell harvester (Cambridge Technology Inc.) for counting in a LKB 1215 liquid scintillation beta counter.

IL-6

IL-6 was assayed using the IL-6-dependant murine myeloid cell line 7TD1 (Van Snick et al, 1986). Duplicate serial dilutions (1:2) of macrophage culture supernatants were

incubated with 2000 7TD1 cells in 200 µl RPMI-NS. After 3 days, the number of viable cells was estimated colorimetrically by assesing the conversion of the tetrazolium salt MTT to formazan in a modification of a method described by Mossman (1983). Briefly, 25 µl of 4 mg/ ml MTT (Sigma) in RPMI was added to each well. After incubation for 4 h at 37°C, media was aspirated carefully and formazan product was solubilised by addition of 100 µl of 95% ethanol, and absorbance was measured at 570 nm using a multiwell ELISA plate reader.

5.2.3 Bioassay Design

Standard curves were constructed for each bioassay by serially diluting 1000U of recombinant cytokine and measuring the cellular proliferation (GM-CSF and IL-6; Figure 5.1) or cytotoxicty (IL-1 and TNFα; Figure 5.2) within the cytokine-specific cell line. Minimum detectable amounts for each assay were, GM-CSF: 1 U/ml (50 U defined as the concentration of GM-CSF stimulating half maximal FD5/12 cell proliferation; equivalent in our hands to 50 CFUc U/ml, where 50 U/ml stimulates half maximal colony development in a bone marrow assay), IL-6: 5 U/ml (50 U/ml defined as producing half maximal 7TD1 growth), and IL-1 and TNFα: 10 U/ml (50 U/ml defined as 50% cytolytic activity).

5.2.4 Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from testicular or peritoneal macrophage cultures 24 hours after plating, or from whole testes according to a modification of the method described by Chomczynski and Sacchi (1987). As an additional positive control for rat cytokine mRNA expression, mRNA was also prepared from rat spleen cells stimulated with 4 mg / ml PHA for 40 h followed by 10 ng/ ml phorbol mystic acid (PMA) for 6 h. Briefly, approximately 10^6 testicular or peritoneal macrophages or activated spleen cells, or 200 mg of testicular tissue, were disrupted in 2 ml of 'solution A' (2 M guanidinium thiocyanate, 0.05 M b-mercaptoethanol, 12.5 mM Na citrate pH 7.0, combined in a 1:1:0.1 ratio with water-saturated phenol and 2 M Na acetate pH 4.0). Phase separation was effected by addition of 0.2 ml of chloroform-isoamyl alcohol mixture (49:1), and the

mixture was vortexed and incubated at 4°C for 10 min. Samples were centrifuged at 10,000 g for 20 min at 4°C, and the RNA precipitated from the aqueous phase by addition of an equal volume of isopropanol (-20°C, 1-2 h). The pellet was washed in 75% ethanol, dried *in vacuo*, dissolved in H_2O and RNA was quantitated by measurement of absorption at 260 nm.

First strand cDNA synthesis was achieved by reverse transcription (RT) of RNA primed with oligo dT, employing a Superscript RNase H- reverse transcriptase kit (Gibco, Grand Island, NY). Approximately 1 mg of RNA in 10 ml of water was incubated with 1 ml of oligo dT (500 mg/ ml; Gibco) at 70°C for 10 min, then chilled on ice for 5 min. Following addition of 4 ml of 5 x RT buffer (250 mM Tris-HCl pH 8.3, 300 mM KCl, 15 mM $MgCl_2$), 2 ml 0.1 M DTT, 2 ml 10 mM dNTP's (10 mM each dATP, dGTP, dCTP and dGTP), and 1 ml of RT enzyme the reaction was allowed to proceed for 90 min at 43°C, prior to RNA-cDNA denaturation and enzyme inactivation at 94°C for 5 min, and quenching on ice for 5 min. The cDNA was made up to 50 ml with water and stored at -20°C.

Primer pairs specific for cytokine cDNAs were designed with the aid of Primer Designer software using published murine cytokine cDNA sequences (Table 5.1). The PCR amplification employed reagents supplied in a Taq DNA polymerase kit (Bresatec, Adelaide, Australia) according to a protocol based on that described by Arcellana-Panlilio and Schultz (1993). Each 50 ml reaction volume consisted of 67 mM Tris-HCl pH 8.8, 17 mM $(NH_4)_2SO_4$, 0.2 mg/ ml gelatin, 0.45% Triton X-100, 2.5 mM $MgCl_2$,, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 2 U Taq DNA polymerase, 2 mM 3' and 5' primer, and 5 ml of cDNA, overlayed with 50 ml of paraffin oil. The conditions for amplification were 5 min at 94°C; followed by 38-48 cycles (see Table 5.2.1) of denaturation for 1 min at 94°C, annealing for 1 min at 58-66°C (see Table 5.2.1), and extension for 1 min at 72°C; followed by a final extension for 7 min at 72°C. Reaction products were analysed by electrophoresis through 2% agarose (electrophoresis grade, Gibco BRL) gel containing 0.5 mg / ml EtBr in TAE buffer (40 mM Tris, 20 mM Na acetate, 1 mM EDTA, pH 7.2), and the size of PCR products were determined by comparison to molecular weight markers (Bresatec).

Table 5.1. Primer sequences and PCR conditions for cytokine cDNAs.

cytokine	5' primer	3' (complimentary) primer	product size	PCR conditions (annealing temp ^{o}C, # cycles)
IL-1α	GAGCAAGCTTAAGAAGAGACGGCTGA	GAGAGAATCCAGCGCTCACGAACAGT	342bp	58°C/48
IL-1β	GAGCAAGCTTTACCTATCCTGTGTAA	GAGAGAATCCGTACTGATGTACCAGT	170bp	58°C/45
IL-6	GTCTGAATTCTCCTCTGGTCTTCTGG	GTCTATCGATGGTCTTGGTCCTTAGC	228bp	58°C/45
GM-CSF	GGAAGCATGTAGAGGCC	GGGCAGTATGTCTGGTA	222bp	66°C/45
TNFα	GAGCAAGCTTCTCCTCTCTACCATCA	GAGAGAATCCCCAAAGTAGACCTACC	182bp	60°C/42
Actin	CGTGGGCCGCCCTAGGCACCA	TTGACCTTAGGGTTCAGGGGGG	243bp	58°C/40

5.2.5 Statistical Analysis

The Students t-test was used to compare control and experimental groups. Values of $P>0.05$ were not considered significant.

5.3 Results

5.3.1 Bioassay Specificity

Thymidine uptake, MTT metabolism and viability of each cytokine-responsive cell line were found not to be affected by the addition to growth media of LPS, IFNγ or indomethecin in the presence or absence of cytokine (Table 5.2). To avoid interassay variability, all supernatants were assayed in one assay. All cytokine assays were performed in flat-bottomed 96-well microtitre trays (Costar, Cambridge, MA) and standardized against recombinant cytokines.

The L929, A375, FD5/12 and 7TD1 cell lines used in the bioassays are reported to be specifically responsive to rodent TNFα, IL-1, GM-CSF and IL-6 respectively (Matthews and Neale, 1987; Nakei et al, 1988; Keslo and Owens, 1988; Van Snick et al, 1986). However, since production of cytokines by the rat testes is not well characterised, the ability of cytokine-specific antibodies to neutralise the bio-response of cell lines to testicular macrophage supernatants was investigated. Goat αGM-CSF polyclonal antibody at a dilution of 1:100,000 neutralised the FD5/12 cell proliferative response to 1000 U / ml of recombinant murine GM-CSF, and similarly neutralised activity in supernatants from unstimulated peritoneal and testicular macrophage cultures (Figure 5.1). The bioactivity of 1000 U / ml of recombinant human IL-6 activity in the 7TD1 assay was reduced approximately 7-fold by 2.5 μg/ml of rat αIL-6 mAb, and the activities of stimulated peritoneal and testicular macrophage culture supernatants were similarly neutralised (Figure 5.1). The cytotoxic activities of recombinant IL-1 and

Table 5.2 Bioassay Specificity

Indomethacin (indo: 10μg/ml), LPS (10μg/ml) and/or IFNγ (200U/ml) had no significant effect on the viability of any of the cytokine-responsive cell lines tested, nor did they significantly alter the half maximal (50%) proliferative (FD5/12 and 7TD1) and cytotoxic (A375 and L929) points in the presence of the recombinant cytokines.

Cytotoxic assays: Where rIL-1 and rTNFα represent values for half maximal cytolytic activity

	Control	+LPS	+IFN	+LPS+IFN	+indo
A375 (A_{570})	1.55±0.13	1.62±0.14	1.53±0.16	1.41±0.15	1.47±0.18
+rIL-1 (%50)	1.02±0.13	0.96±0.09	0.91±0.11	1.05±0.15	1.08±0.11
L929 (A_{570})	1.28±0.18	1.44±0.16	1.21±0.15	1.26±0.19	1.34±0.13
+rTNFα (%50)	0.62±0.08	0.66±0.13	0.72±0.11	0.61±0.08	0.58±0.06

Proliferative Assays: Where rIL-6 and rGM-CSF represent values for half maximal colony development

	Control	+LPS	+IFN	+LPS+IFN	+indo
7TD1(A_{570})	0.22±0.02	0.20±0.02	0.22±0.02	0.24±0.02	0.19±0.02
+rIL-6 (%50)	0.53±0.06	0.57±0.05	0.49±0.04	0.52±0.06	0.58±0.06
FD5/12(dpm)	201±28	183±24	211±18	197±32	168±30
+rGM-CSF(%50)	62034±5134	56734±5403	63117±4718	59906±5921	57390±4981

±SEM values: n=4, no significant differences.

Table 5.3 Effects of Collagenase Isolation on Cytokine Secretion.

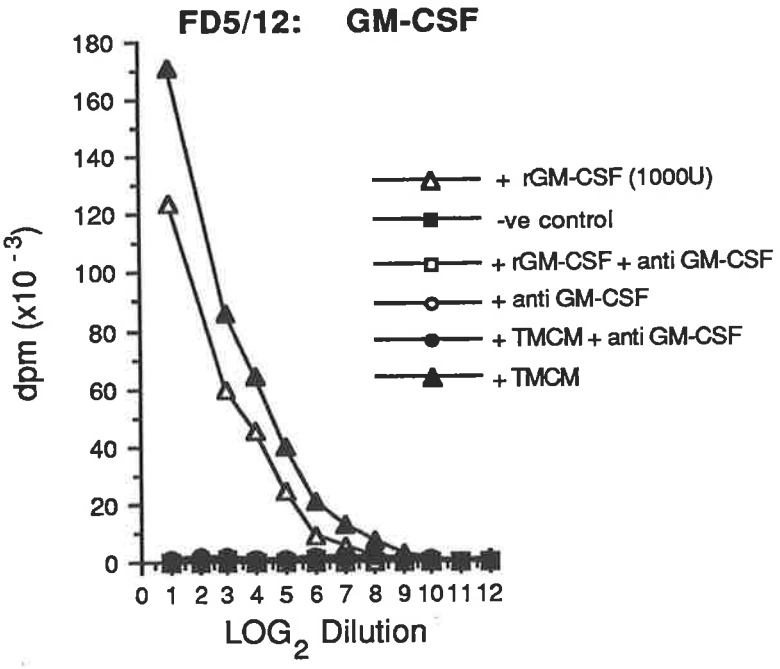
The effects of collagenase on basal cytokine secretion by testicular and peritoneal macrophages compared with the mechanical isolation procedure which uses no enzymatic digestion of testicular tissue.

	Cytokines Expressed as U/10^6 cells			
	IL-1	TNFα	IL-6	GM-CSF
Testicular Macrophage mechanical isolation	23±2.5	22±3.5	108±16	203±24
Testicular Macrophage collagenase isolation	19±2.1	17±2.1	94±12	231±33
Peritoneal Macrophage mechanical isolation	47±3.8	49±6.1	91±14	21±3.1
Peritoneal Macrophage collagenase isolation	52±6.4	41±4.3	121±19	28±4.2

±SEM values: n=4, no significant differences in cytokine production between

Figure 5.1

One thousand units of recombinant GM-CSF (rGMCSF) or recombinant IL-6 (rIL-6) were used to create a standard curve for the FD5/12 and 7TD1 bioassays respectively. Antibodies to GM-CSF (αGMCSF, 1:250 000) and IL-6 (αIL-6, 1:250 000) specifically neutralise the activity of both rGM-CSF and rIL-6. The activity of testicular macrophage conditioned medium (TMCM) from unstimulated cultures and cultures activated with IFNγ and LPS was neutralised activity in the FD/5/12 and 7TD1 assays respectively.


FD5/12: GM-CSF
dpm (x10 -3)
180
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
LOG2 Dilution
+ rGM-CSF (1000U)
-ve control
+ rGM-CSF + anti GM-CSF
+ anti GM-CSF
+ TMCM + anti GM-CSF
+ TMCM

