Isolation and Characterisation of the Immunosuppressive Peptides in the Rat Testis

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Dedication

To my mother ***Naimah – Kelayu*** and to my late father ***Dowa Umbu Rezi – Karuni,*** who passed away on 21 November 2004 during the peak of finishing the writing of this thesis, to whom this thesis is dedicated.

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 another person, except where due reference is made in the text of the thesis.

Sulaiman N. Depamede

December, 2004

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Abstract

The rodent testis is recognised as an immune-privileged site in which allogeneic tissue grafts can survive for long periods of time, possibly indefinitely. Theories developed to date suggest that the testis contains specific immunosuppressive factors that inhibit lymphocyte activation in this site (for review see Maddocks and Setchell, 1990, Streilein, 1993, Filippini et al., 2001). However the nature of these factors has not been well characterised to date.

The present study was conducted in an attempt to isolate and characterise the immunosuppressive factors in the rat testis, and to determine the probable mechanism by which immunosuppression is achieved. A crude testicular extract was used as the source material for the investigations described.

Immunosuppressive activities were assessed using a Con A-induced splenic T cell proliferation bioassay. The splenic T cells were isolated using a percoll density gradient separation which resulted in T cells expressing mainly α/βT cell receptors as determined by FACS analysis. Crude testicular extract suppressed mitogen (Con A or PHA) induced splenic T-cell proliferation. Removal of significant amounts of steroid from testicular homogenate by dextran-charcoal extraction did not abolish the immunosuppressive activities. Separation of this crude testicular extract using a Sephadex G 25 PD-10 column resulted in three molecular weight fractions: Mr > 5, Mr 1-5 and Mr < 1 kDa. The immunosuppressive activity was observed in fractions of Mr > 1

kDa, with the strongest immunosuppressive activity present in the Mr 1-5 kDa fraction. Using a Superdex Peptide PC 3.2/30 column, the strongest immunosuppressive activity was found to have a molecular weight of around 3.5 kDa.

This result contrasts with previous reports of similar investigations on rodent testis immunosuppressive activities, and attempts to further purify these immunosuppressive factors were focused on the low molecular weight substances. The semi-purified (low molecular weight) immunosuppressive activities were found to be relatively heat and pH stable, but were sensitive to trypsin, suggesting they were most likely peptidic in nature.

Further analysis involved ion-exchange chromatography, high performance liquid chromatography (HPLC) and reversed phase high performance liquid chromatography (RP-HPLC) methods. The use of RP-HPLC employed a μRPC C2/C18 column with either trifluoroacetic acid (TFA, 0.1%, v/v) or heptafluorobutyric acid (HFBA, 1%, v/v) and acetonitrile (0-80% v/v) as the elution buffer. This resulted in reduced immunosuppressive activity. Similar results were also observed with the high molecular weight fractions and such effects have also been reported by others working in this area (Saxena et al., 1988). The results suggested that the immunosuppressive factors are relatively unstable under the purification conditions employed and may be oligomeric in nature.

Since the proliferation of activated T cells is related to the production of IL-2 and the expression of the IL-2-receptor, investigations were undertaken on the

production of IL-2 by activated T cells cultured in the presence of testicular immunosuppressive factors. The results show that both the crude and high molecular weight, but not low molecular weight immunosuppressive factors inhibited IL-2 production. In this context, at least two immunosuppressive mechanisms are present in the testis extracts. The high molecular weight factors suppress the proliferation of activated T cells via the inhibition of IL-2 secretion, which has also been reported previously by Pöllänen et al., (1990) and attributed to a TGF-β-like protein named 'protectin' (Pöllänen et al., 1988).

However inhibition of activated T cell proliferation by low molecular weight (LMW-TE) factors did not affect IL-2 production, and was shown in the present study to induce specific programmed cell death (apoptosis) which was not due to general cytotoxic effects.

Although the TE immunosuppressive peptides suppressed both $CD4^+$ and $CD8^+$ T cell subsets, the strongest suppression was found to be extended to the $CD4^+$ T cell subset. It is possible that LMW-TE could possibly be involved in the down regulation of $CD4^+$ T cell populations in the testis as it has been previously reported that in the rat testis $CD8^+$ T cells are more frequently observed compared to $CD4^+$ T cells (Hedger et al., 1998b, Tompkins et al., 1998).

From the present study it can be concluded that the mechanisms regulating the status of the testis as an immunologically privileged site involve complex, multiple and possibly redundant mechanisms to both inhibit an autoimmune

attack on the haploid germ line on the one hand and yet to also allow immunologic responses against pathological events to take place on the other. The presence of high and low molecular weight peptides in the rat testis as reported in the present study contribute to these events directly or indirectly. However, the specific nature of the various components of the regulatory pathways that maintain the unique environment of the testis are still to be elucidated.

Acknowledgement

It is pleasure to acknowledge here the many people who have helped me over the period of completing the work presented in this thesis.

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Preface

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

Abstracts:

Depamede S.N., Kern, S., and Maddocks, S (1994). Immunosuppressive activity in the rat testis that is not temperature or pH labile. Proc.Aust.Soc.Reprod.Biol. 26:69.

Depamede S.N., and Maddocks., S (1995). Characterisation of 1-5 kDa Immunosuppressive factors from the rat testis. Australasian Society for Immunology 1995 Meeting Handbook. Abst. 198.

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
AEBSF	4-[2-aminoethyl] benzene sulfonyl fluoride
Ag	Antigen
APC	Antigen-presenting cell
AVP	Arginine vasopressin
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CD	Cluster of differentiation (e.g. CD4)
Con A	Concanavalin A
CsA	Cyclosporine A
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorter
GM-CSF	Granulocyte macrophage colony stimulating factor
HPLC	High performance liquid chromatography
IFN	Interferon
Ig	Immunoglobulin (e.g. IgG)
IGF	Insulin-like growth factor (e.g. IGF 1)
IL	Interleukin (e.g. IL-2)
kD	Kilo Dalton
LAK cell	Lymphokine-activated killer
lpr	Lymphoproliferation
M	Molar (e.g. 1.5 M or 1500 mM NaCl)
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
Mr	Relative molecular weight
mRNA	Messenger RNA
MSH	Melanocyte stimulating hormone (e.g. α-MSH)
MTT	3-[4,5 dimethyltiazol-2yl]-2,5 diphenyl tetrazolium bromide
NGF	Nerve growth factor
NK cell	Natural killer cell
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PKC	Protein kinase C
RPMI 1640	Royal Park Memorial Institute 1640
TcR or TCR	T cell receptor for antigen
TE	Testis extract or testicular extract
TGF	Transforming growth factor
TNF	Tumour necrosis factor

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1. Introduction and Review of Literature

1.1. Introduction

The word testis originates from Latin, meaning 'witness' or 'spectator' (Setchell, 1978) due to the 18th Century belief of the bystander position played by the testis in reproduction. The testis accomplishes two main functions, the production of male gametes (spermatozoa) necessary for reproduction and the production of male sex hormones (androgens) necessary for the continued production of spermatozoa and the development and maturation of the sexual organs. Moreover, the testis is becoming recognised for other functions including producing immunomodulatory factors that are active in the male and female reproductive tract.

The mammalian testis, especially the rodent testis, along with the brain, anterior chamber of the eye, and the hamster cheek pouch has been considered to be an immunologically "privileged" site (Barker and Billingham, 1977) due to the evidence that in these sites auto-, allo- and even xenografts of various endocrine tissues survive much longer than in other sites of the body (see Maddocks and Setchell, 1990, Streilein, 1993 and 1995).

The mechanism of transplant survival in the testis has not yet been established, however accumulating evidence shows that there may be specific factors, which are secreted locally (Maddocks and Setchell, 1990; Bellgrau et al., 1995, Filippini et al., 2001), that provide the testis with its "immunoprivileged" status. These factors have the ability to suppress mitogen-induced proliferation of lymphocytes *in vitro* (Pöllänen et al., 1988, 1989a, b).

However, the nature of these factors and the support for the concept that these factors are involved in regulating the immune system and are involved in fashioning the immunologically privileged status of the testis are largely unknown (Pöllänen et al., 1993 and Selawry and Cameron, 1993). It has been proposed that cytokines especially TGFβ (Pöllänen et al., 1993; Cupp et al, 1999) which has anti-inflamatory effects (Merly et al., 1998), and Fas Ligand or Fas-L that has immunoprotective ability (Bellgrau et al., 1995 and 1998; Grith et al., 1995; Sanberg et al., 1996, a, b, and 1997; Saporta et al., 1997) may provide the testis with its immunoprivileged status. However some conflicting results have been reported subsequently. Suarez-Pinzon et al., (2000) revealed that Sertoli cell-derived TGF-β1 but not Fas-L mediated the protective effect of Sertoli cells against autoimmune β-cell destruction in NOD mice. Furthermore Restifo (2000 and 2001) suggested that Fas-L was not a single mediator of immune privilege, suggesting that the hypothesis that Fas-L enabled tumor (O'connell et al., 1999) or grafted (Bellgrau et al., 1995) cells to counterattack the immune system needs to be reconsidered.

The aims of the studies described in this thesis were to isolate and characterise immunosuppressive factors in the rat testis and to determine their possible physiological and immunological roles in the testis.

1.2. The Mammalian Testis

In 1841, von Kolliker reported the testis to consist of three functional compartments, namely the seminiferous tubules, the interstitial compartment, and the capsule of the testis or the tunica albuginea. In the early part of that

century the seminiferous tubules were found to contain germinal and somatic elements, while the interstitial compartment contained primarily Leydig cells, named after Franz Leydig who investigated the cells in several species of mammals in 1850, several years after von Kolliker. In more recent times with the advent of higher power microscopy immune cells including macrophages, blood and lymphatic vessels, and nerves have been identified within the testis.

1.2.1. The seminiferous tubules

The seminiferous tubules, which constitute up to 90% of the testicular volume in rodents (Fawcett, 1973) are the site where spermatozoa are produced. These tubules are long convoluted cylindrical two-ended structures (Clermont and Huckins, 1961), opening at each end into the rete testis (Roosen-Runge, 1961), from where the spermatozoa and the fluid in which they are suspended (seminiferous fluid) are carried into the epididymis (see Fig. 1. 1). The tubular walls are formed by a well-defined boundary tissue that is composed of four layers (Setchell, 1978). In rodents the first layer on the inner surface is a non-cellular layer of the tubules, and is followed by a cellular layer which consists of smooth muscle-like or myoid cells. Next, there is an outer non-cellular layer consisting mainly of collagen-like fibres, and finally, on the outside is an outer cellular layer (review see Setchell, 1978, see also Setchell et al., 1994).

The lymphatic endothelial cells together with their nuclei exist on the outside surface of tubules (Setchell, 1978).

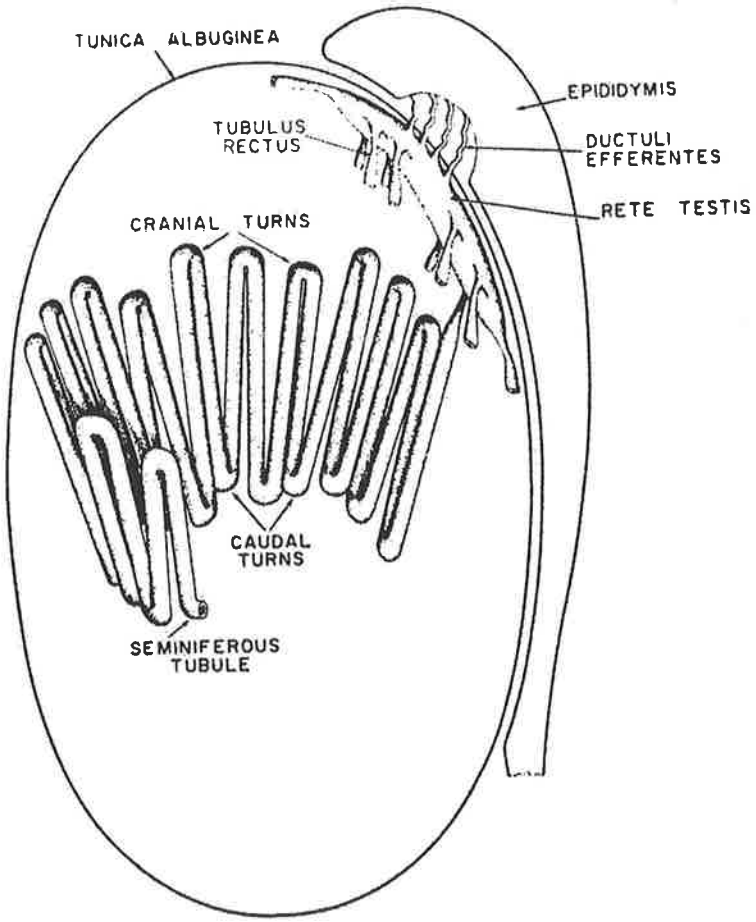


Figure 1.1 Diagrammatic representation of the arrangement of one of the seminiferous tubules and the rete testis in the testis of a rat. (Reproduced from Clermont and Huckins, 1961).