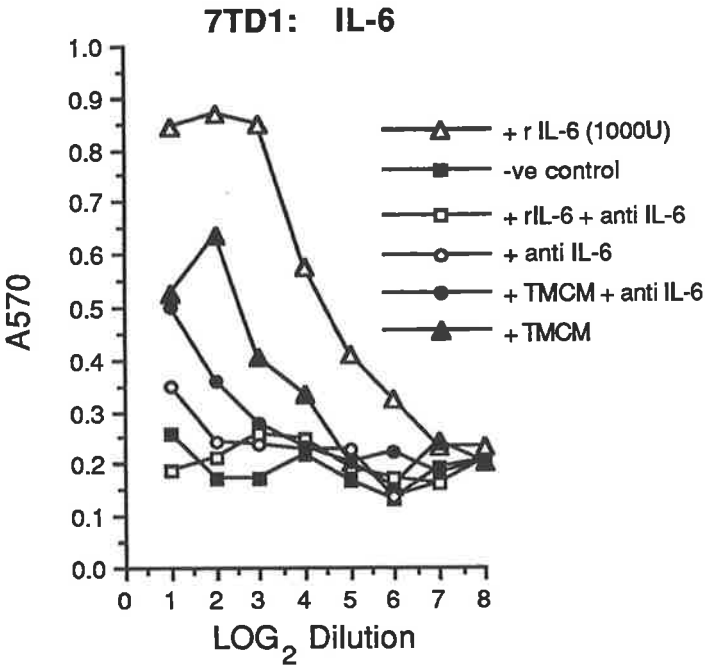
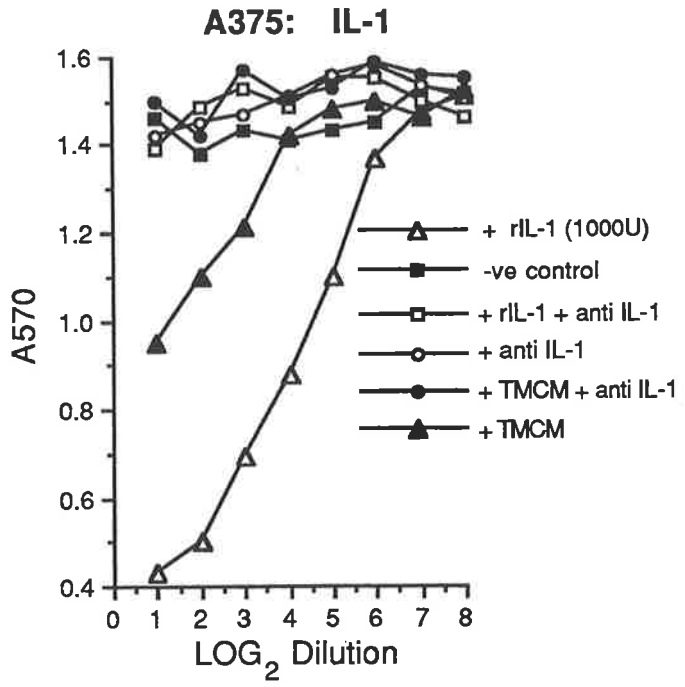
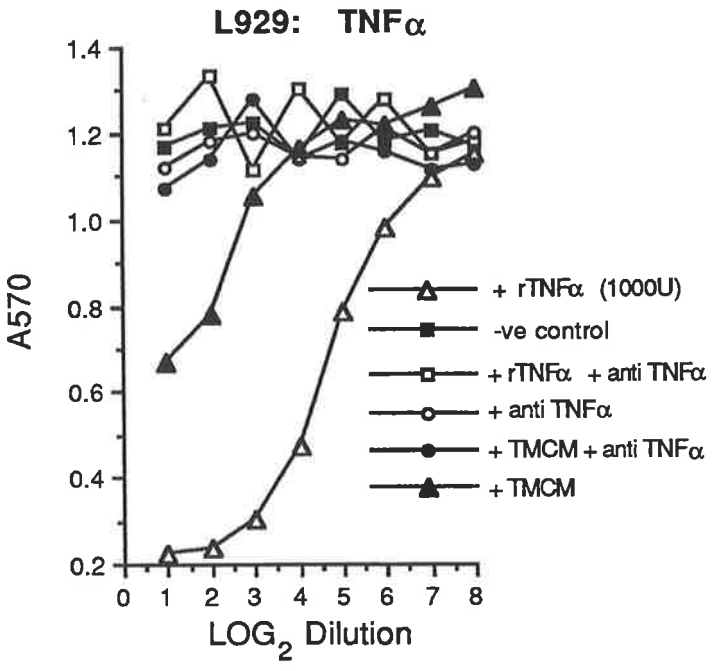

7TD1: IL-6
A570
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8
LOG2 Dilution
+ r IL-6 (1000U)
-ve control
+ rIL-6 + anti IL-6
+ anti IL-6
+ TMCM + anti IL-6
+ TMCM

Figure 5.2

One thousand units of recombinant IL-1 (rIL-1) or recombinant TNFα (rTNFα) are used to create a standard curve for the A375 and L929 bioassays respectively. Antibodies to IL-1 (αIL-1, 1:250 000) and TNFα (αTNFα, 1:250 000) specifically neutralise both the activity of rIL-1 and rTNFα. The activity of testicular macrophage conditioned medium (TMCM) from cultures activated with IFNγ and LPS, in A375 and L929 bioassays repectively was also neutralised.


A375: IL-1
A570
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0 1 2 3 4 5 6 7 8
LOG2 Dilution
+ rIL-1 (1000U)
-ve control
+ rIL-1 + anti IL-1
+ anti IL-1
+ TMCM + anti IL-1
+ TMCM


L929: TNFα
A570
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0 1 2 3 4 5 6 7 8
LOG2 Dilution
+ rTNFα (1000U)
-ve control
+ rTNFα + anti TNFα
+ anti TNFα
+ TMCM + anti TNFα
+ TMCM

cytokines, and further support the likely identity of the bioactivities produced by testicular macrophages *in vitro*.

5.3.2 Cytokine Production by testicular macrophages in vitro.

To investigate the cytokine secretion profile of testicular macrophages, highly enriched cultures of macrophages were prepared by the three step protocol described above. Specific cytokine bioassays were employed to measure the IL-1, TNFα, IL-6 and GM-CSF content of supernatants of cells cultured for 48h alone or in the presence of the macrophage activators LPS and IFNγ. The 'basal' and 'activated' cytokine output of testicular macrophages was compared to that of macrophages harvested from the peritoneal cavity and cultured with and without activation. The results presented are the

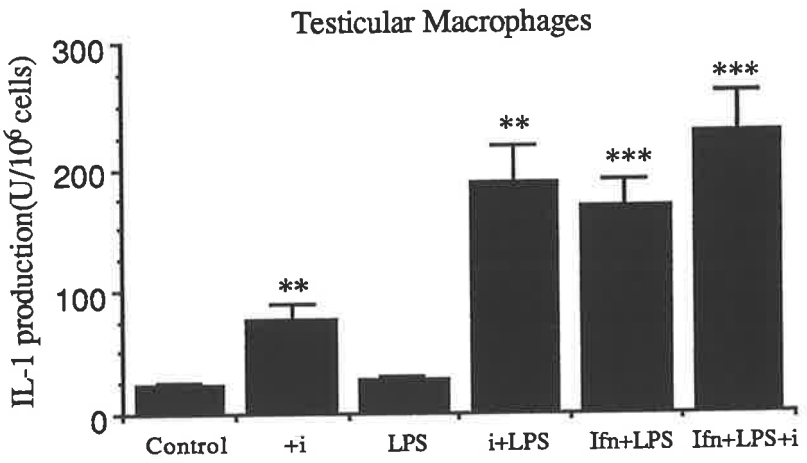
TNFα in the A375 and L929 assays respectively were neutralised by specific antibodies against these cytokines (50μg / ml rabbit αIL-1α neutralised 100U/ ml recombinant IL-1α, and rabbit αTNFα diluted 1:100 neutralised 100U/ml TNFα)(Figure 5.2). The cytotoxic activities of supernatants from activated peritoneal and testicular macrophage cultures were similarly neutralised by αTNFα and αIL-1α antibodies at the same concentrations. These results confirm the specificity of the bioassays for individual rat

mean of 4 separate experiments (3 animals/ isolation). The secretion of cytokines by both testicular and peritoneal macrophages was shown not to be affected by exposure to collagenase (Table 5.3)

For basal cytokine secretion, the most striking finding was that GM-CSF output from testicular macrophages was approximately 740% of that from peritoneal macrophages, whereas production of IL-1,TNFα and IL-6 by the testicular macrophage was similar to or slightly less than for peritoneal macrophages (50%, 60% and 130% respectively of peritoneal macrophage levels)(Figure 5.3 to Figure 5.6).

Figure 5.3

Interleukin-1 (IL-1) concentrations in testicular macrophage (TM) and peritoneal macrophage (PM)-conditioned medium after 48 hours culture, in the presence of various combinations of indomethacin (+i), LPS (+LPS) or IFNγ (Ifn). Values are the mean±SEM of 4 samples. *=P<0.05, **=P<0.01, ***=P<0.001 compared with the Control (basal) concentrations.


Testicular Macrophages
IL-1 production(U/10^6 cells)
300
200
100
0
**
**
***
***
Control
+i
LPS
i+LPS
Ifn+LPS
Ifn+LPS+i

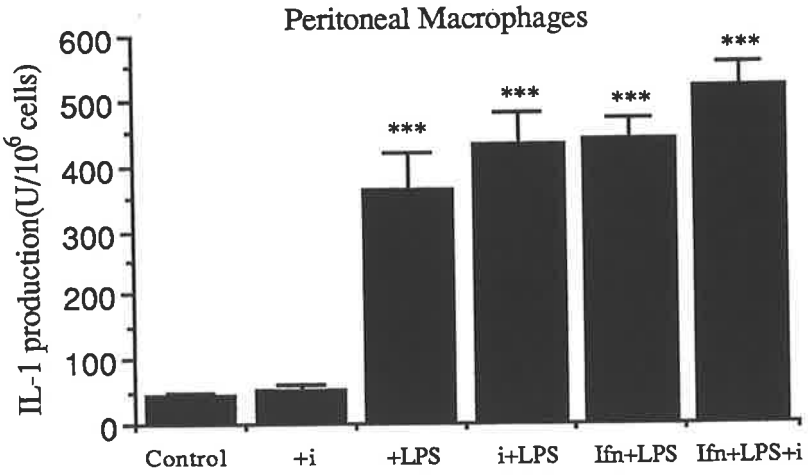
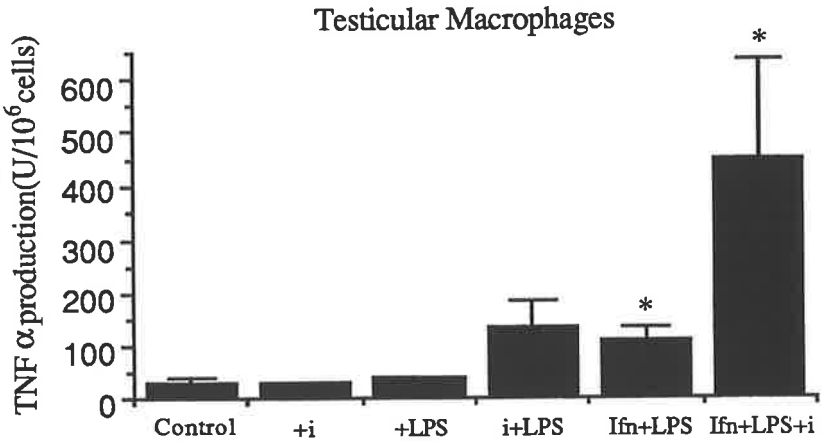

Peritoneal Macrophages
IL-1 production(U/10^6 cells)
600
500
400
300
200
100
0
***
***
***
***
Control
+i
+LPS
i+LPS
Ifn+LPS
Ifn+LPS+i

Figure 5.4

Tumour necrosis factor α (TNFα) concentrations in testicular macrophage (TM) and peritoneal macrophage (PM)-conditioned medium after 48 hours culture, in the presence of various combinations of indomethacin (+i), LPS (+LPS) or IFNγ (Ifn). Values are the mean±SEM of 4 samples. *=P<0.05, **=P<0.01, ***=P<0.001 compared with the Control (basal) concentrations.


Testicular Macrophages
TNF α production(U/10^6 cells)
600
500
400
300
200
100
0
*
*
Control
+i
+LPS
i+LPS
Ifn+LPS
Ifn+LPS+i

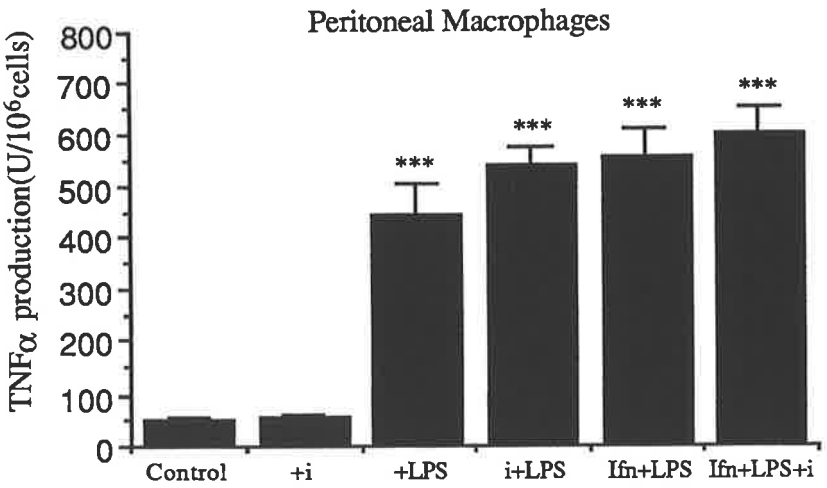
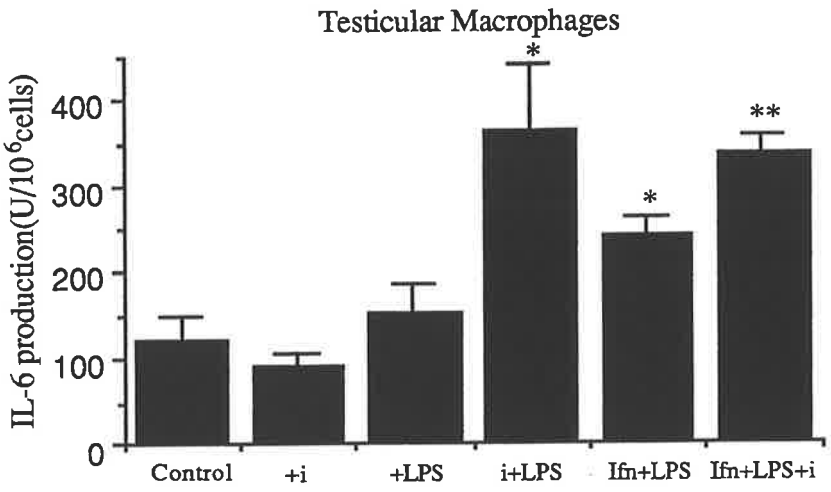

Peritoneal Macrophages
TNFα production(U/10^6cells)
800
700
600
500
400
300
200
100
0
***
***
***
***
Control
+i
+LPS
i+LPS
Ifn+LPS
Ifn+LPS+i

Figure 5.5

Interleukin-6 (IL-6) concentrations in testicular macrophage (TM) and peritoneal macrophage (PM)-conditioned medium after 48 hours culture, in the presence of various combinations of indomethacin (+i), LPS (+LPS) or IFNγ (Ifn). Values are the mean±SEM of 4 samples. *=P<0.05, **=P<0.01, ***=P<0.001 compared with the Control (basal) concentrations.


Testicular Macrophages
IL-6 production(U/10 6cells)
0
100
200
300
400
*
*
**
Control
+i
+LPS
i+LPS
Ifn+LPS
Ifn+LPS+i

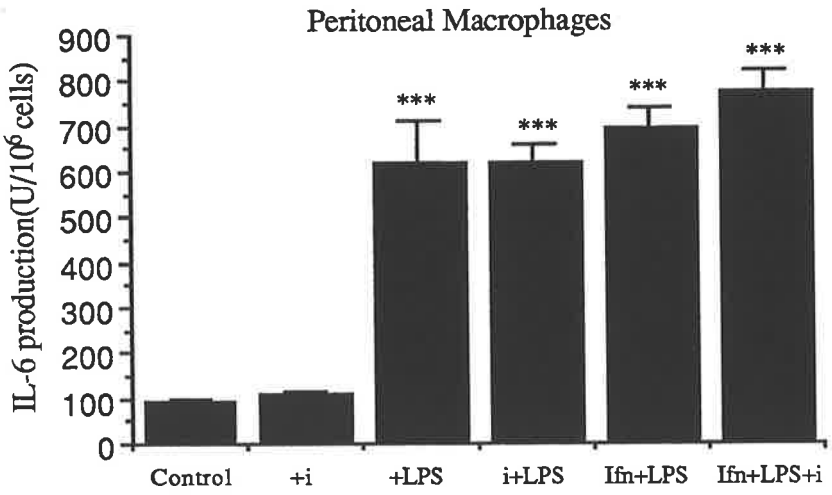
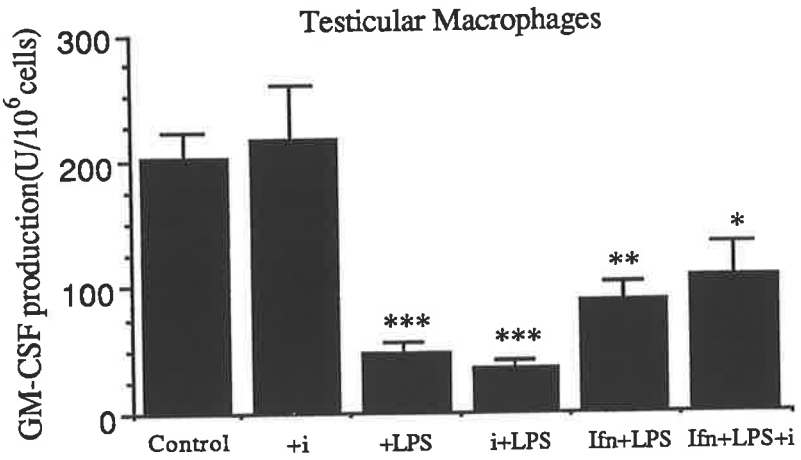
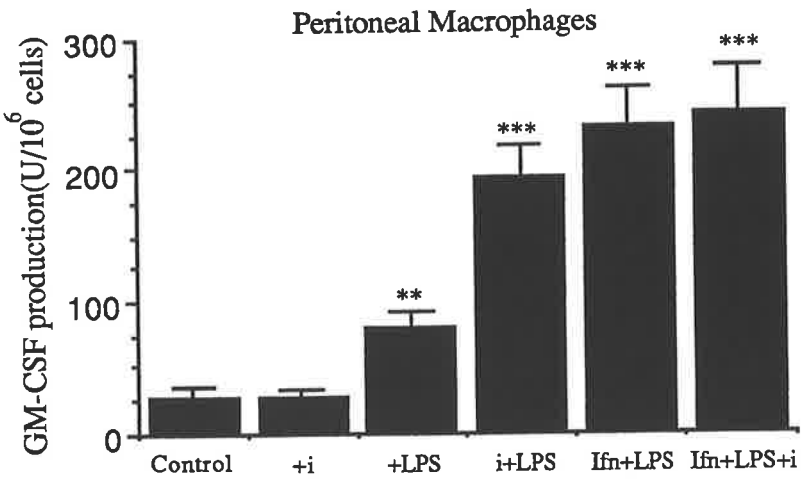

Peritoneal Macrophages
IL-6 production(U/10⁶ cells)
0
100
200
300
400
500
600
700
800
900
***
***
***
***
Control
+i
+LPS
i+LPS
Ifn+LPS
Ifn+LPS+i

Figure 5.6

Granulocyte macrophage-colony stimulating factor (GM-CSF) concentrations in testicular macrophage (TM) and peritoneal macrophage (PM)-conditioned medium after 48 hours culture, in the presence of various combinations of indomethacin (+i), with LPS (+LPS) or IFNγ (Ifn). Values are the mean±SEM of 4 samples. *=P<0.05, **=P<0.01, ***=P<0.001 compared with the Control (basal) concentrations.


Testicular Macrophages
GM-CSF production(U/10^6 cells)
300
200
100
0
***
***
**
*
Control
+i
+LPS
i+LPS
Ifn+LPS
Ifn+LPS+i


Peritoneal Macrophages
GM-CSF production(U/10^6 cells)
300
200
100
0
**
***
***
***
Control
+i
+LPS
i+LPS
Ifn+LPS
Ifn+LPS+i

Most interestingly, the two macrophage populations responded quite differently to activation with LPS, alone or in combination with IFNγ. As expected, production of all four cytokines by peritoneal macrophages was markedly enhanced following incubation with LPS (660%, 840%, 660% and 340% of basal levels for IL-1, TNFα, IL-6 and GM-CSF respectively), and was similar or further enhanced when cells were incubated with both LPS and IFNγ (1020%, 1050%, 740% and 860% of basal levels respectively). In contrast, the secretion of IL-1, TNFα and IL-6 by testicular macrophages was not significantly altered by incubation with LPS (110%, 120%, 120% of basal levels respectively). This refractoriness to LPS activation was at least partially reversed when testicular macrophages were incubated with the potent macrophage activator IFNγ in addition to LPS. TNFα and IL-6 outputs increased to 340% and 200% of basal levels respectively, and the increase in IL-1 release was similar to that seen for peritoneal cells (730% of basal level).

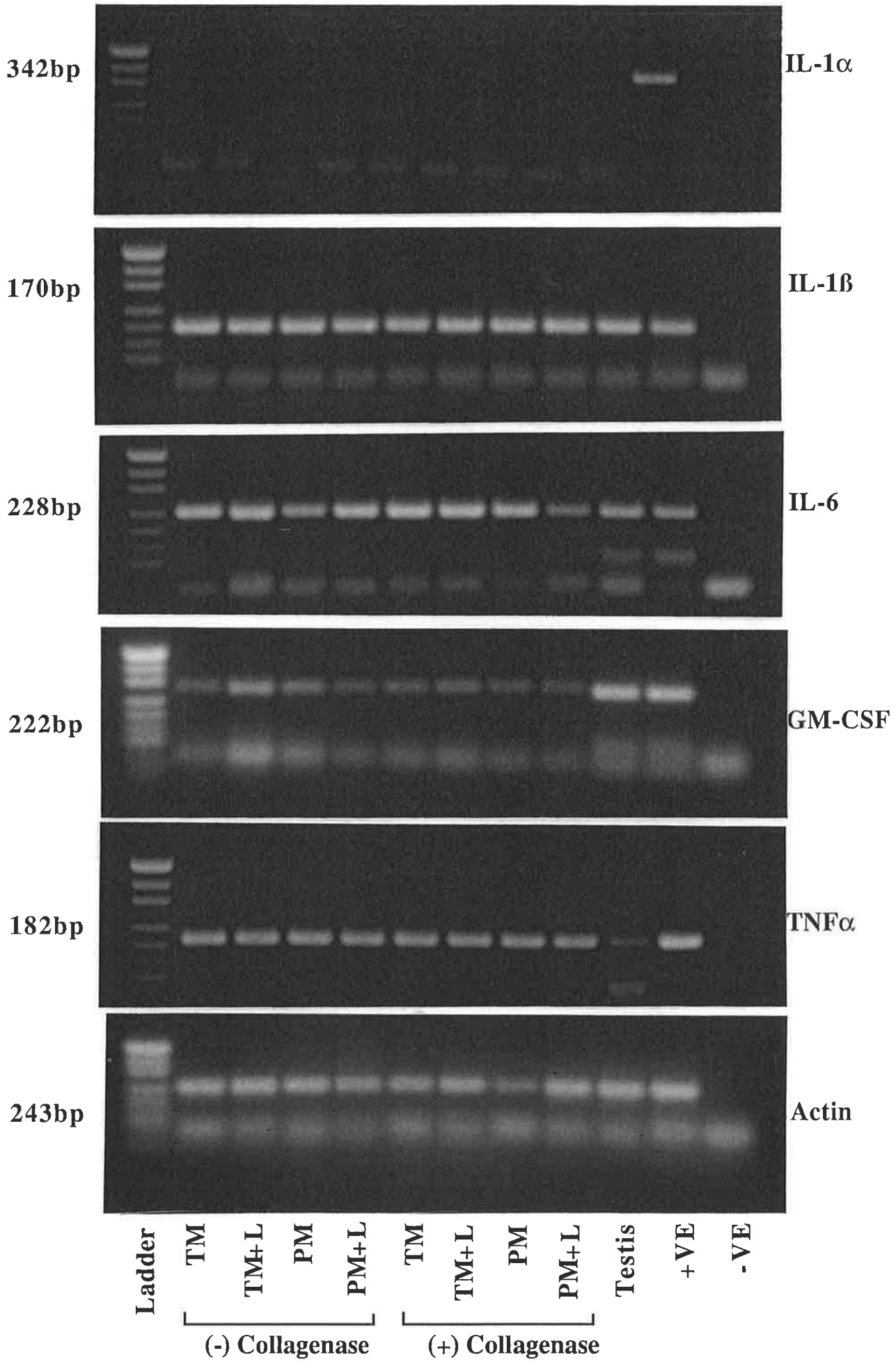
Most unexpectedly, incubation of testicular macrophages with LPS caused a marked decrease in GM-CSF output (to 20% of basal level). IFNγ partially overcame the inhibition by LPS of GM-CSF release from testicular macrophages (from 20% of basal levels with LPS to 45% of basal levels with LPS plus IFNγ).

5.3.3 The effect of indomethacin on cytokine production by testicular macrophages

Testicular macrophages are known to be a potent source of prostaglandins in vitro (Chapter 4). To determine whether the altered cytokine profile of resting testicular macrophages or their diminished responsiveness to LPS activation was dependant upon an autocrine action of prostaglandin, the effect of indomethecin on the cytokine content of supernatants from macrophages was also measured. Whereas indomethecin had no effect on the basal output of cytokines from peritoneal macrophages, the basal IL-1 output from testicular macrophages was found to be elevated approximately 300% in the presence of indomethecin (Figure 5.3). Indomethecin was also found not to have any significant effect on the cytokine output from LPS, or LPS plus IFNγ activated peritoneal cells, but

Figure 5.7

Analysis of RT-PCR amplification products for IL-1α, IL-1ß, IL-6, GM-CSF, TNFα and actin mRNA. Testicular (TM) and peritoneal (PM) macrophages were obtained with (+collagenase) or without (-collagenase) collagenase digestion, and cultured alone or with LPS stimulation (TM+L, PM+L) for 24 hours. A whole testis extract (testis), positive control (activated spleen cells) and a negative control were also analysed, and a puc-19 molecular weight ladder was included to determine the size of the PCR products.


342bp
IL-1α
170bp
IL-1ß
228bp
IL-6
222bp
GM-CSF
182bp
TNFα
243bp
Actin
Ladder
TM
TM+L
PM
PM+L
TM
TM+L
PM
PM+L
Testis
+VE
-VE
(-) Collagenase
(+) Collagenase

did act to at least partially reverse the failure of testicular macrophages to respond to LPS activation.

The IL-1, TNFα and IL-6 output of these cells increased to 820%, 420%, 300% of basal levels in the presence of LPS and indomethecin (Figures 5.3, 5.4 and 5.5). The effect of indomethecin on LPS-induced cytokine output by the testicular macrophages was similar in the presence of IFNγ, with the IL-1, IL-6 and particularly the TNFα contents of supernatants from cells incubated with both activators being elevated a further 20%, 25% and 250% by the inclusion of indomethecin. These data indicate that prostaglandins are important mediators in the inhibition of IL-1, TNFα and IL-6 output from LPS activated testicular macrophages. In contrast, prostaglandins appear not to be involved in the altered regulation of GM-CSF release from testicular compared with peritoneal macrophages, since indomethecin failed to influence the high basal output of GM-CSF from testicular cells, or to alter the effects of LPS, or LPS and IFNγ on this cytokine (Figure 5.6).

5.3.4 Expression of cytokine mRNAs by testicular macrophages and intact testes

To investigate whether production of cytokine bioactivities by testicular macrophages was accompanied by expression of the corresponding mRNAs, cDNA was prepared from cultured cells and subjected to RT-PCR using primer pairs specific for IL-1α, IL-1β, TNFα, IL-6 and GM-CSF cDNAs, in addition to actin which served as a control for cDNA integrity. Amplicons of the predicted sizes were generated, using primers for cytokines including IL-1α, IL-1β, TNFα, IL-6 and GM-CSF, from cDNAs prepared from testicular and peritoneal macrophages. Expression of IL-1α mRNA by unstimulated or LPS-activated testicular macrophages was low and variable, however it was readily detected in whole testis tissue. Expression of all cytokine mRNAs was independent of exposure to collagenase during macrophage purification, and of the addition of LPS to cultures. Furthermore, mRNAs for IL-1β, TNFα, IL-6 and GM-CSF (to a lesser and variable degree) were also detected in intact testes (Figure 5.7).

5.4 Discussion

These studies indicate that macrophages harvested from the rat testes have a novel cytokine secretion profile and an altered responsiveness to activators *in vitro*, compared with macrophages from the peritoneal cavity. Specifically, testicular macrophages were found to have a high basal production of GM-CSF (8-fold greater than peritoneal cells), and a refractoriness to the typical LPS-induced increase in IL-1, TNFα and IL-6 output. These studies confirm and extend previous reports that testicular macrophage release of TNFα responds poorly to LPS activation (Hutson, 1992; Moore and Hutson, 1994; Xiong and Hales, 1993).

The mechanisms underlying the dampened reponse of the testicular macrophage to LPS remain unclear. The finding that treatment of testicular macrophages with LPS caused a reduction in their GM-CSF output indicates that these cells are responsive to LPS, but that specific blocks or alterations in signalling pathways distal to receptor activation may operate for other cytokines. An autocrine role for prostaglandins in inhibiting the LPS response is suggested by the finding that indomethecin reverses, at least partially, the block in LPS-induced upregulation of IL-1, TNFα and IL-6 release. The findings that testicular macrophages are a rich source of prostglandins (Chapter 4), and that prostaglandin E is implicated in the down-regulation of IL-1 and IL-4 production by human monocytes (Hart et al, 1989), support this proposal. IFNγ was also found to partially overcome the inability of testicular cells to respond to LPS, and reduced the LPS-induced decline in GM-CSF synthesis. That these effects of IFNγ are mediated through the prostaglandin axis is suggested by the finding that IFNγ acts to reduce prostaglandin production by testicular macrophages (Chapter 4). However, the failure of indomethecin to alter basal or LPS and IFNγ regulated GM-CSF release suggests that the action of IFNγ in regulating this cytokine may be independant of prostaglandins.

The degree to which these findings can be extrapolated to the *in vivo* behaviour of testicular macrophages is not clear. The possibility that cytokine synthesis by

macrophages is altered as a consequence of the harvesting protocol cannot be excluded, and indeed other investigators have reported an effect of collagenase on testicular macrophage activation (Moore and Hutson, 1994). However results in the present study do not support this as the profile of cytokine bioactivities produced by crude preparations of testicular macrophages isolated by mechanical dispersal of the testis was similar to that produced by testicular macrophages exposed to collagenase. Furthermore, the secretion of cytokines by peritoneal macrophages has been found not to be effected by exposure to collagenase after lavage (Table 5.3).