1.2.2. Germinal elements and Spermatogenesis

The germinal elements in the tubules consist of four germ cell types namely gonocytes, spermatogonia, spermatocytes, and spermatids. The term gonocytes has been reserved for the primordial germ cells (Courot et al., 1970). At maturity, when spermatogenic activity starts, the term gonocytes is used to specify the germ cells up to their differentiation into spermatogonia (Courot et al., 1970). Spermatogonia are then defined as those germ cells, which arise from the gonocytes and are contained in the parietal layer of the seminiferous tubules. Their last division gives rise to the primary spermatocytes (Courot et al., 1970, Russell et al., 1990).

The spermatocyte is a cell undergoing meiosis during sperm formation. In this stage there are two main events. The primary spermatocytes are produced by the last spermatogonial mitosis, and are the cells which undergo the first meiotic division. Cells which undergo the second meiotic division are termed the secondary spermatocytes. The first-division and the second-division cells can be distinguished by the size of the metaphase plate (Courot et al., 1970).

Spermatids are the haploid cells arising from the meiotic division of the secondary spermatocytes. These haploid cells will give rise to spermatozoa without dividing, through morphological transformation and modification of their intracellular components (Courot et al., 1970). From an immunological point of view, these cells are categorised as "non-self" as there is substantial evidence that these cells are immunogenic or auto-antigenic (Tung, 1977, Alexander and Anderson, 1987) due to their ability to initiate an immune

response in ectopic sites and the evidence that they would normally be attacked by the host immune system, were they not sequestered in the testis. Moreover antibodies against these components and spermatozoa can induce male infertility (Huang et al., 1981; Bandhauer, 1982; Naz et al., 1984; Eddy, 1988, Mazumdar and Levine, 1998; Bronson, 1999). However, under "normal" conditions these cells exist in the seminiferous tubules in the gonad without any response from the host immune system.

1.2.3. Somatic elements

There are two types of somatic cells in the seminiferous tubule, the supporting cells and the Sertoli cells. The supporting cells are somatic elements derived from the coelomic cells of the gonadic crests (Courot et al., 1970). These cells are organised into sex cords, which include the gonocytes shortly after sexual differentiation (Courot et al., 1970) and the appearance of these cells do not change until spermatogenesis is established, regardless of whether the spermatogenesis takes place shortly after birth such as in the rat and the mouse, or much later in life such as in man, elephant, sheep, cattle, and monkey (Courot et al., 1970).

The second somatic element in the seminiferous tubules is the Sertoli cell. These cells, named after Enrico Sertoli (1865), are a major somatic cell type within the seminiferous tubules (Tung, 1993), which synthesise and secrete a number of factors, including a large number of proteins (Griswold, 1998). These factors are thought to be essential for spermatogenesis (Kissinger et al., 1982, Griswold, 1998), and may be related to the important Sertoli cell

function of providing nutritive support to the various germinal cells, which transit the seminiferous epithelium (Griswold et al., 1988; Griswold, 1998). Sertoli cells are also known to secrete some factors or peptides that might contribute to immunoregulation in the testis (see section 1.6). The details of the structure of a Sertoli cell can be seen in Figure 1.2.

During the postnatal period, the Sertoli cells undergo changes in morphologic appearance and biochemical activity (Gondos, 1980). During the period until puberty, the Sertoli cells lie immediately inside the boundary tissue of the tubules and surround the undeveloped germinal cells (Setchell, 1978) and provide physical structure to the germinal epithelium (Foster, 1988). From this, the concept that Sertoli cells provide a "nurse" function within the germinal epithelium was proposed due to their close physical contact with the germ cells and their protection of spermatozoa (Foster, 1988; Griswold, 1998). Ireland et al., (1987) suggested that the germ cells may in turn be involved in the local regulation of Sertoli cell function within the seminiferous epithelium. Interactions between Sertoli cells and germ cells have been described as essentially a symbiotic relationship that involve cell-to-cell contact and paracrine communication (for review see de Kretser, 1990; Skinner, 1991). The interaction is dependent on the cyclic changes in Sertoli cells and the seminiferous epithelium, and to the provision of the blood-testis barrier, which is important for protection of germ cells in the seminiferous tubules (see the following section). In addition, Sertoli cells have also been suggested to play an active role in the transfer of spermatocytes from the basal compartment to

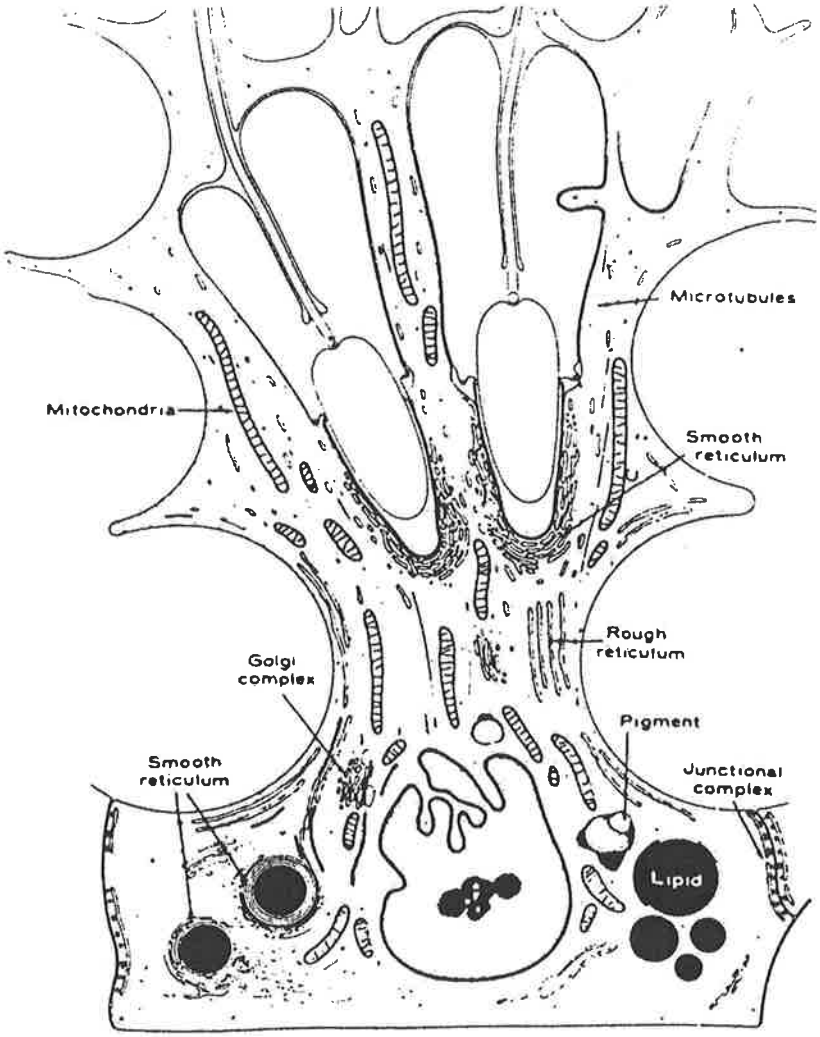


Figure 1.2 Diagrammatic representation of some details of the structure of a Sertoli cell showing cellular arrangements and testicular compartments. (Reproduced from Fawcett, 1975).

the adluminal compartment of the seminiferous epithelium (Russell et al., 1990).

1.2.4. The Blood-Testis Barrier

In the rat, between the sixteenth and nineteenth days after birth, junctional complexes can be found between adjacent Sertoli cells (Vitale et al., 1973; see also Russell et al., 1990) (see Figure 1.2). These junctions are thought to be the principal component of the "blood-testis barrier" (Setchell, 1978). This barrier resides above the spermatogonia but below the spermatocytes and hence it divides the seminiferous epithelium into "basal" and "adluminal" compartments (Neaves, 1977; Setchell, 1978; Russell et al., 1990).

The blood-testis barrier has a wide variety of important biological and possibly immunological functions. These functions include the maintenance of a unique fluid environment supporting spermatogenesis by precluding the transfer of cells and larger hydrophilic substances across the seminiferous tubules, and the partial endocrinological shielding of the seminiferous epithelium (Neaves, 1977). Through this the blood-testis barrier provides an immunological function as it protects against circulating antibodies entering the seminiferous tubules beyond the Sertoli junctions, and also isolates germ cells and germ-cell antigens from the body's immunological system (see also Section 1.3)

1.2.5. Interstitial Compartment

The interstitial compartment of the testis occupies the space built between a bundle of seminiferous tubules (see Figure 1.3). This tissue consists of Leydig

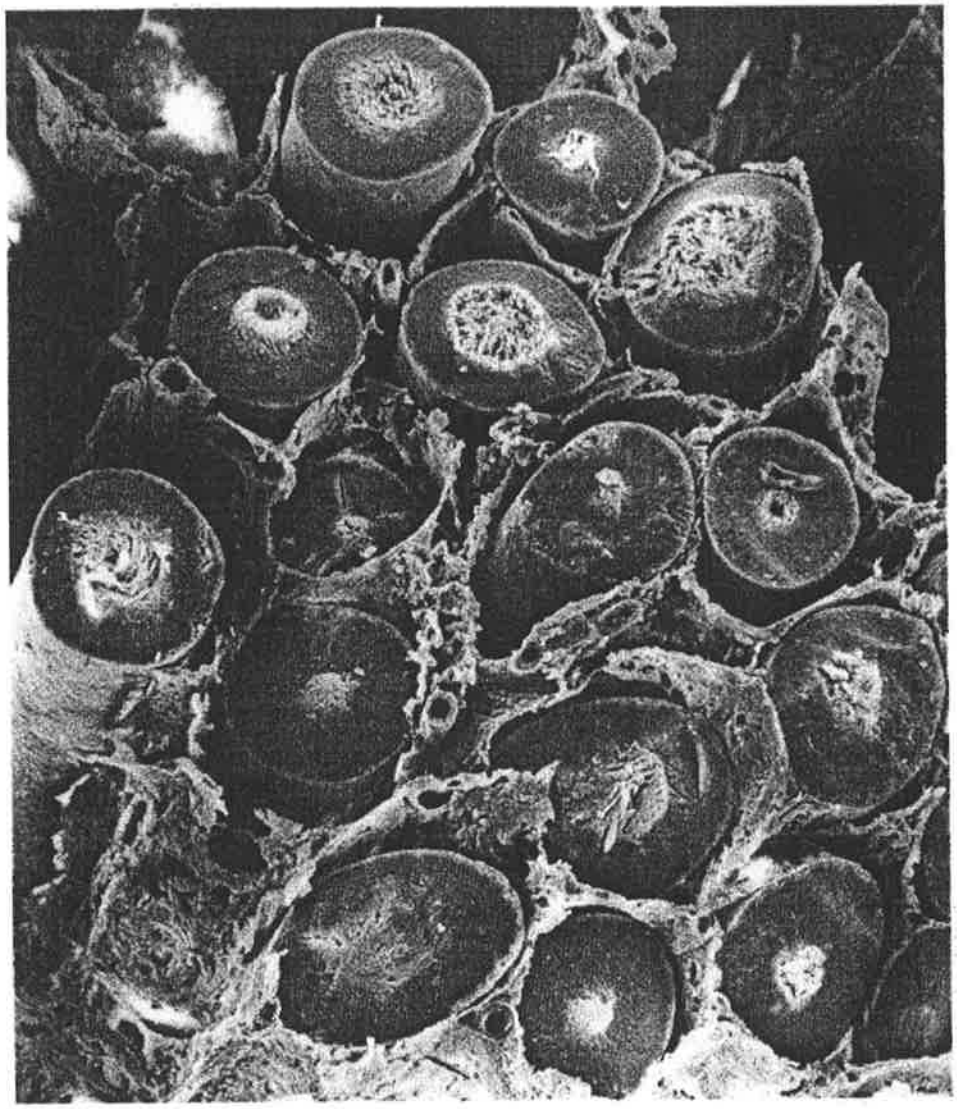


Figure 1.3 A scanning electron micrograph of a cut surface of a rat testis. Note the individual seminiferous tubules embedded in the interstitial tissue. (Reproduced from Setchell et al., 1994).

cells, macrophages, the blood vessels, lymphatic elements, and nerves. Of the latter, none are thought to penetrate the tubules (Setchell, 1978).

1.2.6. Leydig cells

The mammalian Leydig cell is a relatively large, polyhedral, epitheloid cell, and is surrounded by a typical plasma membrane which has many specializations including junctional complexes, projections, and surface indentations (Connell and Connell, 1977). The Leydig cell is the only cell in the interstitial tissue, which has a nucleus with exaggerated thickness when examined with the light microscope (Hooker, 1970). Leydig cells also have a large smooth endoplasmic reticulum.

Bouin and Ancel (1903, cited by Setchell, 1978) suggested that the function of the Leydig cells was producing the male sex hormone. Subsequent studies show that the Leydig cells are responsible for steroid production, especially testosterone, and are the major site of the steroid hormones synthesised de novo in the testis from cholesterol (reviewed in Maddocks and Setchell, 1988). In addition to synthesis of androgen, the Leydig cell is thought to be involved in the production of glycoproteins (Connell and Connell, 1977). Furthermore, other bioactive substances such as β-endorphin, neurophysin, oxytocin, renin, and vasopressin have been reported to be produced by Leydig cells (see Maddocks and Setchell, 1988). These bioactive substances are known to be able to influence the body's immune function (Tizard, 1992; Knigge et al., 2003) providing further mechanisms that might link testicular

immunoregulation to hormonal, paracrine and autocrine mechanisms of control.

1.2.7. Macrophages

In the rat testis, macrophages constitute about 25% of interstitial cells (Miller, 1982 and Niemi et al., 1986). It has also been observed that macrophages resident in the testis are very similar in their morphology and cytology to macrophages in other tissues such as the lung and peritoneum (Miller et al., 1983 and 1984; Bergh, 1987). Hutson (1990) reported that in the rat testis the appearance of macrophages starts at day 19 of gestation, and the numbers increase 15-fold during the first 50 days of postnatal life.

Macrophages in the testis are closely associated with Leydig cells (Fig. 1.4). It has been suggested that testicular macrophages are involved in regulating Leydig cell function (Maddocks and Setchell, 1988). This was supported by findings that these macrophages appear to endocytose portions of Leydig cells (Miller et al., 1983). Hedger and Meinhardt (2000) revealed that the population of rat testicular $CD8^+$ T cell subset is functionally related to either the resident macrophages or Leydig cells. It has been suggested that testicular macrophages are involved in mediating the immunological environment within the testis to protect the developing male germ cells from autoimmune attack, whilst also permitting normal inflammatory responses to occur qualitatively. This is based upon evidence that testicular macrophages are both cytotoxic and phagocytic (Wei et al., 1988) while they also demonstrate significant

immunosuppressive activity (Kern and Maddocks, 1995; Hayes et al., 1996; for recent review see Hedger, 2002).

Whilst the rat testis has been found contain abundant large, round, acid phosphatase positive macrophages (Pöllänen and Maddocks, 1988), the ram testis, in contrast, lacks a comparable population of macrophages and rejects allografts (Maddocks and Setchell, 1988b). This supports the suggestions that the presence of testicular macrophages in the rat testis could possibly contribute to the immune privilege of this tissue (see also Kern et al., 1995 and Hedger 1997).

1.2.8. Blood vessels and Lymph vessels

The relationship between the walls of seminiferous tubules, Leydig cells, and blood and lymph vessels differs between species, and macrophages and the blood vessels are not always closely associated with Leydig cells (Setchell, 1978).

Fawcett et al., (1973) conducted comparative observations on intertubular lymphatics and organisation of the interstitial tissue of the mammalian testis and suggested that the rat testis, along with mouse, guinea pig and chincilla (Fawcett et al., 1973) was categorised by (1) a minimum of interstitial connective tissue and (2) a relatively small volume of Leydig cells (

Figure 1.4). That (3), and mainly in the rat, the visceral layer of endothelium is rudimentary and only occasionally seen with the light microscope, and (4) a continuous sheet of endothelium is lacking over a large part of the surface of

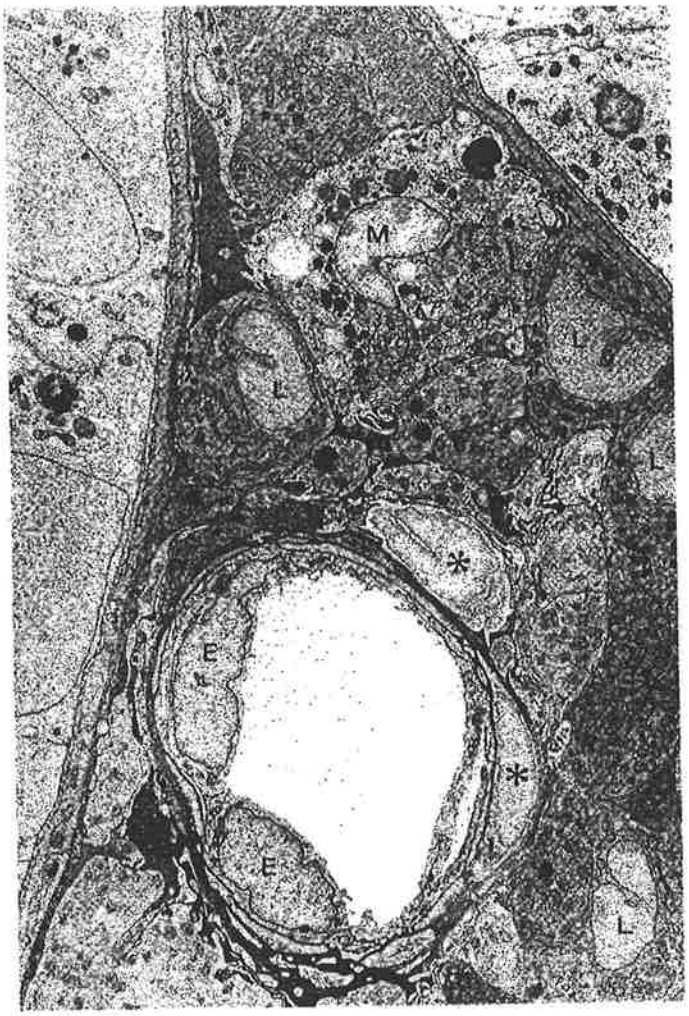


Figure 1.4 Photomicrograph representation of ultrastructure of the intertubular tissue of the adult rat testis, showing Leydig cell nuclei (L), a macrophage (M), vascular endothelial cell nuclei (E), and perivascular interstitial cells (*). The Leydig cells are closely associated with the blood vessels and surrounded by a lymph-filled space. (Reproduced from de Kretser and Kerr, 1994).

the interstitial cell clusters (Fawcett et al., 1973, see also de Kretser and Kerr, 1994). This region of the testis is important for this thesis because this is where foreign tissue grafts transplanted into the rodent testis are usually placed (see Maddocks and Setchell, 1990). The area is rich in efficient lymphatic drainage (Fawcett et al., 1973 a, b; see also Holstein et al., 1979); a point that has attracted specific investigation because it was thought to distinguish the immune privileged status of the testis apart from other tissues that have been regarded classically as being immune privileged (for further review, see 1.3 below). With an intact lymphatic drainage, grafts are usually rejected from sites accessible to the host immune system (Reeves and Todd, 1991; Roitt et al., 1994). Interestingly, as mentioned previously, in the testicular interstitial space antigen-rich sperm are protected from immune responses. Furthermore a prolonged tissue-graft survival in this site has been reported (Whitmore et al., 1985), although in fact T cells and other immune effector cells are normally not restricted from the site (Hedger and Meinhardt, 2000).

1.2.9. The capsule of Testis

The outermost part of the testis is a tough fibrous capsule, known as the tunica albuginea (Setchell, 1978). The tunica albuginea comprises three tunics that enclose the testis. The tunica vasculosa is located nearest to the parenchyma; the tunica albuginea proper lies in the middle between the tunica vasculosa and a visceral tunica vaginalis, which is the outermost layer (Setchell, 1978). A proposed function of the capsule is to maintain the correct hydrodynamic pressure inside the testis, so that the movement of fluid out of and back into the capillaries can be regulated (Setchell, 1978).

1.3. Immunological Aspects of the Mammalian Testis

In the testis, the development and differentiation of the spermatogenic cells, which are known to be immunogenic, occurs well after neonatal tolerance induction. Yet these cells normally exist in the testis without eliciting a host immune response. In addition, foreign antigens grafted into the testis are usually not rejected (Whitmore and Gittes, 1979; Selawry and Whittington, 1984; Whitmore et al., 1985).

Clearly then, the testis is a unique site given the nature of its immune responsiveness. In this section, the "immune privileged" status of the testis is reviewed.

Since the turn of the century, testes and sperm have been shown to provoke an immunogenic response in animals (reviewed by Goldberg and Thomas, 1976). Metchnikoff and then Landsteiner as early as 1899 demonstrated the antigenicity of spermatozoa in heterologous species (Glynn and Holborow, 1965; Shulman, 1974; Menge and Behrman, 1980). It has also been observed that during mammalian spermatogenesis cell surface antigens are expressed temporally (Millete and Bellve, 1977).

The results of several investigations have shown that both spermatozoa and seminal plasma are highly antigenic in man (Well et al., 1956; Shulman, 1972 and 1974; Menge and Behrman, 1980; Bernstein et al., 1981; Mazumdar and Levine, 1998; and Bronson, 1999), and in experimental animals (Marcus et al., 1975; Bradley et al., 1999). Furthermore, it has been found that some sperm surface molecules are highly immunogenic, and antibodies for these

components can induce infertility (Eddy, 1988; Primakoff et al., 1990; Snow and Ball, 1992; Shetty et al., 1999; Auer et al., 2002).

Tung and Fritz (1978) first observed specific surface antigens on rat pachytene spermatocytes, and in 1979 Tung and colleagues reported an autoantigen of testicular cells in guinea pig present during the late stages of spermatozoa differentiation. Handley et al., (1991) suggested that in the rat, the majority of the sperm autoantigens are already present on testicular spermatozoa. While some of them may be expressed completely on the sperm surface, only during the passage of the sperm through the epididymis do they fully appear. Appearance of these cell surface autoantigens during spermatogenesis in the rabbit and in human have been reported (O'rand and Romrell, 1977; Alexander and Anderson, 1987; see also Bohring and Krause, 2003). These sperm autoantigens have been utilised to demonstrate spontaneous autoimmunity to sperm such as with orchitis, (Tung, 1977 and 1995; Beer and Neaves, 1978; Fisch et al., 1989; Bohring and Krause, 2003).

It has been mentioned in 1.2.4 that one of the functions of the blood-testis barrier is to sequester the developing germ cells within a compartment delimited by permeability barriers (Dym and Fawcett, 1970; Neaves, 1977). Damage to the "barrier", such as a result of infection, vasectomy, or other trauma, may result in escape of the spermatogenic elements from the tubules. At the same time there is an opportunity for the body's immune surveillance mechanisms to enter the seminiferous tubules beyond the "barrier". A number of studies have found that in the majority of vasectomised men, significantly

elevated titres of anti-sperm antibodies can be detected (Hancock, 1981) and antisperm antibodies are also produced in other situations when the blood-testis barrier is damaged (Van Lis et al., 1974; Mancini, 1974; Alexander and Anderson, 1979). Germ cells outside the blood-testis barrier can be shown to be autoantigenic but do not normally invoke host-immune responses within the testis (Salomon et al., 1982, Yule et al., 1988).

In all of these instances it can be seen that germ cells, spermatozoa or components of seminal plasma can be antigenic, however, they are not usually attacked by the host immune system when they are in the male reproductive tract (Hogarth, 1982; Whitmore et al., 1985).

1.4. Concepts for the Testis as an Immunologically Privileged Site

Based on the evidence that immunogenic components in the testis are not normally attacked by the host immune system within the male reproductive organs, and the evidence that auto-, allo-, and even xenografts of tumor transplants survive for extended periods in the testicular interstitial tissue (Table 1.1), most investigators support the concept of the testis (especially the rodent testis) together with the brain, the anterior chamber of the eye and the hamster's cheek pouch (Barker and Billingham, 1977) as an immunologically privileged site.