However, it is interesting to speculate that the deviation in cytokine profile and activation responsiveness of testicular macrophages seen in the present study reflects a tissue-specific macrophage phenotype presumably arising in the testes as the consequence of local microenvironmental forces. A comparison between the protein secretory profiles of testicular and peritoneal macrophages has provided other evidence supporting this concept (Hutson and Stocco, 1989). Cytokines and other mediators can both induce and suppress various macrophage properties reversibly, to give rise to macrophages with alternate tissue-specific phenotypes (Rappolee and Werb, 1992; Gordon et al, 1992). Indeed it is clear that the recruitment and behaviour of macrophages within female reproductive tract tissues is tightly regulated by cytokine gradients originating within local stromal cells, particularly the uterine epithelium (Hunt, 1994; Robertson et al, 1994). In the testes, it may be relevent that Sertoli cells produce high levels of IL-1α (Gerad et al, 1991) which is known to regulate its own production and that of TNFα in macrophages through a mechanism of negative feedback involving prostaglandin synthesis (Kunkel et al, 1986a; Kunkel et al, 1986b)

The physiological significance of cytokine secretion by testicular macrophages remains to be investigated. However, a paracrine role in regulating normal testis function, including steroidogenesis and spermatogenesis has long been suggested by the close association of these cells and the testosterone-producing Leydig cells in the testicular interstitium. In

the adult rat testis the macrophages and Leydig cells display physical associations via cytoplasmic interdigitations (Miller et al, 1983; Bergh, 1985; Bergh, 1987; Hutson, 1992). There is also a strong developmental correlation in the rat testis between macrophages and Leydig cells both in number and cell size prior to and during puberty. There have been many *in vitro* studies of the effects of IL-1 and TNFα on testosterone production by Leydig cells, although much of the data has been inconclusive (Warren et al, 1990; Calkins et al, 1990; Moore and Moger, 1990). However it is of interest that depletion of macrophages from the testes by 'suicide' phagocytosis of cytotoxin is associated with reduced testosterone secretion by the depleted testis (Maddocks et al, 1992; Bergh et al, 1993).

The testis has long been recognised as having dysfunctional afferent immune activity, and this has led to its reputation as an immunologically-privileged organ (Maddocks and Setchell, 1990). The current findings, together with the observations in Chapter 4 that testicular macrophages are not able to support mitogen-activated lymphocyte proliferation *in vitro* , are consistent with the conclusion that poor immunoaccessory function of testicular macrophages is a major factor underlying this immune deficiency. The high rate of basal secretion of GM-CSF by testicular macrophages would also be expected to contribute to an 'immunosuppressive' microenvironment, since GM-CSF has been implicated in regulating the synthesis in macrophages of molecules including IL-1 inhibitor (Mazzei et al, 1990) and PGE_2 which, in addition to having autocrine actions in macrophages, inhibits cytotoxic T-lymphocyte generation. Indeed, administration of rGM-CSF to normal mice has been found to induce an a systemic shift towards an immunosuppressive phenotype in peripheral macrophages, characterised by enhanced PGE_2 release (Fu et al, 1991). Constitutive secretion of GM-CSF from tumors appears to promote a metastatic phenotype through a mechanism associated with macrophage-mediated expansion of T-suppressor cell populations (Tsuchiya et al, 1988). The removal of testicular macrophages by liposome-induced suicidal approach increases the local inflammatory response seen in the testis after hCG administration suggesting a role for macrophage modulation of this response at this site (Bergh et al, 1993). This down-

regulation of local immune responses by resident tissue macrophages may be a general physiological phenomenon, since in the lung tissues, alveolar macrophages appear to suppress the pulmonary immune response to microbial flora (Thepen et al, 1989).

CHAPTER 6: Testicular Macrophage and Leydig Cell Interactions.

Chapter 6. Inter-cellular Communication

6.1 Introduction

While traditionally considered in terms of initiating the immune response, the paracrine activities of macrophage products encompass many important events during cell differentiation and proliferation. The macrophage produces a wide range of bioactive factors which are capable of initiating paracrine communication between cells (Rapolee and Werb, 1992). Cytokines and colony stimulating factors once viewed as fulfilling purely immunologic roles within the body are now known to work outside of this area. While this alternate role for macrophages and their products has been most noticeable in female reproduction (Hunt, 1989; Adashi, 1990), there is comparatively little known of their role in male reproductive function.

These associations and recent studies showing that cytokines can influence testosterone production (Warren et al, 1990; Mauduit et al, 1991, Moore and Moger, 1991) have suggested a functional relationship between the testicular macrophage and Leydig cell. However, while these studies examined the effects of recombinant cytokines added to Leydig cell culture, none of these studies examined the role of testicular macrophage-derived cytokines during co-culutre.

The findings in this thesis so far have shown the testicular macrophage to possess a unique secretory profile, of which most notable was the constitutive production of GM-CSF and prostaglandin. The purpose of the present study is to investigate cytokine production by the testicular macrophage and testosterone production by the Leydig cell during co-culture and in the presence of macrophage and Leydig cell stimulatory factors.

6.2 Methods

6.2.1 Cell isolation and culture

Leydig cells, testicular and peritoneal macrophages were all isolated as described in chapter 2.3. Leydig cells (10^6cells/ml), testicular macrophages ($2.5x10^5$cells/ml) and peritoneal macrophages (2.5x105cells/ml) were then cultured in humidified air with 5%CO_2 at 34°C for 24 hours. Co-cultures ($1.25x10^6$cell/ml) contained Leydig cells in a 4:1 ratio with macrophages as occurs naturally in the rat testis, and were cultured under identical conditions simultaneously with the individual cell preparations.

Cell cultures were split into two groups, one with the cyclooxygenase inhibitor indomethacin (10μg/ml) added, and the other without. Cells were then cultured normally for basal readings, while some were cultured alone with the macrophage stimulants IFNγ and LPS (200U/ml, 10μg/ml), and the Leydig cell stimulant hCG(10IU/ml) or a combination of these two. In an additional experiment testosterone (100ng/ml) and Leydig cell conditioned media (100μl:10%) were added to testicular macrophage cultures in order to investigate the downregulation of testicular macrophage-derived GM-CSF. Media randomly collected and pooled from four cultures at each time interval (2, 6, 12 and 24 hours) was then centrifuged (10000xg for 5 min), decanted, frozen and stored at -80°C until assayed.

The conditioned-medium was then assayed for the cytokines IL-1, IL-6, TNFα and GM-CSF as described in chapter 5.2 , and for prostaglandins E_2 and $F_{2\alpha}$, and the steroid testosterone as described in chapter 2.6. The results presented are the observations from six separate isolations.

6.2.2 Statistical Analysis

The student's T-test and analysis of variance were used to determine significant differences where stated in the text.

6.3 Results

6.3.1 Prostaglandin production

Both the Leydig cell and testicular macrophage constituitively produced prostaglandins in vitro (Table 6.1). In comparison to the testicular macrophage and Leydig cell, the peritoneal macrophage produced no detectable levels of prostaglandin E_2 or $F_{2\alpha}$. The presence of indomethacin blocked prostaglandin production in all cultures to levels below the minimum sensitivity of the assay (Table 4.1). While the addition of hCG to culture did not alter prostaglandin levels in the macrophage conditioned medium, Leydig cells responded to hCG by increasing prostaglandin E_2 and $F_{2\alpha}$ production by 100%.

Likewise, LPS and IFNγ did not alter prostaglandin production by the Leydig cell but altered that observed in cultures of macrophages. In the presence of LPS and IFNγ, the peritoneal macrophages increased production of prostaglandin E_2 and $F_{2\alpha}$ from undetectable levels to 13.7±1.9 and 15.5±2.1ng/ml respectively. In contrast the testicular macrophage increased its production of PGE_2 from 14.2±2.6 to 27.5±2.1 ng/ml while simultaneously downregulating $PGF_{2\alpha}$ production from 18.6±1.8 to 7.2±1.2ng/ml.

In co-cultures of Leydig cells and testicular macrophages a similar effect was observed (Table 6.1) after the addition of LPS and IFNγ whereby the prostaglandin E_2 to $F_{2\alpha}$ ratio was altered from 1:1 to 2:1. The PGE_2 concentrations in these cultures were higher than those observed in any others whereas the $PGF_{2\alpha}$ concentrations did not change as compared to untreated Leydig cell-testicular macrophage co-cultures.

6.3.2 Testosterone Production

Neither of the macrophage populations produced detectable levels of testosterone during culture. Purified Leydig cells were cultured for 24 hours either alone or in co-culture with testicular and peritoneal macrophages. Samples of conditioned media taken from Leydig cell

Table 6.1 Prostaglandin Production

Prostaglandin concentrations in conditioned medium from testicular and peritoneal macrophage and Leydig cell culture supernatants, with or without hCG, and with or without LPS and IFNγ.

	Prostaglandin Concentration [ng/ml]	
Treatment	PGE_2	$PGF_{2\alpha}$
Testicular macrophage	14.2±2.6	18.6±1.8
Testicular macrophage + hCG	15.5±2.1	23.2±3.2
Testicular macrophage + IFN + LPS	27.5±2.1	7.2±1.2
Peritoneal macrophage	<1.0	<1.8
Peritoneal macrophage + hCG	<1.0	<1.8
Peritoneal macrophage + IFN + LPS	13.7±1.9	15.5±2.1
Leydig cell	10.1±1.8	14.3±1.6
Leydig cell + hCG	19.7±2.2*	28.4±2.1*
Leydig cell + IFN + LPS	12.2±2.0	15.4±1.3
Testicular Macrophage + Leydig cell	26.6±3.1	28.8±3.9
Testicular Macrophage + Leydig cell + hCG	41.9±3.4*	50.2±3.2*
Testicular Macrophage + Leydig cell+ IFN+ LPS	52.6±5.1*	24.6±2.2
Peritoneal Macrophage + Leydig cell	8.5±0.9	10.0±1.7
Peritoneal Macrophage + Leydig cell + hCG	12.2±1.1*	14.9±1.8*
Peritoneal Macrophage + Leydig cell + IFN+LPS	16.7±1.7*	19.9±2.1*

Values ±SEM, n=4; *p<0.05 compared with values from basal cultures.

cultures at 2, 6, 12 and 24 hours were used to determine the effects of macrophage-Leydig cell co-culture on testosterone production. The purified Leydig cells were shown to be functionally viable by significantly increasing testosterone production in response to hCG stimulation (10 IU/ml; compare Leydig cell concentrations in Figure 6.2A and Figure 6.3A).

The additon of indomethacin to unstimulated Leydig cells cultured alone, significantly reduced the basal production of testosterone over a 24 hour period (Fig 6.1 & 6.2; ANOVA $F<0.01$), but had no effect overall on levels of testosterone production after the addition of hCG to culture (Fig 6.3 & 6.4).

Basal production of testosterone by unstimulated Leydig cells alone or in co-culture with peritoneal macrophages was significantly higher after 2 hours than that seen in Leydig cell testicular macrophage co-culture (Fig 6.1A). The addition of indomethacin significantly and uniformly reduced testosterone production in both basal cultures of Leydig cells (16 ± 1.4 compared to 12 ± 1.8 ng/ml/10^6cells; Fig 6.1A&B) and in the macrophage-Leydig cell co-cultures.

Priming and triggering the testicular macrophages with IFNγ and LPS increased basal testosterone production by the Leydig cell significantly ($p<0.05$), above that of Leydig cells cultured alone (18 ± 1.4 compared to 15 ± 1.3 ng/ml/10^6cells; Fig 6.2A). Peritoneal macrophages did not display a similar effect. However, the addition of indomethacin in conjunction with IFNγ and LPS to peritoneal macrophage-Leydig cell cultures, significantly inhibited testosterone production at 2 and 6 hours but thereafter was not significantly different (Fig 6.2B).

Testosterone production by Leydig cells stimulated with hCG was significantly inhibited by both macrophage populations (Fig 6.3A&B). The addition of indomethacin to culturehad no significant effect on the marcophage derived inhibition of testosterone

Figure 6.1

Testosterone concentrations in testicular macrophage-Leydig cell (TM-LC), peritoneal macrophage-Leydig cell (PM-LC) and Leydig cells alone (LC) conditioned medium, from cultures without (**A**) or with indomethacin (**B**) over a 24 hour period. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs LC. Leydig cell testosterone production was significantly reduced in the presence of indomethacin (**B**) compared to without indomethacin (**A**); ANOVA F stat, $P<0.01$.

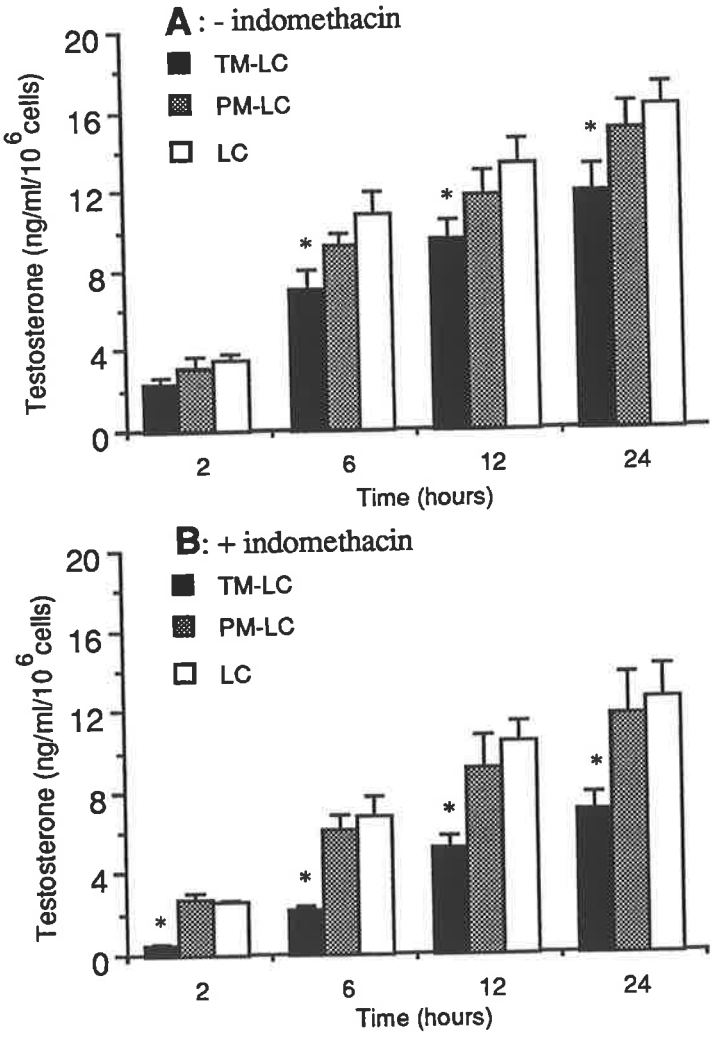

A : - indomethacin
TM-LC
PM-LC
LC
Testosterone (ng/ml/10 6 cells)
20
16
12
8
4
0
2
6
12
24
Time (hours)
B: + indomethacin
TM-LC
PM-LC
LC
Testosterone (ng/ml/10 6 cells)
20
16
12
8
4
0
2
6
12
24
Time (hours)

Figure 6.2

Testosterone concentrations in testicular macrophage-Leydig cell (TM-LC), peritoneal macrophage-Leydig cell (PM-LC) and Leydig cells alone (LC) conditioned medium from cultures stimulated with IFNγ and LPS. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs LC. Leydig cell testosterone production was significantly reduced in the presence of indomethacin (**B**) than observed without indomethacin (**A**); ANOVA F stat, $P<0.01$.