The reason for the acceptance of the normally immunogenic components by the host immune system in the male reproductive tract, as well as for graft survival outside the seminiferous tubules and within the testicular interstitium

Table 1.1 Summary of literature reports of intra-testicular tumor transplants (adapted from Maddocks and Setchell, 1990).

Tissue/graft used	Donor/recipient relationship	Graft duration	Animal used	% success rate
Brown pearce tumour	Xenografts	Variable: 12 days to more than 2 months	• Rabbit to mice	50-100
			• Rabbit to hamster	50-100
			• Rabbit to rat	50-100
			• Rabbit to guinea pig	0
V_2 carcinoma	Allograft	Terminated at 12 weeks	Rabbit	100
Wilms tumor	Allografts	Terminated at 14 days	Wistar rat	100
	Allografts	Terminated at 21 days	Lewis rat	0
	Allografts	Terminated at 21 days	Fischer rat	100
	Allografts + testosterone or oestradiol	Terminated 21 days	Lewis rat	60-80
			Fischer rat	50-70
Insulinoma	Allografts:			
	• Normal	> 50 days	Rat	6
	• Diabetic	20-30 days		
Teratocarcinoma	Isografts	Unknown. Cells continue to grow and give rise to tumors.	Mice	Unknown

remains obscure. However, a number of concepts have been proposed to explain why such grafts might enjoy a prolonged and sometimes indefinite survival in the rodent testis (see also Hedger et al., 1990; Hedger, 1989 and 1997; Maddocks and Setchell, 1990; and more recently Hedger and Meinhardt, 2000).

1.4.1. Blood-testis Barrier Protection

As previously mentioned (section 1.2.2.2), the structural basis for the blood-testis barrier is formed by the tight junctions between adjacent Sertoli cells. This "barrier" segregates the interior of each seminiferous tubule into a basal and an adluminal compartment, and it has been suggested that the barrier acts to protect the sperm and germ cells from the systemic immune system and prevent an antoimmune response (Dym and Fawcett, 1970). Further evidence of the existence of a functional blood-testis barrier was provided by Setchell and Waites (1975). Using an intra-vascular infusion of vital dyes, which led to staining of most cells in the body with a notable exception in the brain due to the existence of the vascular blood-brain barrier, Setchell and Waites (1975) found a comparable situation in the testis, where cells in the interstitial tissue were stained but dyes did not stain cells inside the seminiferous tubules.

While interstitial fluid in the testis contains greater concentrations of albumin and gamma-globulin than most other tissues in the body, the "blood-testis" barrier prevents antibodies from entering the seminiferous tubules beyond the Sertoli cell tight junctions (Setchell and Brooks, 1988; Pöllänen and Setchell, 1989).

Given these findings, the blood-testis barrier might protect the "immunogenic" germ cells in the adluminal compartment. However, the barrier does not protect the spermatogonia or preleptotene spermatocytes present in the basal part of the seminiferous epithelium outside the Sertoli cell tight junctions (Yule et al., 1988). Furthermore, seminiferous tubule damage in men is not always correlated to the presence of anti-sperm antibodies (Hargreave et al., 1982), and several immune responses are inhibited *in vitro* and *in vivo* by testicular cells (Hurtenbach et al., 1980; Born and Werkele, 1982; Hurtenbach and Shearer, 1982).

Also of significance is that most of the foreign grafts placed into the testis are transplanted into the interstitial tissue and thus are located outside, and are unprotected by the blood-testis barrier. These findings all point to the fact that additional mechanisms (other than the blood-testis barrier) must exist in the testis to afford immune protection, and the role of the local factors within this site might afford the rat testis its status as an immunologically privileged site (see also Hedger, 1997; Hedger and Meinhardt, 2000).

1.4.2. The Lack of Lymphatic vessels

Grafts are usually only rejected from sites accessible to the host immune system (Roitt et al., 1994) with an intact lymphatic drainage (Reeves and Todd, 1991). When the immune system is excluded from an organ or tissues, the site thus becomes immunologically privileged. Barker et al. (1969), Yoffey and Courtice (1970) and Santiago-Delpin (1983), suggest that the reason some tissues, such as anterior chamber of the eye and the cheek pouch of the

hamster, are immunologically privileged sites, may be due to the small number, or even absence, of both blood and lymphatic vessels. A similar explanation was offered by Medawar and Russel (1958) for the testis. However, the testicular interstitial tissue where foreign grafts are usually transplanted in the testis possesses numerous lymphatic connections with a very effective lymphatic drainage (Fawcett et al. 1973; Head et al. 1983b; Maddocks and Setchell, 1988); see

Figure 1.4. Clearly, any proposal that immune privilege arises from poor lymphatic drainage does not account for graft survival in the testis. This theory is not now accepted for the eye either (see Rocha et al., 1992). Recent studies have revealed that ocular fluids contain a potpourri of immunosuppressive and immunoregulatory factors that suppress T-cell proliferation and the secretion of proinflammatory cytokines (Niederkorn, 2002). Niederkorn (2002, 2003) suggests that the ocular immune privilege is sustained by factors such as Fas ligand (Fas-L or CD95L), which induces apoptosis of infiltrating inflammatory cells that suppress immune cell proliferation and purge immune cells that enter the eye.

1.4.3. Concept of Specific Immunosuppressive Factors

Various theories have been advanced to explain the phenomenon mentioned above, and Maddocks and Setchell (1990) suggested that "... the most likely explanation at present seems to be that the testis contains specific immunosuppressive factors that inhibit lymphocyte activities in this site". In

addition, Hedger (1997) also reviewed possible local mechanisms responsible for inhibition of immune cell function in the testis.

Head et al., (1983a,b) and Head and Billingham (1985) suggested that one of the factors likely to be important were the steroids (androgens) produced in the testis. On the other hand, Grossman et al., (1979), Raveche et al., (1980) and Sason and Mayer (1981), suggested that androgens can not be responsible because lymphocytes do not contain androgen receptors. Furthermore, Maddocks (1987), Maddocks and Setchell (1988a), then Selawry and Whittington (1988) suggested that steroids produced by the Leydig cells do not reach high enough concentrations to create immunosuppression in the testis. However this issue remains open since there have been several recent studies demonstrating novel androgen actions mediated via membrane receptors on both macrophages and lymphocytes (Hedger, 2005, personal communication).

Early studies have shown the presence of immune cell inhibiting factors in the mouse, bovine, and human seminal plasma (Marcus et al., 1978; Ablin et al., 1980; Anderson and Tarter, 1982). Murine germ cells have also been reported to induce immune tolerance (Hurtenbach et al., 1980 and 1982), and Leydig cells have also been reported to be able to suppress lymphocyte proliferation *in vitro* (Born and Werkele, 1982). Numerous other reports by Pöllänen et al., (1988, 1989), Sainio-Pöllänen et al., (1991), De Cesaris et al., (1986), Wyatt et al., (1988), Emoto et al., (1989, 1990), Hedger et al., (1990), and De Cesaris (1992), followed by Korbutt et al., (1997), and Sanberg et al., (1997) also support the hypothesis that certain factors are produced in the rodent testis to

create the immune privilege status in this site. A number of studies up to the late 1990s have also suggested that Sertoli cells, which express the T cell-cytotoxic Fas ligand (Bellgrau et al., 1995 and 1998) are responsible for the immune privileged phenomenon of the testis (Hemedinger et al., 2002).

1.4.4. Mechanisms of Immunosuppression

The presence of immunoprivileged sites, such as the anterior chamber of the eye, the brain, the testis, and that they support allograft survival has stimulated interest in the development of an immune suppressant in order to permit graft transplantation at other sites. Many immunosuppressive agents have been established, and some of them are now in widespread clinical use (Table 1.2, Thomson et al., 1992).

Possible mechanisms of graft survival could be studied from an understanding of the mode of action of the immunosuppressant. The mode of action of current immunosuppressive agents has been discussed broadly by Thomson et al., (1992) and Schreiber and Crabtree (1992). Corticosteroids generally have effects on leukocyte circulation. In humans, when corticosteroids are administered, the number of circulating eosinophils, basophils, and lymphocytes declines abruptly while the number of neutrophils increases. It has been found that thymic atrophy and triggering of T-cell apoptosis occurs when corticosteroids are administered in high doses. Lower doses on the other hand, suppress the ability of T cells to produce interleukin 2 (IL-2) and hence their responses to mitogens such as concanavalin A and phytohemagglutinin is reduced (Tizard, 1992). Most immunosuppressive drugs have been designed to

inhibit cell division, and to act on various stages of nucleic acid synthesis and activity (Schreiber and Crabtree, 1992). Due to their high toxicity and lack of specificity in their actions, the three cytotoxic agents in Table 1.2 now have been replaced in the treatment of allograft rejection by three very specific and relatively non toxic agents, cyclosporine A (Cs A), tacrolimus (FK506) and rapamycin (also known as sirolimus). Cyclosporine A and FK506 exert their pharmacological effects by binding to members of a family of intracellular proteins known as the immunophilins and inhibit the activity of calcineurin, a major signaling pathway in T cells, at nanomolar concentrations (Brazelton and Morris, 1996). Rapamycine has quite distinct mode of action from either CsA or FK506. Although it binds with immunophilins, the rapamycin:immunophilin complex has no effect on calcineurin activity (Bierer et al., 1990).

Cyclosporine A, which comprises 11 amino acids and has a molecular weight of 1202.6 Da, has its main site of biochemical action in the $CD4^+$ T cell (Thomson et al., 1992). Furthermore, mitogenic T cell responses to anti-CD3, concanavalin A and phytohemagglutinin are also known to be inhibited by CsA (Thomson et al., 1992).

Preliminary *in vitro* and *in vivo* studies showed that FK 506 was very effective; with activity usually 10-100 times lower in concentration compared with CsA in suppressing immune responses (Goto et al., 1987; Kino et al., 1987a, 1987b). Further studies also showed that FK 506 was very effective in suppressing both alloantigen- and T-cell mitogen-induced lymphocyte

proliferations (Thomson et al., 1992, see also Allison, 2000, Gorantla et al., 2001).

Table 1.2 Immunosuppressive drugs in widespread clinical use

Agents	Mechanism
Cytotoxic Agents	
Azathioprine	Purine biosynthesis inhibitor
Cyclophosphamide	A1kylating agent
Methotrexate	Folic acid antagonist
Corticosteroids	
Methylprednisolone	Multiple effects on neutrophils macrophages and T cells
Prednisolone	Multiple effects on neutrophils macrophages and T cells
Prednisone	Multiple effects on neutrophils macrophages and T cells
T lymphocyte suppressants	
Cyclosporine A	Inhibition of TcR-mediated $CD4^-$ T cell activation
FK506	Inhibition of T cell activation via TcR-CD3 complex and CD2
Rapamycin	Forming complexes with intracellular binding protein, immunophilins
Anti-CD3 monoclonal antibody (OKT3)	Depletion of $CD3^+$ T cells

(Modified from Thomson, et al., 1992, see also Garantla et al., 2000).

Thomson et al., (1992) cite Bierer et al., (1991), as suggesting that like CsA, the ability of FK 506 to inhibit T cell activation is mediated both through the

TcR-CD3 complex and via another cell surface molecule, CD2. They have also suggested that FK 506 influences T cell activation during the early events and that these could include Ca^{2+} mobilisation, protein kinase C (PKC) activation, cytokine gene transcription, cytokine secretion and cytokine-receptor expression, most of which occur within the first two hours of activation (Thomson et al., 1992; for a review on the genetic regulatory events guiding T cell activation, see Crabtree, 1989). This mode of action by FK 506 in suppressing T-cell mitogen-induced lymphocyte proliferation is illustrated in Figure 1.5 (Crabtree, 1989; Thomson et al., 1992).

In this figure, Thomson et al., (1992) also indicate the possible targets for FK 506 (and CsA). By using this illustration, it may be possible to develop a similar suggestion for the process of how cells, molecules or factors involved in the physiological suppression can inhibit the immune response. This in turn, could then explain why an allograft put in an area of the body such as a privileged site can survive, and explain how the immune responses are suppressed locally.

Saporta et al., (1997) and more recently Dufour et al., (2003) reported that xenotransplantation of porcine Sertoli cells into the rat brain were able to survive at least two months without immunosuppression. As mentioned previously, the brain together with the anterior chamber of the eye have also been considered to the classic immunologically privileged sites (Barker and Billingham, 1985) since allografts transplanted in these sites survived longer than allografts transplanted in the peripheral sites.