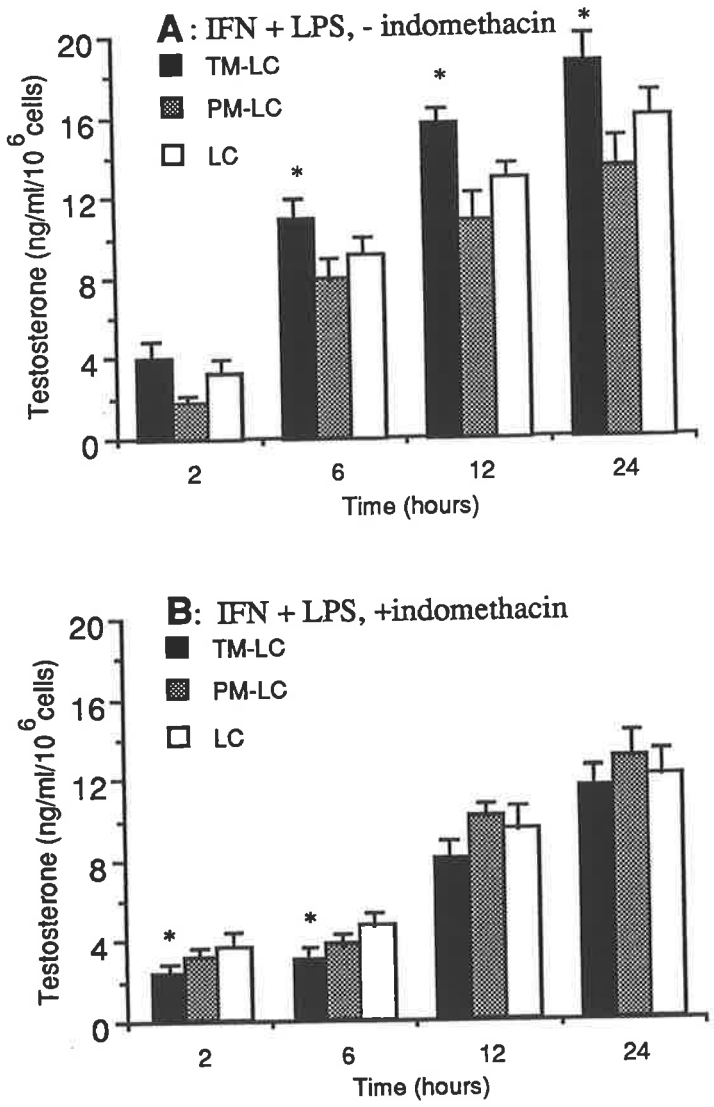

A : IFN + LPS, - indomethacin
TM-LC
PM-LC
LC
Testosterone (ng/ml/10 6 cells)
Time (hours)
B: IFN + LPS, +indomethacin
TM-LC
PM-LC
LC
Testosterone (ng/ml/10 6 cells)
Time (hours)

Figure 6.3

Testosterone concentrations in testicular macrophage-Leydig cell (TM-LC), peritoneal macrophage-Leydig cell (PM-LC) and Leydig cells alone (LC) conditioned medium from cultures stimulated with hCG, without (**A**) or with indomethacin (**B**) over a 24 hour period. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs LC.

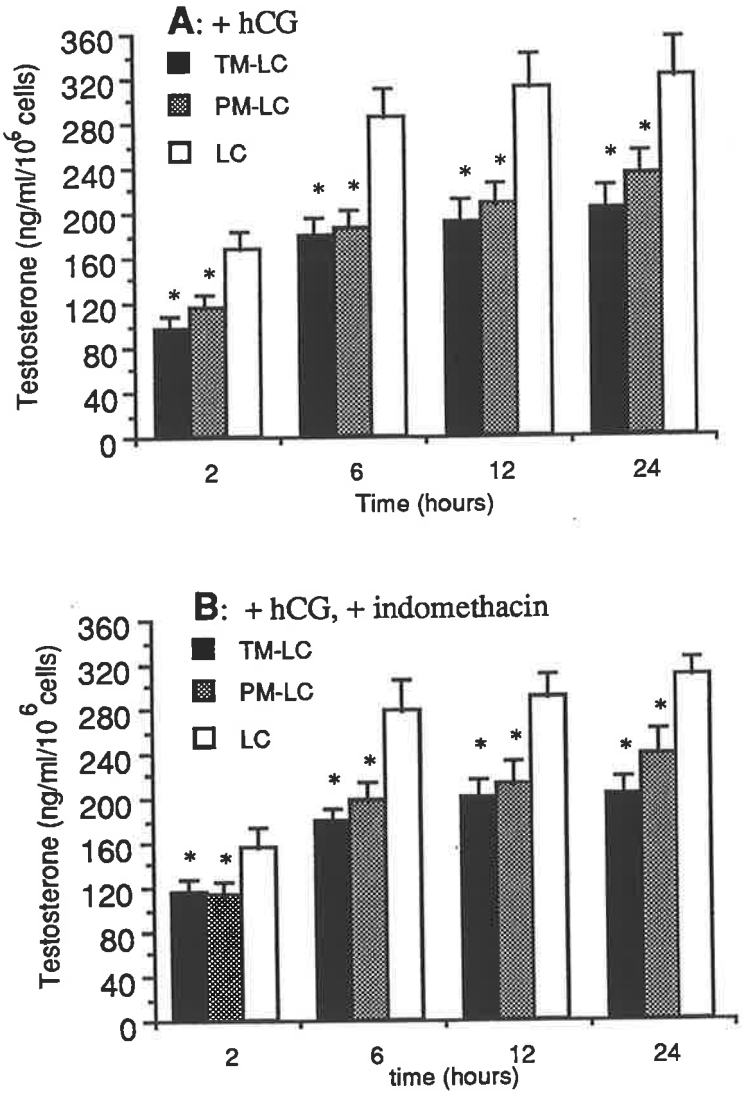
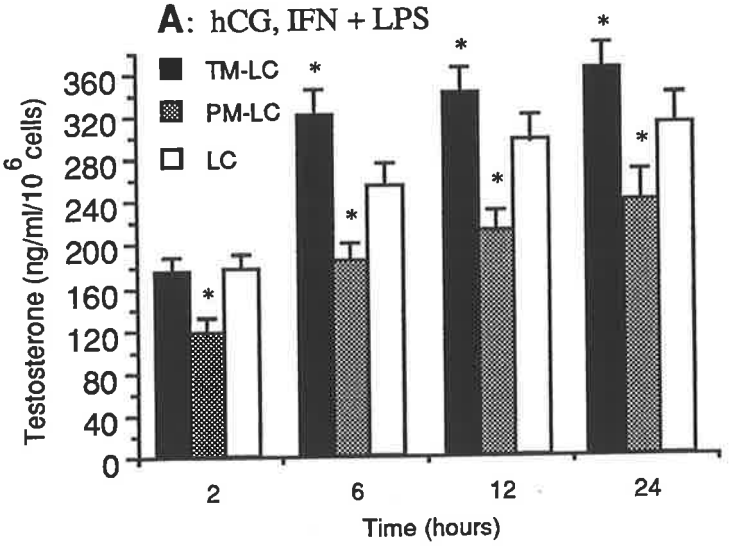
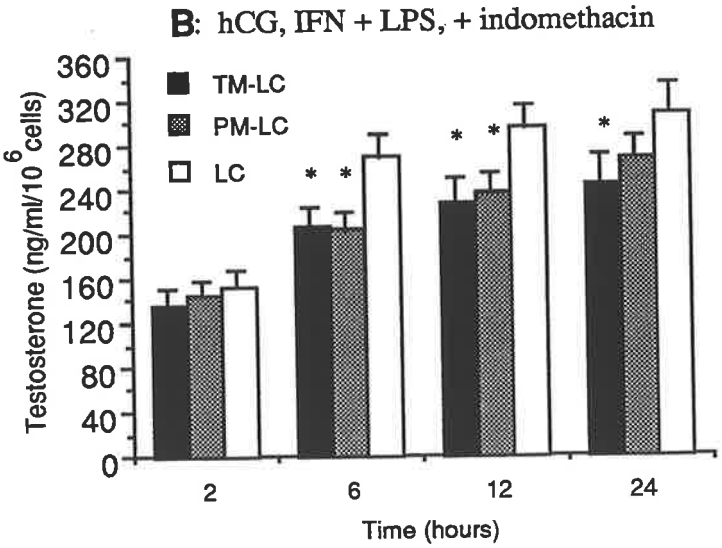

A: + hCG
TM-LC
PM-LC
LC
Testosterone (ng/ml/10^6 cells)
360
320
280
240
200
160
120
80
40
0
2
6
12
24
Time (hours)
B: + hCG, + indomethacin
TM-LC
PM-LC
LC
Testosterone (ng/ml/10^6 cells)
360
320
280
240
200
160
120
80
40
0
2
6
12
24
time (hours)

Figure 6.4

Testosterone concentrations in testicular macrophage-Leydig cell (TM-LC), peritoneal macrophage-Leydig cell (PM-LC) and Leydig cells alone (LC) conditioned medium from cultures stimulated with hCG, LPS and IFNγ. Cells were culturedwithout (**A**) or with indomethacin (**B**) over a 24 hour period. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs LC.


A: hCG, IFN + LPS
TM-LC
PM-LC
LC
Testosterone (ng/ml/10^6 cells)
360
320
280
240
200
160
120
80
40
0
2
6
12
24
Time (hours)


B: hCG, IFN + LPS, + indomethacin
TM-LC
PM-LC
LC
Testosterone (ng/ml/10^6 cells)
360
320
280
240
200
160
120
80
40
0
2
6
12
24
Time (hours)

production under these circumstances. The inhibition of hCG-stimulated testosterone production was overcome after 2 hours and significantly increased when IFNγ and LPS were added to cultures of testicular macrophages and Leydig cells (358±23 compared to 296±27 ng/ml/10^6cells; Fig 6.4A), whereas peritoneal macrophages continued to inhibit testosterone production over the 24 hours. Interestingly, the additon of indomethacin to these hCG, IFNγ and LPS treated cultures (Fig 6.4B), nullified the increased testosterone production observed in Fig 6.4A. While testosterone production was still significantly lower in macrophage-Leydig cell co-cultures, the effect was not as great as observed in cultures without IFNγ and LPS.

6.3.3 Cytokine Production

The production of cytokines by the testicular macrophage alone and in co-culture with Leydig cells was measured by bioassay of conditioned medium at 2, 6, 12 and 24 hours of culture. Peritoneal macrophages produced negligable amounts of the cytokines assayed for, unless activated, and did not respond to the presence of Leydig cells or hCG in culture (Table 6.2).

The production of cytokines IL-1 and TNFα by the testicular macrophage was unchanged by co-culture with Leydig cells (Fig 6.5 & 6.6). The amounts secreted basally were minimal, and not affected by Leydig cell stimulation with hCG. The addition of LPS and IFNγ to culture significantly increased production of IL-1 and TNFα by the testicular macrophages especially after 12 hours of culture.

The Leydig cell produced appreciable quantities of IL-6 in culture although none of the other cytokines assayed for were detected in the conditioned medium (Fig 6.7). Leydig cell production of IL-6 after 24 hours was greater than that seen in macrophage cultures (87±6 versus 61±8 U/10^6 cells), and the production appeared to be constituitve and increased in a linear fashion over 24 hours.

Table 6.2 Cytokine Production by the Peritoneal Macrophage

Peritoneal macrophages produced negligible amounts of the cytokines IL-1, IL-6, TNFα and GM-CSF when cultured alone, in the presence of Leydig cells, with hCG or in the presence of hCG stimulated Leydig cells. When activated with IFNγ and LPS, the peritoneal macrophage increased production of all four cytokines significantly ($P<0.001$).

	Cytokine Production (U/10^6cells)			
Peritoneal Macrophages	IL-1	IL-6	TNFα	GM-CSF
basal	34±6.5	26±4.2	35±3.8	14±2.9
+IFN + LPS	372±46*	640±67*	515±63*	215±32*
+Leydig cells	28±3.6	33±3.5	27±4.2	12±1.4
+hCG	27±3.1	20±2.4	30±2.7	11±1.6
+Leydig cells + hCG	36±4.2	22±1.8	28±3.3	16±2.4

±SEM values: n=4, *=P<0.001 compared with basal levels

Figure 6.5 IL-1 production

IL-1 concentrations over a 24 hour period in conditioned medium from (**A**) testicular macrophages alone or (**B**) in co-culture with Leydig cells. The legend is as follows; (hCG), (L) and (IFN) denotes the addition of hCG, LPS and IFNγ to culture. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs basal.

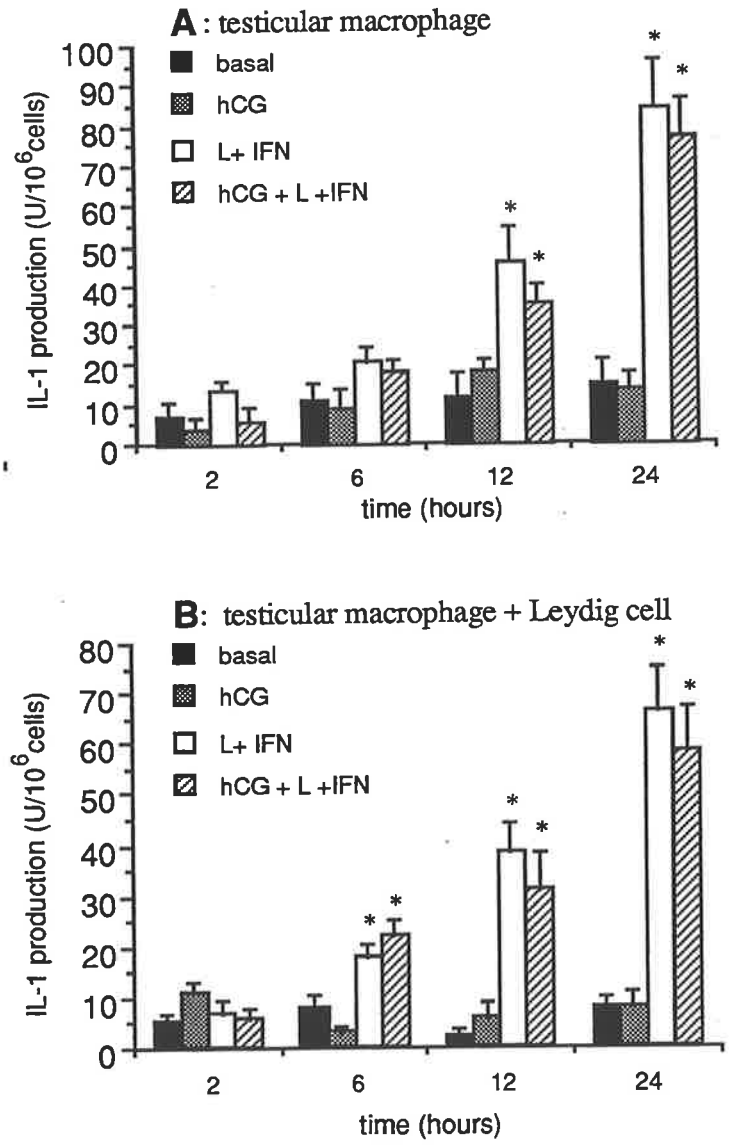

A : testicular macrophage
basal
hCG
L+ IFN
hCG + L +IFN
IL-1 production (U/10^6cells)
100
90
80
70
60
50
40
30
20
10
0
2
6
12
24
time (hours)
B: testicular macrophage + Leydig cell
basal
hCG
L+ IFN
hCG + L +IFN
IL-1 production (U/10^6cells)
80
70
60
50
40
30
20
10
0
2
6
12
24
time (hours)

Figure 6.6 TNFα production

TNF concentrations over a 24 hour period in conditioned medium from (**A**) testicular macrophages alone or (**B**) in co-culture with Leydig cells. The legend is as follows; (hCG), (L) and (IFN) denotes the addition of hCG, LPS and IFNγ to culture. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs basal.

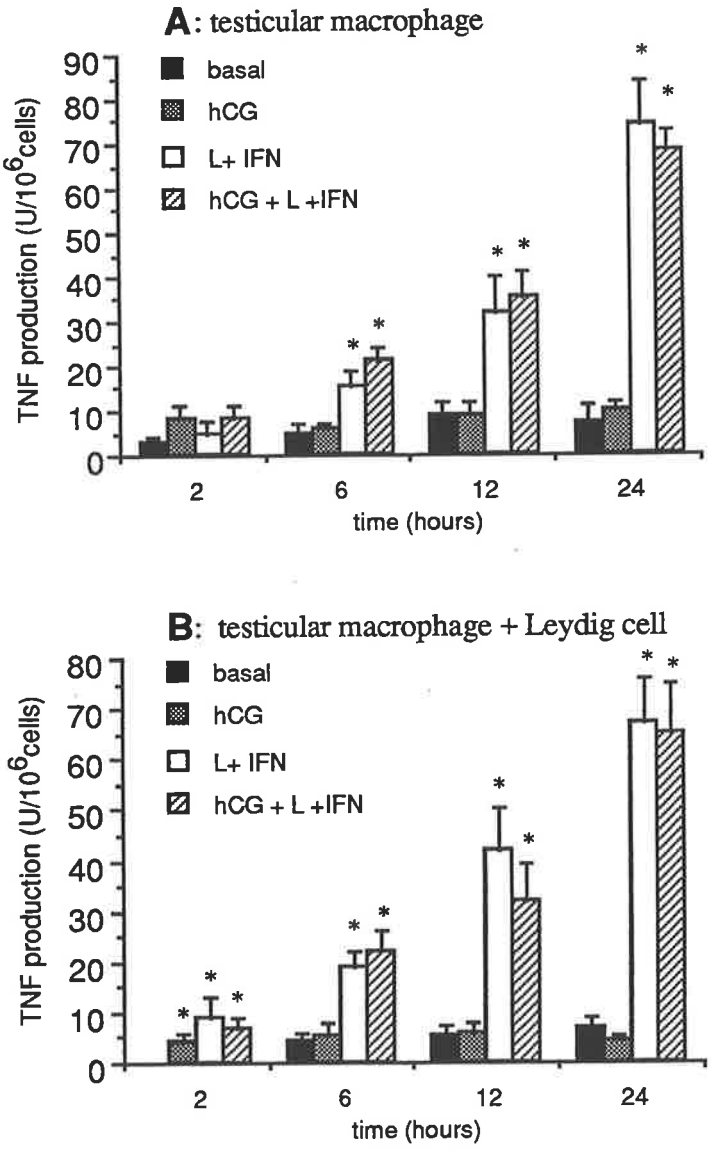
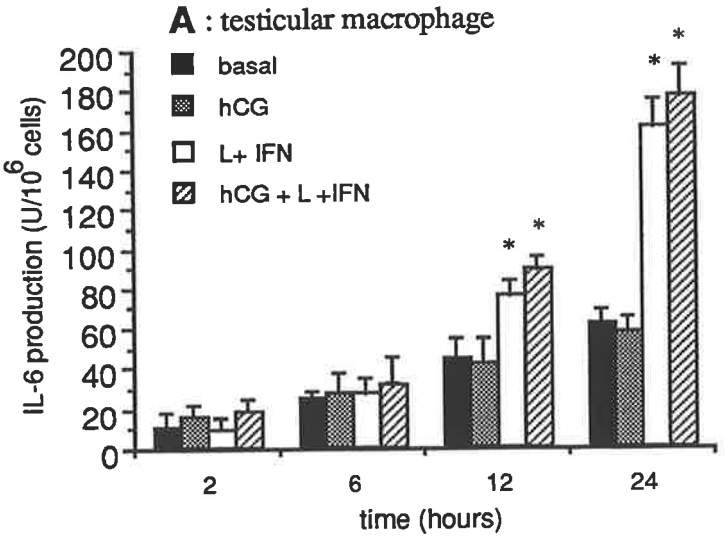
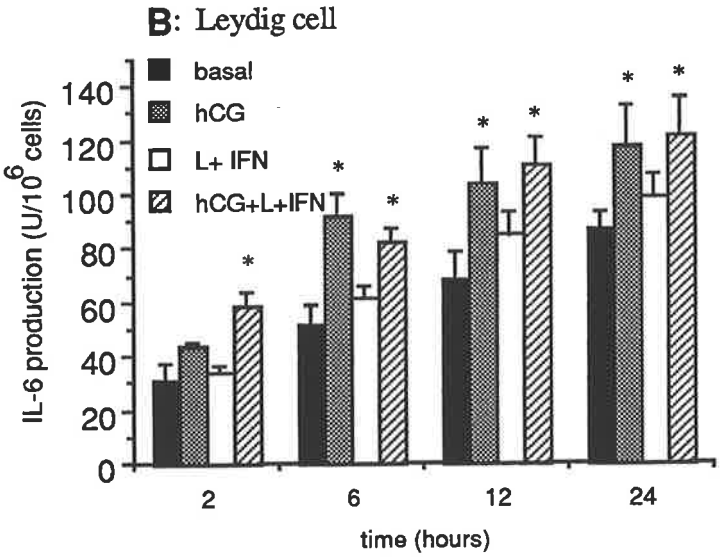
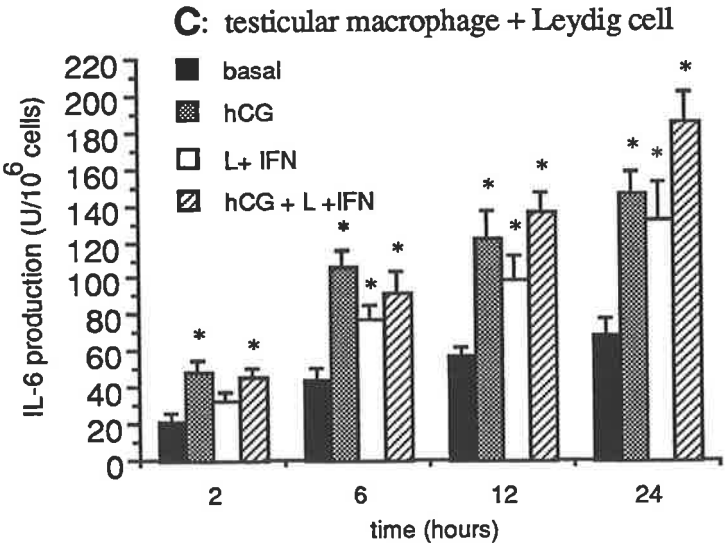

A: testicular macrophage
basal
hCG
L+ IFN
hCG + L +IFN
TNF production (U/10^6cells)
90
80
70
60
50
40
30
20
10
0
2
6
12
24
time (hours)
B: testicular macrophage + Leydig cell
basal
hCG
L+ IFN
hCG + L +IFN
TNF production (U/10^6cells)
80
70
60
50
40
30
20
10
0
2
6
12
24
time (hours)

Figure 6.7 IL-6 production

IL-6 concentrations over a 24 hour period in conditioned medium from (**A**) testicular macrophages alone, (**B**) Leydig cells alone or (**C**) in co-culture with Leydig cells. The legend is as follows; (hCG), (L) and (IFN) denotes the addition of hCG, LPS and IFNγ to culture. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs basal.


A : testicular macrophage
basal
hCG
L+ IFN
hCG + L +IFN
IL-6 production (U/10^6 cells)
200
180
160
140
120
100
80
60
40
20
0
2
6
12
24
time (hours)


B: Leydig cell
basal
hCG
L+ IFN
hCG+L+IFN
IL-6 production (U/10^6 cells)
140
120
100
80
60
40
20
0
2
6
12
24
time (hours)


C: testicular macrophage + Leydig cell
basal
hCG
L+ IFN
hCG + L +IFN
IL-6 production (U/10^6 cells)
220
200
180
160
140
120
100
80
60
40
20
0
2
6
12
24
time (hours)

GM-CSF was again shown to be constituitively produced by the testicular macrophage over the 24 hour period (Fig 6.8A). While culture with hCG had no direct effect on GM-CSF, its production was significantly downregulated during culture with IFNγ and LPS after 24 hours of culture from 141±13 to 22±4 U/10^6 cells.

When the testicular macrophages were cultured with Leydig cells (Fig 6.8 B), production of GM-CSF was significantly lower after 6 hours of culture (from 141±13 to 98±8 U/10^6 cells) suggesting that the presence of the Leydig cells may regulate GM-CSF

As observed for IL-1 and TNFα, IL-6 production by the testicular macrophage was low unless stimulated with LPS and IFNγ. IL-6 produced by testicular macrophage-Leydig cell co-cultures was not significantly different to that of Leydig cells alone over the first six hours of culture. Culture with hCG significantly increased IL-6 production in both testicular macrophage and Leydig cell co-cultures. The increase in production was mainly seen in the first six hours of stimulation, where hCG stimulation increasd IL-6 production by 200% in Leydig cell cultures and 100% in the co-cultures with testicular macrophages (Fig 6.7 A&B). IL-6 production in the testicular macrophage-Leydig cell co-cultures, largely reflected the observations made of macrophages and Leydig cells alone.

production. More interestingly however, the addition of hCG to the co-culture resulted in the significant downregulation of GM-CSF after just six hours of culture (68±5 compared to to 27±4 U/10^6 cells). This is in comparison to the effects of IFNγ and LPS which were not mediated until after 12 hours of culture. Futhermore, this effect appears to be the direct result of a Leydig cell derived factor induced by hCG and dependant on cell-cell contact (Figure 6.8). The addition of Leydig cell conditioned medium stimulated with hCG, testosterone, or hCG on its own when added to testicular macrophage culture had no effect on GM-CSF production (Table 6.3).

Figure 6.8 GM-CSF production

GM-CSF concentrations over a 24 hour period in conditioned medium from (**A**) testicular macrophages alone or (**B**) in co-culture with Leydig cells. The legend is as follows; (hCG), (L) and (IFN) denotes the addition of hCG, LPS and IFNγ to culture. Values are the mean±SEM of 6 samples. *=$P<0.05$ vs basal.

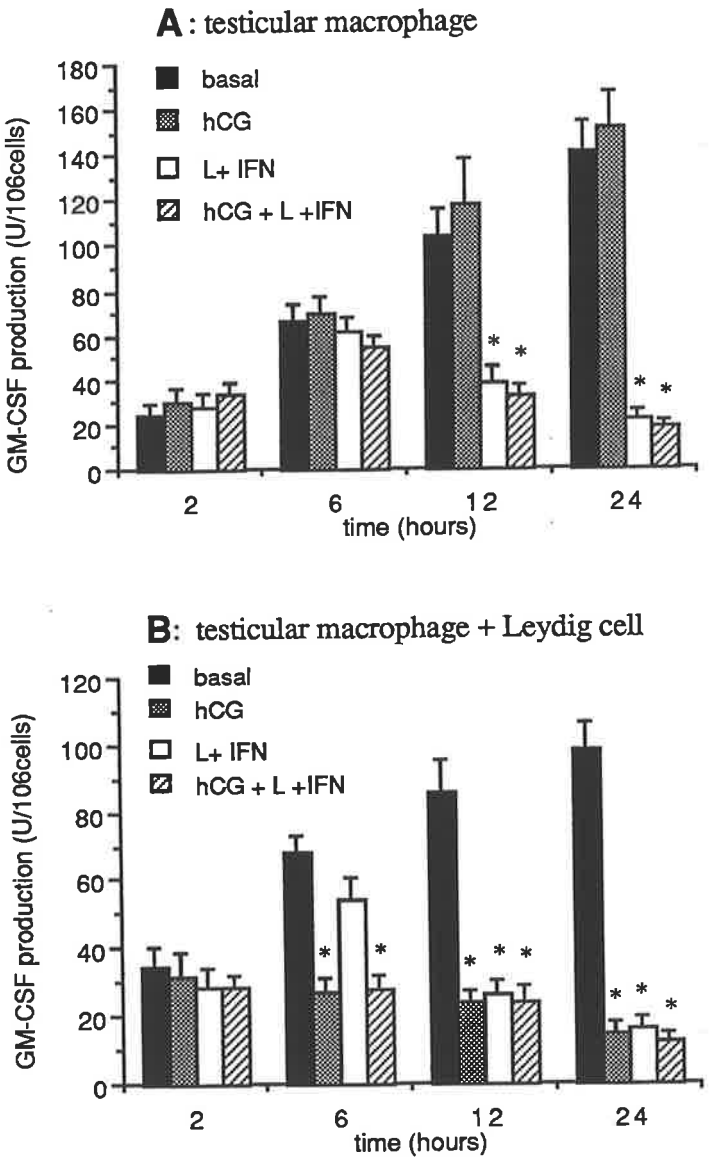

A : testicular macrophage
basal
hCG
L+ IFN
hCG + L +IFN
GM-CSF production (U/106cells)
180
160
140
120
100
80
60
40
20
0
2
6
12
24
time (hours)
B: testicular macrophage + Leydig cell
basal
hCG
L+ IFN
hCG + L +IFN
GM-CSF production (U/106cells)
120
100
80
60
40
20
0
2
6
12
24
time (hours)

Table 6.3 The effect of hCG, testosterone and Leydig cell conditioned medium on testicular macrophage GM-CSF production.

Testicular macrophage GM-CSF production basally, in the presence of hCG (10 IU/ml), testosterone (100ng/ml), Leydig cell conditioned medium (LCCM) or Leydig cell conditioned medium from cultures stimulated with hCG (LCCM±hCG).

	GM-CSF Production (U/10^6cells)				
Time (hours)	Basal	hCG	testosterone	LCCM	LCCM+hCG
2	24±5	30±6	34±5	25±8	28±6
6	67±7	70±7	85±9	75±11	58±6
12	104±16	118±20	123±16	112±19	98±18
24	141±13	152±15	149±21	139±20	122±24

Values±SEM. n=5: no significant differences observed

The results presented in this study support the concept that the testicular macrophage and Leydig cell may be functionally linked. Testicular macrophages displayed the capacity to regulate Leydig cell testosterone production in vitro, suppressing production in some cases and significantly increasing testosterone production after immuno-stimulation. That the peritoneal macrophage could not emulate the testicular macrophages ability to increase Leydig cell testosterone production, suggests that the testicular macrophage produces factors specifically for this purpose.