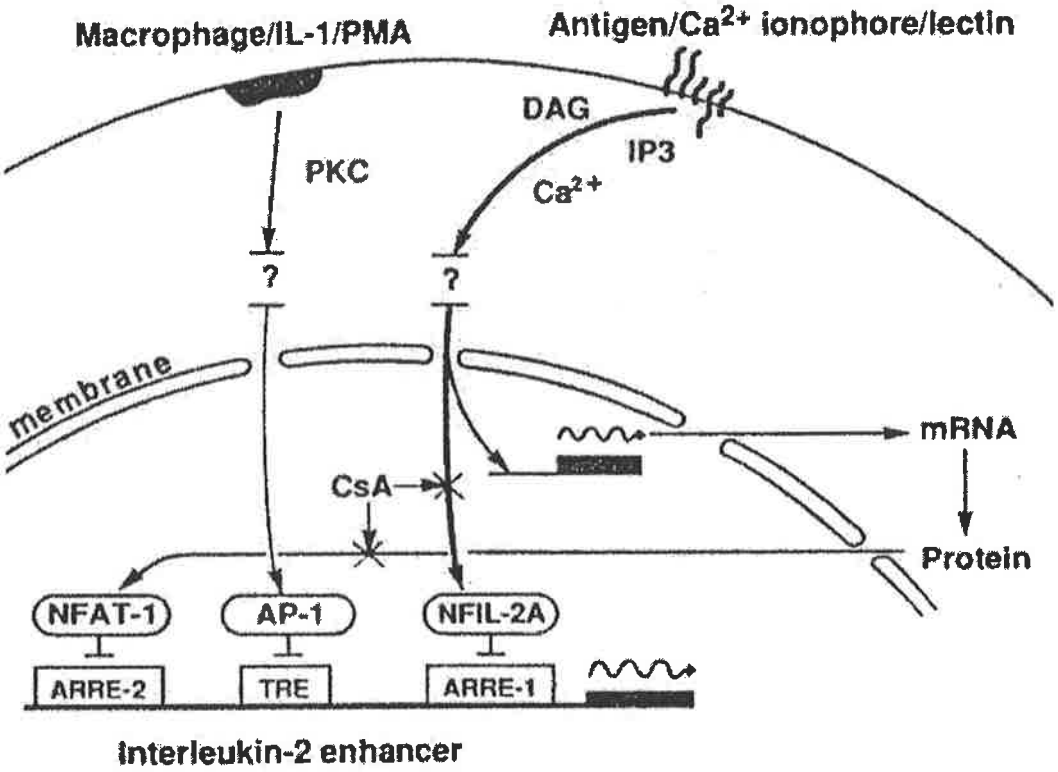


Figure 1.5 Diagrammatic representation of a tentative model of the pathways of T lymphocyte activation, showing transcription regulatory proteins and their binding sites. Possible targets for immunosuppressive drugs FK 506 (and CsA) action are also indicated (Reproduced from Thomson et al., 1992).

Note: DAG: diacylglycerol; Ca^{2+}, IP3 (inositol 1,4,5-triphosphate), and PKC protein kinase C are postulated to be second messengers in transmitting signals initiated at the antigen receptor. NFIL-2: nuclear factor interleukin-2; NFAT: nuclear factor of activated T cells; ARRE: antigen receptor response element; TRE: the phorbol myristate acetate response element (For further review, see Crabtree, 1989).

The survival of xenotransplantation of porcine Sertoli cells in the rat brain without immunosuppression could be seen as the ability of Sertoli cells to produce an immunosuppressive factor as suggested elsewhere (Selawry and Cameron, 1993; Bellgrau et al., 1995 and 1998). Whether the mechanism of the survival of Sertoli grafted in the brain shown by Saporta and Dufuour and colleagues was due to the suppression of immuno-competent cells by Sertoli cells or their products that was similar to those of Cyclosphorine A or FK 506 targets as proposed in Figure 1.5 is still unclear; or it might also be possible due to the nature of the brain itself as an immune privileged site.

1.5. Apoptosis

Apoptosis is a physiological form of cell death required to guarantee the balance of the cell division through the degree of cell death in multicellular organisms. The role of apoptosis as another mechanism of maintaining a site to be immune privilege has been proposed recently. As suggested elsewhere the primary reason for the testis being an immunologically privileged site is likely to be to avoid the potential auto antigens of the spermatogenic cells in the testis from provoking destructive autoimmune attacks (Turek et al., 1996).

It has been reported that apoptosis could regulate body self-tolerance by controlling immune cell proliferation and preventing unregulated proliferation of auto-reactive cells. Takahashi et al., (1994) and Bellgrau et al., (1995 and 1998) reported that Sertoli cells express constitutively the ligand of Fas (CD95) molecules which could induce immune tolerance in the testis because apoptosis of activated T cells occurs when the Fas (CD95) molecules bind to T

cells. However apoptosis in the T cells could also arise due to the absence of stimuli needed for their growth and function eg. IL-2 dependent T cells undergo apoptosis in the absence of IL-2 (Duke and Cohen, 1986).

1.6. Possible Immunoregulatory Peptides in the Testis

Several peptide factors in the testis have been reported which share functional similarities with peptides which have been shown to affect the functions of immune cells *in vivo* or *in vitro* (Pöllänen et al., 1990).

The lymphocyte regulating peptides analogous to those observed in the testis and their predicted relative molecular weight are presented in

Table 1.3. From this table it can be seen that Sertoli cells, Leydig cells and macrophages in the testis secrete peptides which are able to act on lymphocytes. Ontogeny studies of immunosuppressive activity in the rat testis conducted by Pöllänen et al., (1992) showed that the maximum immunosuppressive activity is present in the rat testis at 44-60 days old. Furthermore, Martinova et al., (1993) reported that in the rat testis, immunoregulatory proteins are already being synthesised by the Sertoli cells by the time of blood-testis barrier formation. The secretions of cultured Sertoli cells have been reported to inhibit mitogen-induced T-cell proliferation *in vitro* due to the presence of immunosuppressive molecules (Whitmore et al., 1985; Wyatt et al., 1988; de Cesaris et al., 1992).

Table 1.3 Immunoregulatory effects and molecular weight of lymphocyte regulating peptides analogous to those observed in the testis.

Factor and producing cell	Mr (kDa)	Effect(s)	References
IGF I (Sertoli, peritubular)	7.7 (active peptide) 30; 50 (binding proteins)	Inhibition of lymphocyte proliferation	Hunt and Eardley, 1986
IL-1 (Sertoli)	17.5	Induction of IL-2 receptor, IL-2 and other lymphokines expression	Mizel, 1982
Inhibin (Sertoli)	20; 31, heterodimer	Stimulation of mitogenesis of thymocytes	Hedger et al., 1989
Activin (Sertoli, Leydig)	13; 31, homodimer	Inhibition of mitogenesis of thymocytes	Hedger et al., 1989
TGF-β (Sertoli, peritubular)	25, homodimer	Inhibition of ConA-, anti-T3 and anti-Ly6C but not ionomycin+ PMA-induced proliferation of T cells	Moog et al., 1988
		Inhibition of IL-2-induced up regulation of IL-2 and transferrin receptors in activated T cells	Kehrl et al., 1986
		Inhibition of IL-2 production and down-regulation of IL-2 receptors in T cells	Lin and Siekierka, 1988
NGF-β (spermatocytes, spermatids)	13.3; 28	Stimulation of ConA-mediated mitogenesis of lymphocytes	Thorpe et al., 1986
		Stimulation of IL-2 receptor expression on cultured lymphocytes	Thorpe et al., 1987
AVP (Leydig)	1.1.-active peptide 25-30 binding protein	Stimulation of γ-interferon production by Lyt-2^+ splenocytes	Torres and Johnson, 1988a,b

Table 1.3. (Continued)

Factor and producing cell	Mr (kDa)	Effect(s)	References
Substance P (Leydig)	<5	Stimulation of T Lymphocyte proliferation Inhibition of ConA-stimulated proliferation of PBL *in vivo*	Payan et al., 1983; Stanisz et al., 1986,1987; Scicchitano et al., 1988. Wilson et al., 1988
ACTH (Leydig)	<5	Inhibition of superoxide induction in macrophages	Johnson and Smith, 1988
α-MSH (Leydig)	<5	Inhibition of inflammatory and immunostimulatory effects of IL-1	Cannon et al., 1986
GM-CSF (Macrophage, male germ cells)	34-37	Induction of suppressive responses, increase sperm motility.	Kern et al., 1995; Zambrano et al., 2001 and Vilanova et al., 2003

Adapted and modified from Pöllänen et al., (1990).

It was also reported that Sertoli cells produce clusterin which was shown to have tolerogenic abilities (Bailey and Griswold, 1999). Arginine vasopressin (AVP) is another substance produced by Leydig cells, and this has been reported to be able to stimulate the production of γ-interferon by Lyt-2^+ splenocytes (Torres and Johnson, 1988a,b).

Testicular macrophages have also been implicated in regulating immune function locally in the testis. Takemura and Werb (1984) have reported that macrophages secrete several immunosuppressive factors, and the resident testicular macrophages may also secrete immunosuppressive factors such as GM-CSF and prostaglandin (Kern et al., 1995).

1.7. Partial Characterisation of Immunosuppressive factors in the Testis

Pöllänen and his group have identified several characteristics of immunosuppressive factors in the testis. They have carried out extensive investigations into the immunosuppressive activity in the testis. According to this group, an immunosuppressive factor, which they have named "protectin", was heat- and pH labile (Pöllänen et al. 1988) and also increased cyclic adenosine monophosphate concentrations in the lymphocytes (Pöllänen et al. 1991). More recent studies indicate possible associations of protectin with the G-protein receptor (Hurley, 1999). Studies on the levels of protectin activity in scrotal and abdominal testes of hyphophysectomised and hormone-treated rats suggested that the levels of testicular immunosuppressive activity increase when testosterone production decreases (Sainio-Pöllänen et al., 1991).

Based on gel filtration of the extracellular fluid of the rat testicular interstitial tissue, four classes of protectin have been reported: protectin A and B corresponding to about 400 kDa and 200 kDa, respectively, and protectin C and D corresponding to about 65 kDa and 25 kDa respectively (Pöllänen et al, 1990). Subsequent studies by Pöllänen et al., (1992), revealed further characteristics of the rat testicular immunosuppressant and reported that it was not cytotoxic and that the immunosuppressive activity is due to a non-denatured agent. The immunosuppression was not due to trypsin-like proteases, and the testicular fraction with a molecular weight higher than 5 kDa (Mr > 5 kDa) was not cytotoxic.

Other characteristics of testicular immunosuppression have also been reported. De Cesaris et al. (1992) found an immunosuppressive factor that was heat and freeze stable, but which was denatured by trypsin. Emoto et al., (1991) found that the immunosuppressive activity of mouse testicular extracts was denatured by DNase, RNase or Pronase treatment; and that, after pronase treatment, the suppressive activity was increased (Emoto et al., 1991).

Furthermore Wyatt et al., (1988) found that heat treatment did not inactivate the immunosuppressive activity of rat Sertoli cell proteins, as did Emoto et al., (1990) who used a water-soluble fraction of mouse seminal vesicle fluid. Those immunosuppressive factors could be different from the factors reported by Pöllänen and co-workers in 1988 but may still correspond to the 25 kDa immunosuppressive factor reported by Sainio-Pöllänen and colleagues in 1990 from abdominal testes, and the testes of hyphophysectomised rats.

Immunosuppressive activities have also been observed in extracts of the brain, kidney and liver (Pöllänen et al., 1989). However, Emoto et al., (1989) reported that fluid from liver, spleen and kidney of mice did not have similar immunosuppressive effects to their testicular extract. Furthermore, Pöllänen et al., (1989) also reported that the testicular immunosuppressive substances could be precipitated with ammonium sulphate. However no further investigation with this precipitated fraction has been reported.

Whilst immunosuppressive activity in the testis of less than 5 kDa has been reported, such as arginine vasopressin found by Nicholson et al., (1984), investigations on the characterisation of immunosuppressive factors or

peptides in the testis, particularly the rat testis, have focused on the factors from high molecular weight classes. This is due largely to evidence that most of the low molecular weight substances give rise to cytotoxic effects, or need carriers to optimise their effects.