It appears that regulation of Leydig cell steroidogenesis may at least in part, be under the control of testicular macrophage derived prostaglandin. Arachidonic acid and its lipoxygenase and cyclooxygenase metabolites, such as leukotrienes and prostaglandins, have long beeen suggested as mediators of Leydig cell steroidogenesis. The prostaglandins PGE_2 and $PGF_{2\alpha}$ are known to be powerful regulators of ovarian

6.4 Discussion

steroidogenesis (Evrard et al, 1978; Kato, 1982; Band et al, 1986; Olofsson and Leung, 1994) and recently have been implicated as regulating the transcription of steroidogenic enzymes in porcine granulosa cells (Li et al, 1993). $PGF_{2\alpha}$ and PGE_2 are the two most prevalent prostaglandins in the testis (Reddy et al, 1992), and the Leydig cell has been shown to express receptors for both of them (Orlicky and Williams-Skipp, 1992; Erichsen et al, 1988; Sebokova and Kolena, 1978)

These two prostaglandins have also been shown to alter Leydig cell steroidogenesis both in vivo and in vitro (Bartke et al, 1973; Saksena et al, 1975; Chantharaksri and Fuchs, 1980; Bilinska and Wojtusiak, 1988; Sawada et al, 1994), and their concentrations in interstitial fluid have been shown to increase markedly after LH or hCG stimulation (Abayasekara et al, 1990). Although Leydig cells have been shown to produce these prostaglandins after LH stimulation (Hauor et al, 1979; Cooke et al, 1991), it is unlikely

that they are the main site of production. Studies by Abayasekara et al, (1990) have shown that concentrations of these prostaglandins in interstitial fluid appear unaltered even after the removal of Leydig cells with EDS. The observations made in Chapter 4 and the results in Table 6.1, would strongly suggest that the source of these prostaglandins is primarily the testicular macrophage and that the testicular macrophage may regulate Leydig cell steroidogenesis using these factors.

When Leydig cells stimulated with hCG were co-cultured with testicular macrophages immuno-activated with IFNγ and LPS, a significant increase in testosterone production was observed. This co-incides with the reduction of $PGF2\alpha$ production and an increase in PGE_2. The observed increase in testosterone production suggests that the alteration of the PGE_2:$PGF2\alpha$ ratio from approximately 1:1 to 4:1 (Table 4.1) by the immuno-activated testicular macrophage is responsible for this effect and it also supports reports that PGF2α downregulates testosterone production (Chantharaksri and Fuchs, 1980; Singh and Domonic, 1986; Bilinska and Wojtusiak, 1988; Sawada et al, 1994). That the inhibition of prostaglandin production with indomethacin reversed this effect and resulted in significantly reduced testosterone production when compared to Leydig cells alone further supports this fact.

The addition of indomethacin to culture significantly reduced total testosterone production in basal cultures of Leydig cells alone, and had a similar effect on testosterone concentrations in Leydig cell co-cultures stimulated with hCG. In unstimulated Leydig cell co-cultures the peritoneal macrophages had no significant effect on testosterone production irrespective of the presence of indomethacin. In comparison co-cultures of testicular macrophages and Leydig cells displayed significantly decreased testosterone concentrations regardless of the presence of indomethacin. This observation agrees with a previous study by Lombard-Vignon et al, (1991) which reported Leydig cells as having decreased testosterone production in the presence of testicular macrophages.

When co-cultured with hCG-stimulated Leydig cells, both macrophage populations significantly decreased testosterone production irrespective of the presence of indomethacin. These observations again agree with the published literature (Sun et al, 1993), and perhaps indicates the presence of an inhibitor of steroidogenesis from outside the cyclooxygenase pathway common to both macrophage populations.

Prostaglandin production is also associated with the down regulation of the pro-inflammatory cytokines IL-1, IL-6 and TNFα through a negative feedback mechanism (Kunkel et al, 1986a; Kunkel et al, 1986b). However, the majority of literature studies examining the effects of cytokines on Leydig cell testosterone production in vitro have used IL-1α, IL-1β and TNFα (reviewed Hutson, 1994). In contrast, the observations in this thesis (see chapter 5) suggest that the testicular macrophage normally produces very little IL-1, IL-6 or TNFα except under immunologic activation.

Observations in this chapter (Fig. 6.5 to 6.7) further support this, showing that not only does the testicular macrophage have low basal production of these factors, but their production is not stimulated by hCG or the processes of steroidogenesis. In view of this it would appear unlikely that these factors are normally involved in the macrophage regulation of steroidogenesis in vivo, and the physiological relevance of such studies is perhaps now to be questioned.

The production of IL-6 and GM-CSF by cells of the female reproductive tract emphasises the importance of these factors for female sexual function (Robertson et al, 1992). The production of these factors by cells in the testicular interstitum is therefore not perhaps unexpected. That the testicular macrophage contituitively produces GM-CSF and the Leydig cell IL-6, suggests specific roles for these cytokines in testicular physiology.

Recent evidence indicates that IL-6 may act on Sertoli cells to release products such as transferrin (Boockfor and Schwarz, 1991), although it has recently been reported that

Sertoli cells produce an IL-6-like factor of their own (Syed et al, 1993). That the Leydig cell constituitively produces IL-6 is of interest and has only recently been documented. Brockfort (1994), has shown an increase in IL-6 mRNA which is dose-dependently stimulated by the action of hCG on the Leydig cell, although its role and targets have yet to be identified. The results from the present study show that hCG significantly stimulates IL-6 production in the first 6 hours of culture suggesting that its increased production may be associated with the upregulation of steroidogenesis.

Likewise the constituitive production of GM-CSF by the testicular macrophage also suggests a role for this factor within the testis. Macrophage-derived GM-CSF has been suggested as a paracrine regulator of female reproduction, possibly with a role in tissue remodelling (Robertson et al, 1992). It is therefore not unlikely that it performs an analagous role in the testis regulating the cell proliferation and growth of cells in the interstitum and perhaps inside the tubules.

Basal production of GM-CSF by the testicular macrophage appears to be under Leydig cell regulation. Basal GM-CSF production in the macrophage-Leydig cell co-cultures was significantly lower over 24 hours than when compared to testicular macrophages alone. This suggests a physiological interaction between the testicular macrophage and Leydig cell which may reflect their close physical proximity in vivo (Bergh, 1985; Hutson, 1992), and the ability of the Leydig cell to regulate the basal production of this factor. That GM-CSF regulation may be coupled to Leydig cell steroidogenesis also appears likely. The decrease in GM-CSF production observed after Leydig cell stimulation with hCG suggests that hCG induces a Leydig cell-derived factor capable of downregulating GM-CSF production by the testicular macrophage. This factor is likely to be dependant on cell-cell contact as neither testosterone or Leydig cell conditioned medium added to testicular macrophage cultures altered GM-CSF production. However, as the downregulation of GM-CSF production coincided with an increase in testosterone production it is perhaps unlikely that GM-CSF is directly involved in the processes of steroidogenesis.

It is noteworthy that the downregulation of GM-CSF production coincides with an increase in IL-6 production by the Leydig cell indicating a complimentary relationship between the synthesis of these factors by these respective cells. Moreover, the production of GM-CSF and PGE_2 by macrophages is associated with the induction of suppressive immune responses (Walker et al, 1992; Morrisey and Ireland, 1991; Fu et al, 1991; Tsuchiya et al, 1988) , which down regulate the the production of the pro-inflammatory cytokines IL1, TNFα and IL-6.

From these results it would seem unlikely that either IL-6 or GM-CSF have a direct role in regulating testosterone synthesis, although their production may be regulated by steroidogenesis in a manner similar to macrophages and their factors in the uterus (Finn and Pope, 1986). The observations presented in this chapter demonstrate that altering the prostaglandin profile of the testicular macrophage either through the use of indomethacin or immuno-stimulants is capable of influencing Leydig cell steroidogenesis. The likelyhood of a role for the testicular macrophage in steroidogenesis is further supported by work which demonstrates that the removal of these cells in vivo with Cl_2-MDP, results in reduced testosterone production by the remaining Leydig cells (Maddocks et al, 1992; Berg et al, 1993a,b).

Thus it may be that the unique secretory profile of the testicular macrophage, high in both GM-CSF and prostaglandin assumes an important role within the testis. The constituitive production of these factors may not only be responsible for intercellular communication and the regulation of the Leydig cell, but also to help create and maintain the immune privilege observed in the testis.

CHAPTER 7: General Discussion

7.1 General Discussion

The studies reported in this thesis have primarily centred upon the immunological characteristics of the testicular macrophage and their likely function or action within the testis in an attempt to elucidate the contribution of this cell type to normal testicular physiology and to the apparent immune privilege afforded the testis. By developing a novel three step density-dependant procedure for isolation and purification of rat testicular macrophages based on the work by Dirami et al (1991), it was possible to produce highly purified cultures of testicular macrophages for in vitro work. Although the collagenase digestion of testicular tissue during the isolation of testicular macrophages has been reported to activate these cells (Moore and Hutson, 1994), the isolation procedures used in the course of this thesis have not shown such effects and cytokine mRNA and protein levels were not significantly altered between enzymatically and mechanically digested tissues.

Peritoneal macrophages were chosen for several reasons as a comparison for testicular macrophages. The majority of the peritoneal macrophage population is found in a resting state which is characterised by low secretory phase, low Fc expression and minimal immuno-activity. As a result of their environment and their quiescent nature, they display the more traditional immunological characteristics of macrophage immunobiology unlike the tissue bound resident macrophages of the liver, spleen or lung. The peritoneal macrophage expresses the common macrophage markers, and may be readily immuno-activated to cytotoxic and effector cell status by LPS alone, whereas this is not always the case with resident macrophages from other tissues. Tissue bound macrophages perform local physiological functions and this is usually manifested by a specific secretory phase, which alters basic macrophage characteristics. Therefore in terms of studying macrophage immuno-biology, the two highly defined states of rest and activation were considered to make the peritoneal population a useful comparison for the testicular macrophage. In retrospect, however, it would perhaps have been of interest to have included macrophages of a resident population from another tissue (eg.

lung), in these studies. This would have greatly complicated cell preparation schedules, but should be considered in future studies of this nature.

The testicular macrophage possesses all of the well known macrophage morphological and (immuno)cytochemical characteristics (Chapter 3). The presence of non-specific esterase activity in the cell, the expression of common markers such as CD11b (Mac 1) and W3/25 and the presence of markers specific for macrophage monoclonal antibodies, ED1 and ED2, show it to be similar to many other macrophage types. Observations in this thesis have shown that the in vivo population is largely homologous and stains for mainly ED1 and ED2 in accordance with the published literature (Hutson, 1990; Wang et al, 1994).

While there may be some heterogeneity within the population of macrophages resident in the testis with regard to the expression of ED3, the population appears to be functionally similar, an observation reflected by uniform expression of the Fc receptor. Rat testicular macrophages avidly bound and opsonised IgG-coated sheep erythrocytes without being previously activated. In comparison quiescent peritoneal macrophages which do not normally become phagocytic without exogenous stimulation required at least 12 hours to exercise their phagocytic abilities. That the majority of testicular macrophages appear uniformly and intrinsically phagocytic demonstrates they possess one of the important macrophage characteristics for immuno-competence.

Immunologically, high Fc receptor expression is generally recognised as a response to antibody-bound antigen. This is a response usually only seen in macrophages, and not observed in dendritic cells (Steinman and Nussenzweig, 1980; Mettay et al, 1991). It is not all together unusual that such a high proportion of testicular macrophages express Fc receptors constitutively as macrophages in the liver, spleen and lung are known to also be avidly phagocytic (Johnston, 1988). Whereas in immunological terms it suggests activation, it also points to a tissue specific function, such as the removal of

old cells, and also in lymphoid organs as a way of trapping antigen. Thus the high Fc receptor expression by up to 85 % of the population resident in the testis may be linked with the phagocytosis of defunct Leydig cells as observed by Miller, et al (1983).

The ability to induce lymphocyte proliferation in vitro, was also tested in the course of this thesis. The testicular macrophages inhibited the proliferation of Concanavalin A stimulated lymphocytes in vitro even after culture with LPS by means of a secreted factor. This observation suggests the testicular macrophages produce a factor which when secreted into the testicular interstitium or culture medium is capable of reducing lymphocyte proliferation and may be the basis for the provision of immune privilege in the testis.

It has been previously shown by Emoto et al, (1989) that mouse testicular extract has the capacity to stimulate macrophages non-specifically and to down regulate mitogen induced T-cell proliferation. Additionally, Pollanen et al, (1989, 1990) have described a number of heat and pH-labile factors of high molecular weight, termed protectins, which have been shown to inhibit lymphocyte proliferation in vitro. Although to this point the source of these factors has not been identified, this inhibition does not appear to be due to testosterone or other Leydig cell derived factors (Hedger et al, 1990).

The fractionation of testicular macrophage conditioned medium by centricon membranes (Chapter 4), showed that there was a broad spectrum of inhibition, present at both high, medium and low molecular weights. The current literature (Pollanen et al, 1989; Sainio-Pollanen et al, 1991; Pollanen et al, 1992) suggests that the majority of the immuno-suppresive factors found in testis extract are of very high molecular weights exceeding 60 000MW. The removal of factors below 14 000MW from testicular macrophage-conditioned medium by dialysis significantly reduced, but did not eliminate all of the inhibitory effects, which suggests that some of the observed inhibition is mediated by higher molecular weight factors. Pollanen et al, (1993) has

suggested TGFß (25 000MW) as the main suppressor of immune activity in the testis. The inhibition of lymphocyte proliferation in the presence of fractionated testicular macrophage conditioned medium (factors between 10 000MW and 30 000MW), would seem to coincide with the presence of a immuno-supressive factor this size.

However, the majority of the inhibition was in the low molecular weight fraction (<10 000MW), and it was found that by blocking the cyclooxygenase pathway and hence the production of prostaglandin with the inhibitor indomethacin, normal lymphocyte proliferation was almost completely restored. The presence of a second low molecular weight factor not blocked by the presence of indomethacin is also of interest. Associated work in this laboratory by Depamede and Maddocks (1995), has described a low molecular weight factor that inhibits specifically activated lymphocytes, is present in rat testicular extract, and may contribute to the downregulation of immune response in the testis.

Subsequent studies on prostaglandin production by the testicular macrophage revealed it to produce high basal levels of PGE_2 and $PGF_{2\alpha}$ and it is these that were primarily responsible for the inhibition of lymphocyte proliferation in the presence of testicular macrophages and their conditioned media. It also seems likely that the testicular macrophage is the main source of prostaglandin production in the interstitial tissue. Cooke et al (1991) have shown that even after the removal of Leydig cells with EDS, concentrations of prostaglandin E_2 and $F_{2\alpha}$ in the testicular interstitial fluid remain unchanged. The present data also concurs with the high level of Fc receptor expression by the testicular macrophage, as avidly phagocytic macrophages in other tissues usually produce high levels of prostaglandin (Werb and Rapolee, 1992). Constitutive production of prostaglandin by macrophages has also been associated with the down regulation of the cytokines involved in lymphocyte stimulation and proliferation (Kunkel et al, 1986 a,b). It may be that the maintenance of raised prostaglandin levels

in the testis is another important mechanism by which immune responses are suppressed in the testis.