Kehrl et al., (1986) and Moog et al., (1988) reported that TGF-β could inhibit mitogen-induced T cell proliferation, and Sertoli cells are known to produce this protein (Skinner et al. 1989). TGF- β1 and – β2 were also reported to be present in defined stages of testicular cell differentiation, in the somatic cells and the germ cells (Teerds and Dorrington, 1993). However De Cesaris and co-workers reported that immunosuppressive proteins present in mouse Sertoli cell culture supernatant did not correspond to TGF β-like factors found in the testis (De Cesaris et al. 1992). In contrast Pöllänen and his group reported that the immunosuppressive activity in the extracts of rat seminiferous tubules was a TGF β-like factor because the moiety they found could be abrogated by incubation with monoclonal anti-TGFβ antibodies (Pöllänen et al. 1993).

Whether TGF β related polypeptides are principally involved in the regulation of immunosuppression in the testis is still to be determined. Hedger et al., (1998a) reported that the factor predominantly responsible for the inhibition of T cells proliferation by rat testicular interstitial fluid does not appear to be one of the known mammalian TGF β isoforms since the inhibitory activity was not affected by a polyspecific TGF β antiserum. Bellgrau et al., (1995) and Tageda et al., (1998) suggested that the T cell-cytotoxic Fas ligand expressed on the surface of the Sertoli cells could play important roles in the

orchestrating the immunoprivileged of the rodent testis. However, Suarez-Pinzon et al., (1999) demonstrated that the protection of syngeneic islet β-cells grafts from autoimmune destruction in NOD mice was carried out by TGF β produced by Sertoli cells, and was not due to Fas ligand. Similarly, Hedger and Meinhardt (2000) also suggested that the role of Fas ligand produced by Sertoli cells does not explain the prolonged survival of grafts transplanted within the interstitial tissue (Hedger and Meinhardt, 2000).

Head and Billingham (1985) and Whitmore et al., (1985) have reported that the survival of allografts in the testes of experimentally cryptorchid rats (which causes complete loss of germ cells and reduction in many Sertoli functions), suggests that the importance of Sertoli cells in fashioning the privileged status of the testis is unclear. Hedger (1989 and 2002) have also proposed that the resident macrophages rather than Sertoli cells or germ cells were more important for maintenance the immune privilege of the testis.

1.8. The purpose of this study

It is generally accepted that the rodent testis is a site particularly hospitable to grafts of foreign tissue. The presence of cellular or soluble factor(s), which create a unique local microenvironment, that can regulate or inhibit lymphocyte activation in the testis (Born and Werkele, 1982; Head and Billingham, 1985b; Whitmore et al., 1985, Pöllänen and Maddocks, 1988; Maddocks and Setchell, 1990; Pöllänen et al., 1990; Hedger, 1997 and Hedger and Meinhardt, 2000) has been implied as the reason the rodent testis is immunologically privileged.

Immunosuppressive factor(s) have been shown to exist in the seminal plasma, the testicular interstitial fluid and in testicular extract. However, the specificities, characteristics, and the origin of these factor(s), and how they work in providing the special immunological status of the testis are still somewhat obscure. Furthermore, reports reviewed so far suggest that immunosuppression in the testis is a complex event, and further understanding requires the factors involved to be characterised. The present study was undertaken in an attempt to isolate, and further characterise the factor(s) involved. The possible mechanisms of the immunosuppression involved in this event were also studied. The studies reported in this thesis should contribute additional information to our understanding of the immunology and the physiology of the testis.

2. Materials and Methods

In this chapter general materials and methods used throughout this thesis are described. Additional specific methods or protocols used for specific experiments will be described as appropriate in subsequent chapters.

2.1. Animals

Albino Wistar rats were used as donors of testis, brain, liver, or kidney tissue, and normal rat serum. Inbred DA rats were used as donors of lymphocytes. All rats were purchased from the Laboratory Animal Services department, The University of Adelaide, Adelaide, South Australia. They were adult males, sexually mature, weighing no less than 300g, and housed under standard conditions with food and water provided ad libitum. All rats were sacrificed by CO_2 inhalation. All experimental procedures involving animals in these studies were approved by the Animal Ethics Committee at The University of Adelaide.

2.2. Reagents/Chemicals

RPMI 1640 and Penicillin (5000 IU/ml) with Streptomycin (5000 μg/ml) were purchased from Cytosystems, Castle Hill, NSW, Australia. Heat-inactivated foetal bovine serum, glutamine solution (200 mM, 2.92%), and Monomed-A (serum replacement) were purchased from CSL, Parkville, Victoria, Australia.

Ammonium Chloride, Ammonium Sulphate, Sodium Chloride, Potassium Chloride, Sodium hydrogen carbonate, Iso-propyl alcohol (Propan-2-ol), Phosphoric acid and pH standard solution all were purchased from BDH Chemicals Pty. Ltd., Kilsyth, Victoria, Australia. EDTA

(ethylenediaminetetraacetic acid) was purchased from AJAX Chemicals, Sydney, Australia.

Percoll, PD-10 Sephadex G-25 Columns, G25 (Gradifrac) C26/70 Column, Superdex Peptide PC 3.2/30, Mono S HR 5/5, Mono Q PC 1.6/5, μRPC C2/C18 SC 2.1/10 HPLC columns were purchased from Pharmacia Biotech, Amersham Pharmacia Biotech, Sydney, Australia.

Mouse anti-rat α/β TcR (R73), CD4 (W3/25), CD8 (Ox8) and B cell (Ox33) monoclonal antibodies (mAb), and the fluorescent isothiocyanate-sheep anti-mouse immunoglobulin antibody (FITC-SHAM) were purchased from Serotec Ltd, Oxford, England. Mouse anti-rat mac-1 (CD11b) (WT. 5) and anti-Giardia (IB5) monoclonal antibodies were generously provided by Dr Graham Mayrhofer, Department of Microbiology and Immunology, The University of Adelaide.

Concanavalin A, *Canavalis ensiformis* (Con A), Phytohemagglutinin (PHA), bovine serum albumin (BSA) fraction V powder, chain B-bovine insulin, 3-[4,5 dimethyltiazol-2yl]-2,5 diphenyl tetrazolium bromide (MTT), 4-[2-aminoethyl] benzene sulfonyl fluoride (AEBSF) hydrochloride, trypan blue stain (0.4%), and Coomassie Brilliant Blue G were all purchased from the Sigma Chemical Company, St. Louis, MO, USA.

A fibroblast cell line (Rat-2, ATCC CRL 1764) was generously supplied by Professor Peter Rathjen of the Department of Biochemistry, The University of Adelaide. GA 4.2 hybridoma T cells, the IL-2 dependant CTLL cell line, the I-3 (cell transfected with H-2K^b), the EG 7 (cell transfected with ovalbumin) cell

lines, and SIINFEKL (chicken ovalbumin 257-264) peptide were generously provided by Dr A. W. Purcell, Department of Microbiology and Immunology, University of Melbourne.

Tritiated [^{3}H] thymidine (specific activity 1.04TBq/mM) and Biodegradable Counting Scintillant (BCS) were purchased from Amersham Australia. The BCS scintillant was used in conjunction with a 1215 Rackbeta II (LKB, Wallac, Findland) β-counter

In addition, when the 1450 Microbeta Trilux liquid scintillation and Luminescence (Wallac, Turku, Finland) counter was used, the Wallac Betaplate Scint scintillant and Printed Filtermat A #1450-421 glass fibre filter were used and they were purchased from Perkin Elmer Life Sciences, Australia.

Dialysis tubing (<12000 MW cut-off) was purchased from Selby-Anax, Australia. Amicon and Centricon microconcentrators were purchased from W. R. Grace & Co, Beverley, MA, USA.

Negative selection systems for $CD4^+$ and $CD8^+$ rat T lymphocyte separation kits were purchased from StemSep™, StemCell Technologies, Inc., Vancouver, Canada. The kits contained antibody cocktails for $CD4^+$ (# 3L10142) and $CD8^+$ (#2H156226) rat T lymphocyte and magnetic colloid #04F11840.

Recombinant rat IL-2 (rrIL2, # 502-RL-010) and anti-rat IL-2 antibody (anti-rrIL-2 antibody, # AF-502-NA) were purchased from Research and Development Systems, Inc., Minneapolis, USA.

The Vybrant™ Apoptosis Assay Kit (Kit #2, V-13241 Alexa Fluor® 499 annexin V/propidium iodide) was purchased from Molecluar Probes, Inc., The Netherlands.

2.3. Sample preparations

2.3.1. Preparation of testicular extract

Testicular extracts were prepared as previously described by Pöllanen et al., (1988) with certain modifications, in phosphate buffered saline without Ca^{2+}, Mg^{2+} (PBS^-). The PBS^- components were prepared as follows:

NaCl	8.00 g
KCl	0.20 g
$Na_2 HPO_4$ anhydrous	1.15 g
$KH_2 PO_4$	0.20 g

and were dissolved in one litre Milli-Q water. The PBS^- solution was then pH adjusted to 7.2 using 1 N NaOH or 1 M HCl, and filtered through a 0.22 µm filter (Millipore, Bedford, MA 01730).

The testes of albino Wistar rats were decapsulated, weighed and placed into a pre-chilled (4° C) glass homogeniser with a teflon pestle (1 g wet weight tissue/3 ml PBS). The testes were then homogenised at 4° C with 8-10 strokes of the pestle rotating at 1500 rpm.

The homogenates were initially centrifuged at 250 x g for 15 minutes at 4° C after which the supernatants were collected and centrifuged at 10000 x g for 30 minutes at 4° C to remove cellular debris. The second supernatants were then collected and passed through a combination of Millipore membrane filters of 0.80 and 0.45 μm pore sizes. These filtered supernatants were designated as testicular extract (TE) and stored at -20°C until use.

2.3.2. Preparation of normal rat serum

Adult Albino Wistar rats were euthanised by CO_2 inhalation and blood was immediately collected from the left ventricle of the heart using 5 ml syringes and a 21 G needle. The blood was then transferred into a 5 ml glass tube and allowed to clot at room temperature. After one hour the clot was loosened from the walls of the tube to aid retraction, and the blood was centrifuged at 1000 x g for 15 minutes at 4° C. The resultant serum was then filtered using a combination of 0.45 μm and 0.22 μm pore size filters. Aliquots of the resulting normal rat serum were stored at -20° C until use.

2.3.3. Preparation of brain, kidney and liver extracts

Extracts of brain, kidney and liver were prepared in a similar manner to testicular extracts. However, the kidney and the liver extracts were extremely concentrated and the second supernatants were diluted two fold in PBS and centrifuged one extra time at 10,000 x g for 30 minutes at 4°C. The extracts of brain, kidney and liver were then filtered as described in 2.3.1 and stored at -20° C until use.

2.4. Isolation of splenic T cells

One of the most commonly used methods in the isolation or separation of human and mice peripheral blood mononuclear cells is through nylon wool columns (Papamichael et al., 1976; Gutierrez et al., 1979). This method is also commonly used for isolating T cells from mouse or rat lymphoid tissues with an almost pure population of T cells obtained (Julius et al., 1973; Papamichael et al., 1976, and Johnson and Thrope, 1988).

Gutierrez et al., (1979) reported that centrifugation of monocytes over a discontinuous density gradient of Percoll was a simple and very rapid technique for isolating human peripheral B and T cells. With this technique both B and T lymphocyte populations were found to be highly enriched in one step (Gutierrez et al., 1979). Furthermore, the interaction of cells with receptors that could activate T and/or B cells, can be avoided providing a significant advantage over other methods commonly used (Gutierrez et al., 1979).

Because T cells and their receptors are known to be central to the orchestration of the immune response against foreign antigens (Janeway, 1988, and Janeway et al., 1988a, b), these cells were used to assess the immunomodulatory actions of testicular extracts and other substances used in this study. For this purpose, adult rat splenic-T cells were isolated by a modification of the method of purification of lymphocytes developed by Gutierrez et al., (1979). In addition, the lymphocyte proliferation assay used in this thesis was adapted from Pöllänen et al., (1988).

2.4.1. Preparation of Lysing Solution

A lysing solution was used to lyse erythrocytes in the spleen cell suspensions (see 2.4.3). A variety of lysing solutions have been developed including Geys Hemolytic medium and an ammonium chloride based solution (Johnstone and Thrope, 1988, and Zola, 1989). The stock solution of the ammonium chloride based solution was made up as follows.

Ammonium chloride	8.260 g
Sodium bicarbonate	1.000 g
EDTA	0.037 g

The ingredients were dissolved in 100 ml Milli-Q water and the solution was sterilised by filtering with a 0.22 μm filter (Sterile Millex-GV, Millipore) into a sterile 100 ml vial. Stored at room temperature, the stock solution was subsequently diluted 10 times with sterile Milli-Q water before use.

2.4.2. Preparation of Percoll Concentrations

The density gradients of the Percoll used in this thesis were 1.052, 1.063, 1.075, 1.085 and 1.122 g/ml. These density gradients were made up by diluting neat Percoll with 1.5 M NaCl, based on the calculation of the following formula (Percoll Booklet, Methodology and Applications, Pharmacia, 1985).

$$\mathbf{Vo = \{ V \times [p - (0.1 \times p_{10}) - 0.9] \} / (p_o - 1)}$$

Where:

Vo	= volume of the original Percoll	ml
V	= volume of the final working solution	ml

P	= desired density of the final solution	g/ml
P_o	= density of the original Percoll (1.130)	g/ml
P_{10}	= density of 1.5 M NaCl (1.058)	g/ml

The volume of 1.5 M NaCl used was 10% of the desired volume of working solution, and sterile Milli-Q water was added to raise the final volume to that of the working solution needed (see Table 2.1). All of the preparation was undertaken using sterile procedures.

Table 2.1 The actual amount of ingredients used to prepare the density gradient of Percoll for the final working solution of 40 ml.

Desired density (p) (g/ml)	Working solution (V) (ml)	NaCl (1.5 M) (ml)	Original Percoll (Vo) (ml)	Milli-Q water added (ml)
1.052	40.000	4.000	14.220	21.780
1.063	40.000	4.000	17.600	18.400
1.075	40.000	4.000	21.280	15.720
1.085	40.000	4.000	24.360	11.640
1.122	40.000	4.000	35.760	0.240

2.4.3. Preparation of the rat spleen cells

The spleen was aseptically removed from adult male DA rats euthanized as in 2.3.2, and placed in a petri dish containing RPMI 1640 (10 ml) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), heat-inactivated foetal bovine serum (10%), and L-glutamine (2 mM). This medium was designated as RPMI 1640 complete medium. The spleen was dissected and the contents

flushed into the petri dish by injecting medium into the spleen. The splenic cells were then transferred into a 12 ml tube, and allowed to stand for five minutes. The free cells were then placed in a 50 ml conical tube, (aggregated cells were discarded), and the tube was centrifuged at 500 x g for five minutes at 20° C. While the cells were being centrifuged, the lysing solution was made up by diluting the stock solution 1:10 in sterile Milli-Q water. After centrifugation the supernatant was removed and the cell pellet resuspended in two ml of the lysing solution. The rest of the lysing solution (8 ml) was then added and the tube was left to stand for exactly five minutes at room temperature.

RPMI 1640 (20 ml) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg /ml) (designated as RPMI 1640 without serum) was added, and the suspension was then centrifuged at 500 x g, 20° C for five minutes. The cells were washed once in 20 ml RPMI 1640 without serum, after which the cells were resuspended with one ml of RPMI 1640 complete medium. Finally the cells were counted and their viability was examined by assessing cell exclusion of 0.2% trypan blue (modified from Harlow and Lane, 1988) before undergoing further separation.

2.4.4. Generation of Discontinuous Gradients

The following procedures were modified from Gutierrez et al. (1979). Spleen cells (not exceeding 1 x 10^8 cells/ml) were centrifuged in RPMI 1640 complete medium in a 30 ml polysulfone Oak Ridge centrifuge tube. The supernatant was discarded and the pellet was resuspended in 2 ml of complete medium. A

discontinuous gradient was prepared by aspirating the lowest density gradient (1.052 g/ml, 4 ml volume) into the tube via a single lumen polyethylene tube.

Successive fractions of the higher density solutions (4 ml volumes, 8 ml for the 1.063 g/ml density gradient) were then added beneath the top layer, thereby preventing mixing of the gradients. The cell solution was then placed on top of the gradients (Figure 2.1). The gradient was centrifuged at 2000 x g in a bench-top centrifuge (Beckman GPR centrifuge, Beckman) for 10 minutes at 20° C.

The cells at the interface of each layer were carefully removed and washed twice in RPMI 1640 without serum. Cells remaining in the interface between 1.052 g/ml and 1.063 g/ml Percoll density, were designated as 'fraction I', cells between 1.063 g/ml and 1.075 g/ml Percoll density as 'fraction II', cells collected from the interface between 1.075 g/ml and 1.085 g/ml Percoll density as 'fraction III', and cells collected from the interface between 1.085 g/ml and 1.122 g/ml Percoll density were designated as 'fraction IV'. The cells were then prepared for further analysis and characterisation.

2.4.5. Cell viability

Cell viability of isolated T cells was more than 95% as determined by trypan blue exclusion and very few erythrocytes (less than 5%) were present after erythrolysis treatments. Cell viability was even higher following Percoll fractionation (> 98%).

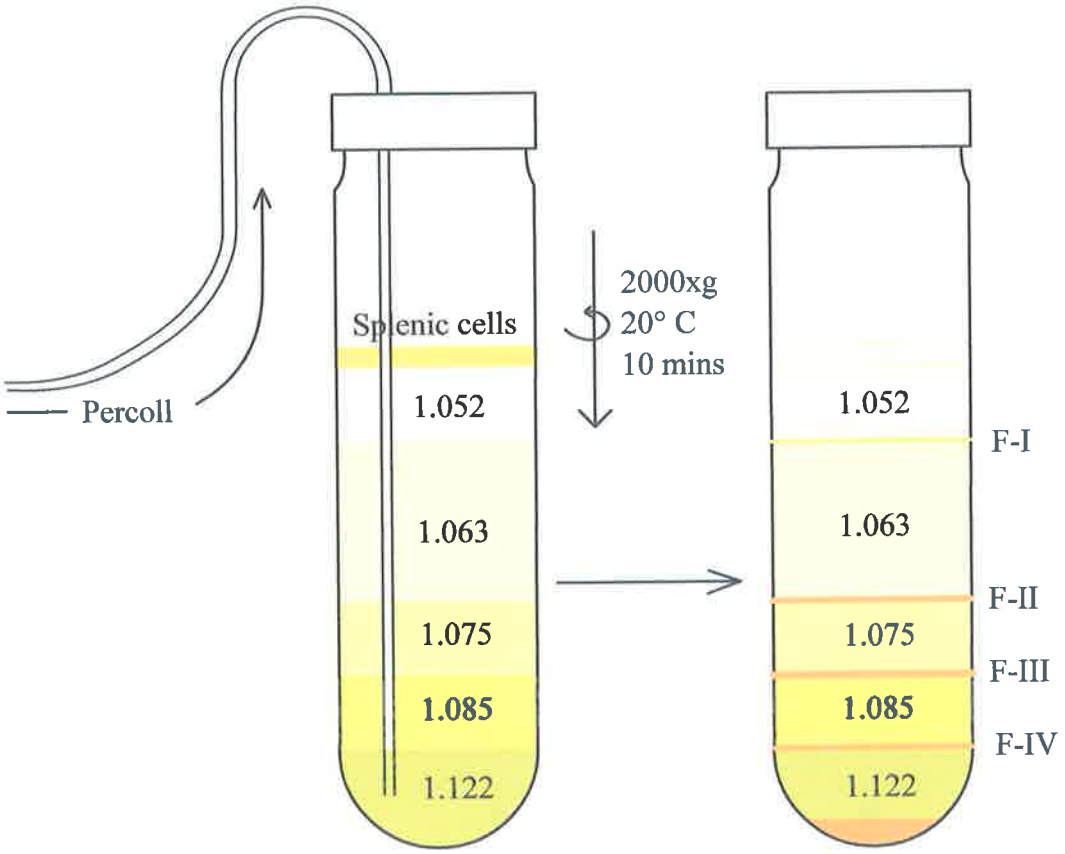


Figure 2.1 Illustration of separation of T and B lymphocytes from spleen cells on a discontinuous gradient of Percoll in the ranges of density 1.052-1.122 g/ml. F-I, F-II, F-III, and F-IV: Fractions I, II, III, and IV.

2.4.6. *Identification of the type of the splenic cells*

After separation on the discontinuous gradient, the five fractions, and an unpurified sample of splenic cells were stained for surface immunoglobulins with a method of single colour FACS labelling (Brideau et al., 1980) in order to determine the cellular constituents.

Six mouse-anti rat monoclonal antibodies were used to label the lymphocyte fractions, namely anti-α/β TcR (R73) (Hünig et al. 1989), anti CD4 (W3/25) (Williams et al., 1977), anti-CD8 (Ox8) (Brideau et al., 1980), anti- B cell (Ox33) (Woollett et al., 1985), anti-Mac-1 (CD11b; WT.5) (Springer et al., 1979), and anti-Giardia (IB5) as a negative control. This enabled identification and quantification of the cell types isolated in each fraction.

Each lymphocyte cell suspension (1 x 10^6 cells) was aliquoted (1 ml) into FACS tubes on ice. The cells were centrifuged at 250 x g at 4° C for seven minutes. Supernatants were completely removed using a Pasture pipette connected to a vacuum pump, and then desired monoclonal antibodies (50 µl each) were added to each pellet. The pellets were then resuspended by gently vortexing. The suspensions were incubated on ice and vortexed every 15 minutes. After 45 minutes, 3 ml of ice-cold PBS containing 1% FCS was added to each tube, the suspension resuspended and centrifuged as above. After removing the supernatants, the cells were washed two times in washing solution (PBS containing 1% FCS and 0.01% sodium azide).

Following these processes, the conjugate (50 µl, FITC-SHAM in a 1: 100 dilution with PBS supplemented with 1% NRS) was added, and the cells were

resuspended and incubated for one hour on ice. After incubation, the cells were washed twice with two millilitres of washing solution. Following the second wash, the pellets were resuspended in one millilitre of fixative (FACS fix, 1% (v/v) paraformaldehyde) solution and stored in a dark room at 4° C for 24 h. The cells were then resuspended and analysed by flow cytometry using a FACS Scan (Becton Dickinson, UK).

Fraction I and II were found to contain mostly B cells and macrophages. Fraction III contained mostly T cells (43.2%) which consisted of CD4 (34.4%) and CD8 (18.6%) positive T cells, B cells (36.5%) and macrophages (10 %) (Figure 2.2). The most "pure" T cells were observed in fraction IV which strongly expressed T cell receptors (76.2%) and which consisted of CD4 (63.6%) and CD8 (20%) positive cells, with 20% B cells, and 0.6% macrophages (see Figure 2.3). The higher proportion of $CD4^+$ T cells compared to $CD8^+$ T cells subset in this study is in complete agreement with the previous study of Westermann and Pabst (1992) who reported that in the blood and other connective tissues $CD4^+$ T cells subset represents the major lymphocyte subset.

2.4.7. Lymphocyte culture

In order to decide which fraction should be used for the lymphocyte proliferation assays in this study, the lymphocytes obtained from each Percoll density fraction and from unfractionated spleen cells were cultured in the presence of Concanavalin A (con A) and phytohemagglutination (PHA). Both of these mitogens are known to stimulate T-cell proliferation (Tizard, 1992).

Figure 2.2. Expression of surface markers on cells in Fraction III.

Freshly prepared fraction III splenic cells were incubated with indicated antibody and stained with FITC-conjugated second antibody prior to FACS analysis.