This hypothesis was further examined by studying the cytokine secretory profile of the testicular macrophage (Chapter 5). The constitutive production of GM-CSF and the impaired production of the pro-inflammatory cytokines IL-1, IL-6 and TNFα may explain the inhibitory action of testicular macrophage conditioned medium on lymphocyte proliferation in vitro. The pro-inflammatory cytokines are essential for the proliferation of lymphocytes, and in contrast to peritoneal macrophages, the testicular macrophage produced very low levels of the cytokines IL-1, IL-6 and TNFα, even after LPS stimulation. Unlike the peritoneal macrophage which responded to LPS alone, by increasing cytokine output, the testicular macrophage required activation with both LPS and IFNγ, in order to stimulate production of these factors. Moreover, even after stimulation with LPS and IFNγ, the testicular macrophage produced less of these cytokines than the peritoneal macrophage. This highlights the differences in regulation of the immune response between the testicular and peritoneal populations.

The reduced synthesis of the pro-inflammatory cytokines and the constitutive production of prostaglandin and GM-CSF by the testicular macrophage may combine to create an immuno-suppressive environment both in vitro, and in vivo in the testicular interstitium (Tsuchiya et al, 1988; Fu et al, 1991; Morrissey and Ireland, 1991). This refractoriness to LPS and the associated inhibition of lymphocyte proliferation is notably shared by the Kuppfer cells of the liver (Lepay et al, 1985a,b), and might suggest that the testicular macrophage is challenged so as to be unable to respond to an immune stimulus and thus can not participate in an immune reaction as would normally be expected of most macrophages.

This hypothesis fits with the current concept of immune privilege in the testis (Maddocks and Setchell, 1990), as the suspension of the immune reaction is seen only

against single-set intra-testicular grafts placed into the interstitium. When the host has been pre-sensitised extra-testicularly to the antigen, an immune response within the testis will usually occur when antigen is present at this site. In these cases the immune reaction in the testis is likely to be initiated by T-lymphocytes already pre-sensitised to the antigen, migrating through the testicular interstitium. The T-lymphocytes in the testis are predominantly those expressing CD8, the cell surface receptor associated with cytotoxic T-cell activity, and elevated numbers of these T-cells in the testicular interstitium have been associated with testicular failures in men (El-Demiry et al, 1987; Pollanen and Maddocks, 1988). This is likely to be due to the immune activity of these T-cells which are capable of secreting the cytokine IFNγ and activating all immunocompetent cells in the region (Ijzermans and Marquet, 1989).

However, after priming and triggering of testicular macrophages with IFNγ and LPS in vitro, the present studies show that the testicular macrophage can in fact be induced to augment lymphocyte proliferation, and even stimulate proliferation to levels seen above the control. This observation indicates that the testicular macrophage is indeed capable of contributing to lymphocyte proliferation during an aggressive immune reaction. Therefore, that the induction of an immune response in the testis may result in testicular failure and host infertility, underlines the importance of immune privilege for normal testicular function and the need to prevent the incidence of immune reactions at this site.

The present study has also demonstrated a differential regulation of the prostaglandin E_2 and $F_{2\alpha}$ secretion by the testicular macrophage following immuno-activation with IFNγ and LPS. While IFNγ on its own or in conjunction with LPS had no effect on the PGE_2 production, $PGF_{2\alpha}$ levels were observed to drop four-fold. Activating the testicular macrophage in this manner seems to create an important change in the ratio of prostaglandins E_2 and $F_{2\alpha}$ from a ratio 1:1 to 3:1 and appears to increase Leydig cell steroidogenesis as a result. Regulatory associations in the ovary between

prostaglandins and steroidogenesis have been demonstrated in the female (Li et al, 1993; Olofsson and Leung, 1994) and an analagous situation in the testis might involve testicular macrophage-derived prostaglandin and testosterone production.

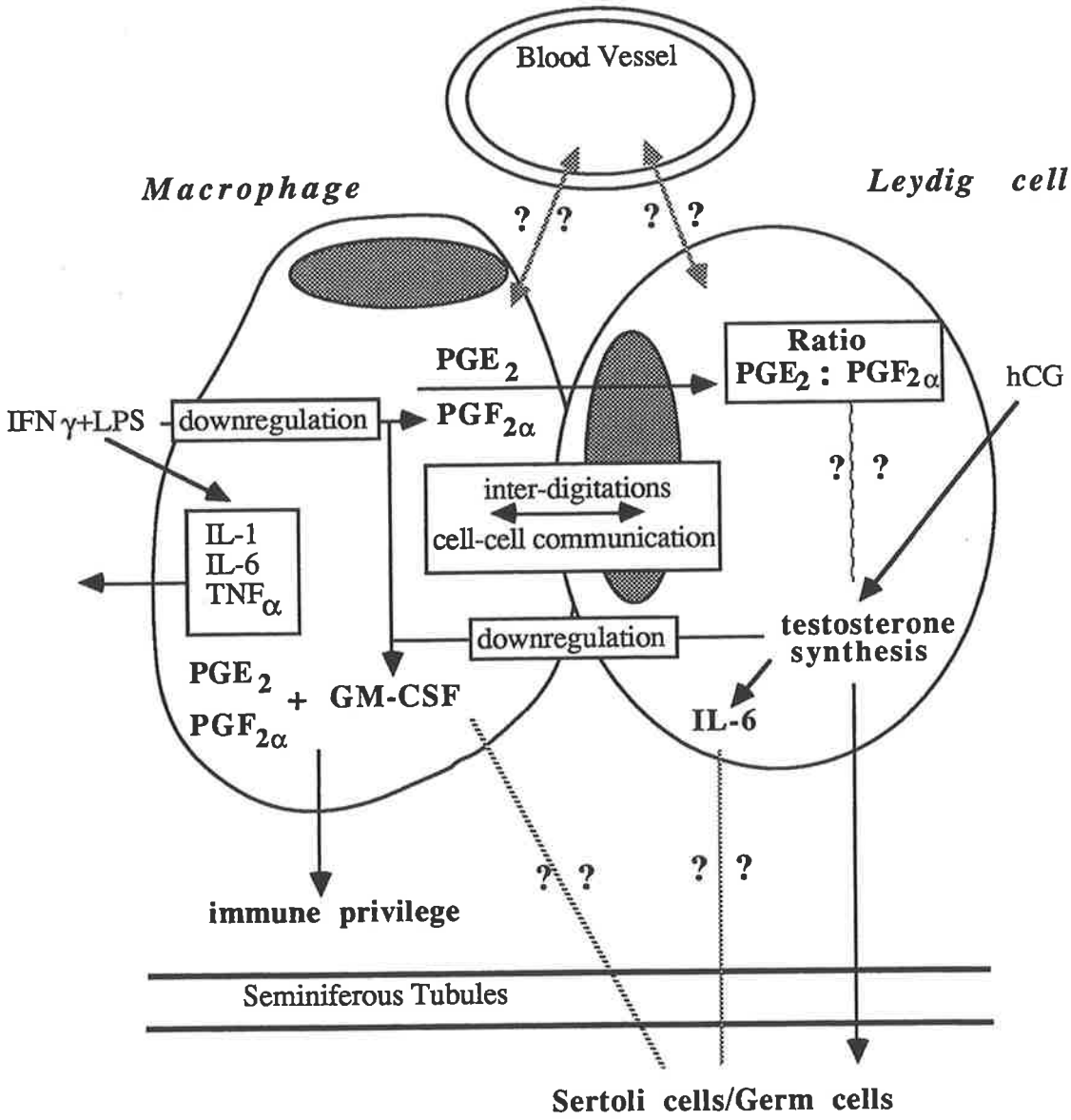
The high levels of PGE_2 still present in the testicular macrophage conditioned medium even after activation with IFNγ and LPS appear to be at odds with the increases in lymphocyte proliferation induced by this conditioned medium at this time. However, the present studies also showed that IFNγ and LPS stimulated production of the lympho-proliferative cytokines IL-1, IL-6 and TNFα, in conjunction with the down regulation of GM-CSF and $PGF_{2\alpha}$.

This would indicate that under normal circumstances the testicular macrophage is immuno-suppressive due to its secretion of prostaglandin and inability to stimulate T cell proliferation the secretion of proinflammatory cytokines. The inhibitory action of the testicular macrophage may only be overcome after "pathological" immuno-activation which causes downregulation of the factors GM-CSF and prostaglandin, increases production of IL-1, IL-6 and TNFα and in so doing reverses the inhibitory effects on lymphocyte proliferation (Figure 7.1). The increased lymphocyte proliferation observed after IFNγ and LPS activation is consistent with both the reduction in prostaglandin and the marked increase in production of IL-1, IL-6 and TNFα by the testicular macrophage. It would therefore appear that the macrophages resident in the testicular interstitium are under tight immunological control which prevents immune reactions in the testis normally, but this may be overcome after priming and triggering of the cell by factors such as IFNγ and LPS.

The studies in this thesis suggest that the immunobiology of the testicular macrophage is somewhat unique, especially with regard to its secretory profile. It not only retains some aspects of immunological function, but may also perform specific functions within the testis as do macrophages resident at other sites (Nathan et al 1980; Johnston

Figure 7.1

A schematic diagram of the immunobiology of the testicular macrophage and cellular interactions with the Leydig cell. Solid lines represent interactions examined in this thesis, while broken lines represent possible targets for the factors involved.


Blood Vessel
Macrophage
Leydig cell
? ? ? ?
PGE 2
PGF2α
Ratio
PGE2 : PGF2α
hCG
IFN γ+LPS
downregulation
inter-digitations
cell-cell communication
? ?
IL-1
IL-6
TNFα
downregulation
testosterone
synthesis
PGE 2 + GM-CSF
PGF2α
IL-6
? ? ? ?
immune privilege
Seminiferous Tubules
Sertoli cells/Germ cells

1988). To this point it remains difficult to define the role(s) the testicular macrophage may have in testicular physiology. However, the production of prostaglandin and GM-CSF by the testicular macrophage and their suppressive effects are unlikely to be primarily for the provision of immune privilege. The Leydig cell or even the cells inside the tubules are likely targets for these products. This view is further reinforced by observations of cytokine and testosterone production and regulation seen in macrophage and Leydig cell co-culture.

While numerous studies have added cytokines such as IL-1 and TNFα exogenously to Leydig cell cultures (reviewed, Hutson, 1994), studies reported in this thesis suggest that these factors are not normally produced by the testicular macrophage in vitro. In view of the observations made here, testicular macrophages were also cultured with Leydig cells in order to best gauge the relationship between these two cell types (Chapter 6). IFNγ and LPS stimulated testicular macrophages in the presence of Leydig cells were shown to be capable of increasing testosterone production.

These results would appear to be in accordance with the few studies conducted on testicular macrophage-Leydig cell co-culture and in particular the study by Lombard-Vignon et al, (1991) which showed an increase in testosterone production after stimulation of the testicular macrophage with LPS. These studies have also demonstrated the testicular macrophage to be the main source of prostaglandin in the testis and that blocking prostaglandin synthesis with indomethacin significantly reduced Leydig cell testosterone production.

The importance of the testicular macrophage in testosterone production has also been highlighted in other studies by the novel removal of these cells from the testicular interstitium with Cl_2 MDP. In the absence of the testicular macrophages and their products, testosterone secretion was observed to decrease (Maddocks et al, 1992; Berg et al, 1993; Gaytan et al, 1994). Furthermore, these studies have demonstrated that the

absence of testicular macrophages seems to increase the vascular inflammatory response seen in the testis after the administration of hCG, thereby suggesting that the testicular macrophage may secrete a factor(s) which mediates vascular permeability in this region.

In view of this evidence it would appear that the testicular macrophage may be functionally coupled to the Leydig cell by the hCG-induced inflammatory response, and this may explain the observed decrease in GM-CSF production seen in vitro after hCG treatment and Leydig cell co-culture. The addition of hCG to culture rapidly induced IL-6 production by the Leydig and a concurrent reduction in GM-CSF production by the testicular macrophage. Notably the effects of hCG on GM-CSF production by the testicular macrophage appear to be indirect and must be the result of Leydig cell derived factors in response to hCG stimulation. That the removal of Leydig cells from the testicular interstitium with ethane dimethanesulphonate prevents the hCG response (Setchell and Rommerts, 1985), demonstrates the vital importance of the Leydig cell in mediating the effects of this factor. The addition of hCG, Leydig cell conditioned medium or testosterone alone to testicular macrophages in culture, does not significantly alter GM-CSF production suggesting that cell-cell contact is required for this interaction. Observations by Hutson (1992a), have described cytoplasmic interdigitations between the testicular macrophage and Leydig cell and this connection may represent a rapid means of intercellular communication and regulation that is significant in the light of the above observations.

The downregulation of testicular macrophage GM-CSF production in co-culture occured simultaneously as IL-6 production by the Leydig cell increased and both of these responses were seen to be due to changes in Leydig cell steroidogenesis. This data suggests that contrary to current opinion, cytokines are unlikely to be involved directly in regulating steroidogenesis in the testis. It would appear more likely that

production of these cytokines is more specifically regulated by steroidogenic mechanisms in the Leydig cell.

The constitutive production of significant quantities of GM-CSF by the testicular macrophage suggests a specific role for it in testicular physiology, and possibly a role analagous to that described in the female reproductive tract. Both GM-CSF and IL-6 are important for the development of haematopoietic cell progenitors and are also produced by macrophages in the uterus, with the speculated function of being important for tissue remodelling (Robertson et al, 1992; Hunt, 1994). As these cytokines are involved in the proliferation and differentiation of cells, their targets in the testis might be speculated to be the rapidly dividing germ cells inside the seminiferous tubles. While the testicular interstitum is an environment where these factors are expected to have a very short half-life, IL-1 has been shown to cross the blood-testis barrier nearly six times as fast as albumin (Banks and Kastin, 1992).

However, both IL-1 and IL-6 are produced inside the seminiferous tubules by the Sertoli cells (Gerad et al, 1991; Syed et al, 1993) and IL-1α has been shown to induce the proliferation of spermatogonia (Pollanen et al, 1989b). As a result, the production of cytokines within the testicular interstitium may conversely be argued to not be directed at targets inside the seminiferous tubules and these factors may be produced for targets within the interstitial tissue. For example, IL-ß has been suggested to regulate the migration of polymorphonuclear leukocytes in the testis and consequently the vascular permeability of the tissue (Bergh and Soder, 1990). Much future work will need to be focussed on these intriguing issues before they can be further resolved.

The experiments reported in this thesis have contributed new information about the complex nature of the macrophage resident in the testicular interstitium. These studies have characterised unique qualities of the testicular macrophage and its immunobiology. In this thesis I have attempted to relate these qualities (Fig. 7.1), to

the roles they may play in both the physiological regulation of the testis with specific regard to steroidogenesis and to the immunology of the testicular environment and the provision of immune privilege.

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