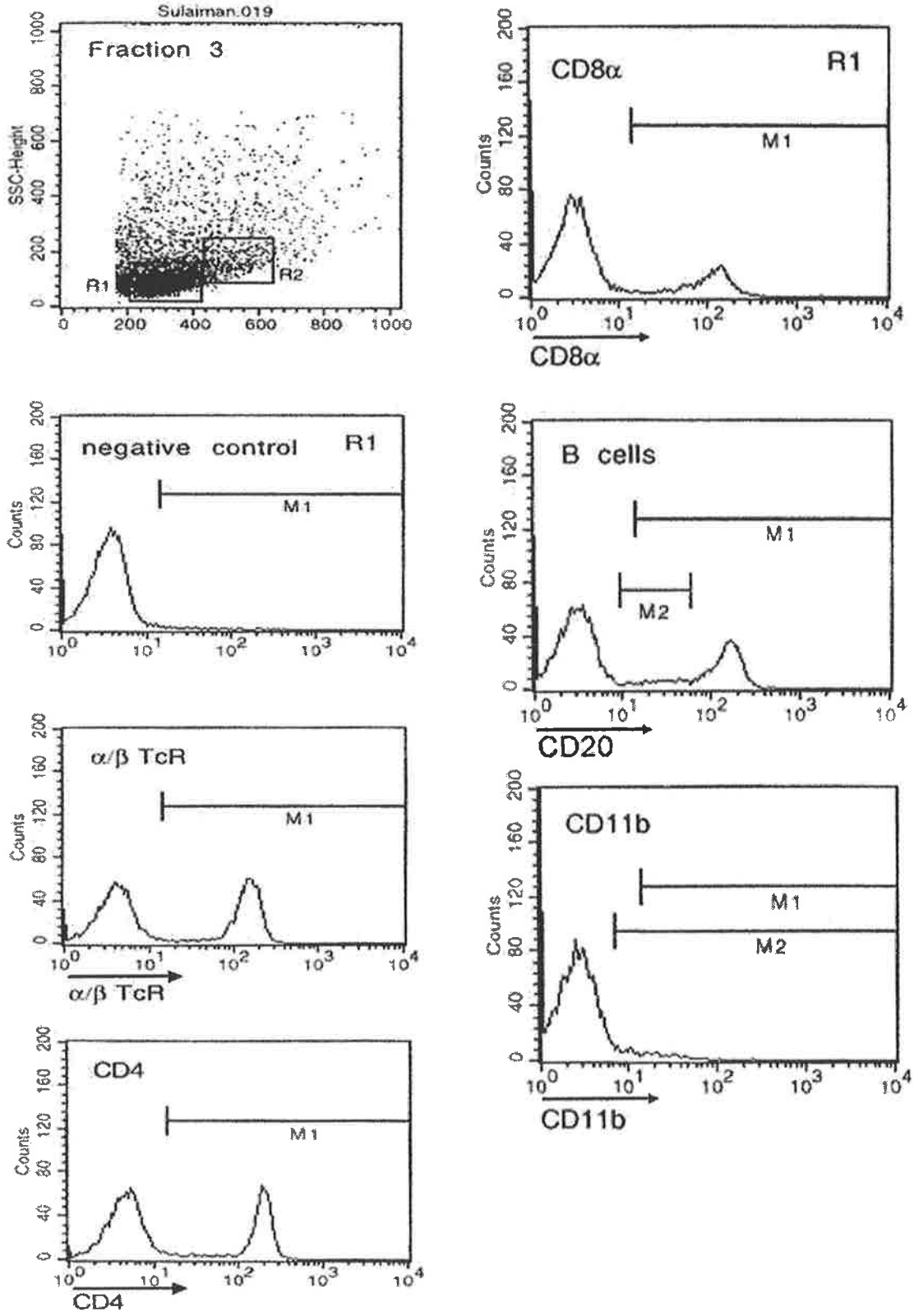


Figure 2.2 Expression of surface markers on cells in Fraction III.

Figure 2.3. Expression of surface markers on cells in Fraction IV.

Freshly prepared fraction IV splenic cells were incubated with indicated antibody and stained with FITC-conjugated second antibody prior to FACS analysis.

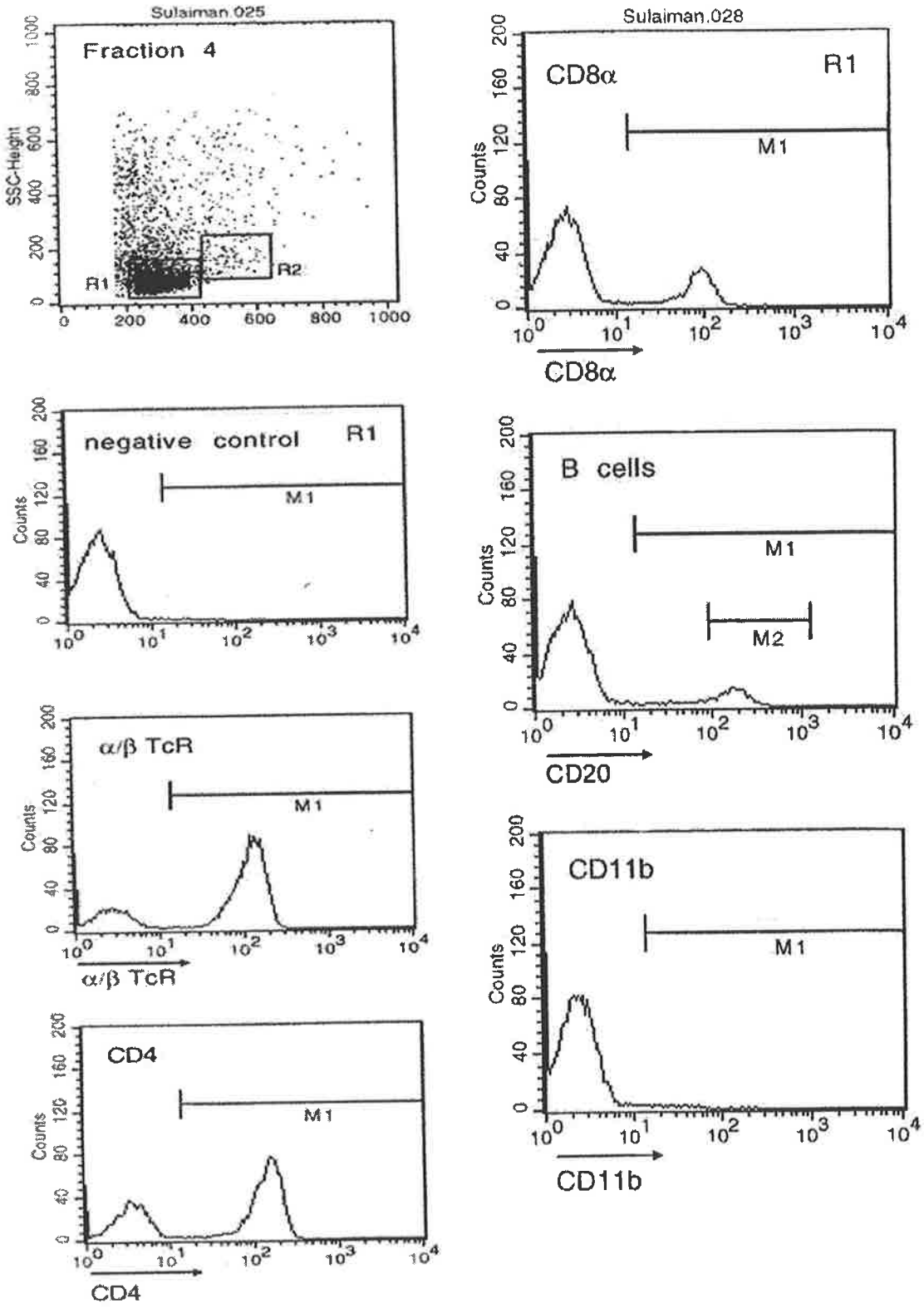


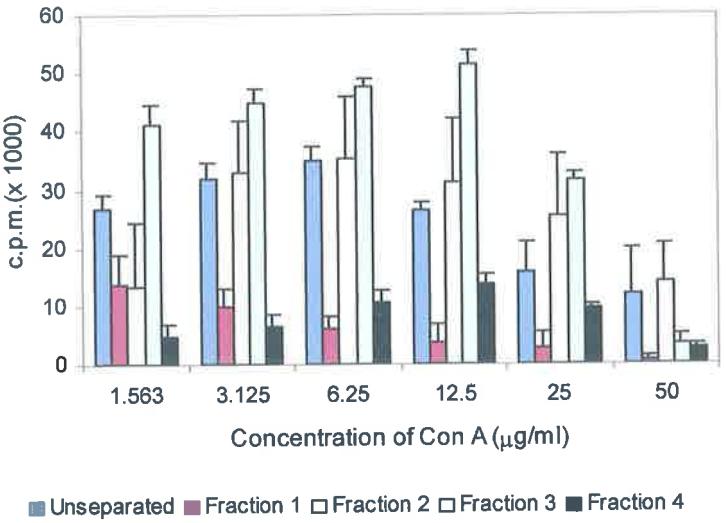
Figure 2.3 Expression of surface markers on cells in Fraction IV.

Lymphocyte fractions were cultured in standard 96-well titre plates with U-shaped wells (Falcon No 3077) as described by Pöllanen et al. (1988) with several modifications. The washed cells from each Percoll fraction and unseparated cells were first counted in 0.2 % trypan blue solution (Sigma) and diluted to 5 x 10^6 cells/ml in complete medium. A 50 µl-aliquot of the cell suspension of each fraction was then pipetted into each well of a culture plate.

The cells were stimulated by adding 50 µl of Concanavalin A (Con A) or PHA solution (various concentrations, 1.6 to 50 µg/ml) in RPMI 1640 without serum. An additional 50 µl-aliquot of RPMI 1640 complete medium was added to each well, and finally, 50 µl of RPMI without serum was added to each well for a final volume of 200 µl. Each treatment was cultured in triplicate and the assays were repeated three times. After 56 h of culture at 37° C in an atmosphere of 5% CO_2 in air, 0.25 µCi of [^{3}H] Thymidine in 25 µl RPMI without serum was added to each well. The cells were then harvested 16 h later onto glass fibre filter discs using a PHD cell harvester (Cambridge Technology Inc., USA). Radioactivity in the filter discs was measured using standard scintillation counting, in a 1215 Rackbeta II (LKB, Wallac, Findland) β-counter.

The combination of macrophages and lymphocytes in fraction III were found to give the optimum response to Con A and PHA while fraction IV which consisted of highly purified T cells and very few macrophages were poorly activated by the mitogens (Figure 2.4). Similar results have also been found by Gutierrez et al., (1979) and Mayrhofer et al., (1986) who revealed that

a)



b)

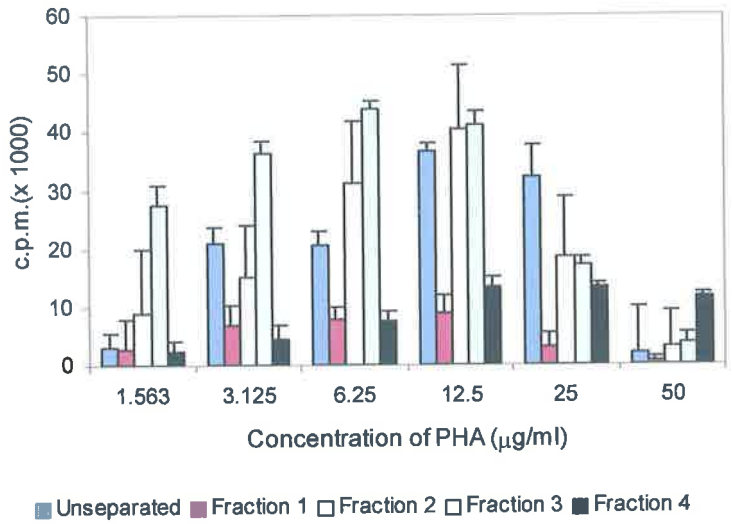


Figure 2.4 Response of unseparated, and fractionated rat splenic cells separated using a discontinues gradient of Percoll, to several concentrations of (a) Con A or (b) PHA. (Values are Mean ± SEM; n=3; c.p.m. = count per minute).

overly purified T cells respond poorly to T cell mitogens. It has previously been demonstrated that macrophages are needed to optimise mitogen-induced T-cell proliferation, and this may be the reason that fraction IV, which consisted of "pure" T cells, gave a poor response to both Con A and PHA mitogens.

In the studies reported in this thesis, the lymphocytes collected from fraction III (designated as "splenic T cells") were used for the lymphocyte proliferation assay to examine the immunosuppressive activity of the rat testicular extracts or of other extracts or substances used in relation with this research.

2.5. Measurement of protein concentration

A protein determination method developed by Bradford (1976) was used to measure the protein concentrations in the samples used in this study. This method involves the binding of an acidic solution of Coomassie Brilliant Blue G-250 to protein. The binding causes a shift in the absorbance maximum from 465 to 595 nm (Reisner et al., 1975; Sedmak et al., 1977).

2.5.1. Preparation of stock protein reagent

Coomasie Brilliant Blue G-250 (100 mg, Sigma) was dissolved in 95% ethanol (50 ml). Into this solution concentrated phosphoric acid (100 ml, BDH AnalaR) and Milli-Q water (50 ml) were added to make a final volume of 200 ml. The solution was transferred to a dried cleaned bottle, wrapped in aluminium foil to protect from light and stored at 4° C until use. For use the stock solution was diluted 1 in 5 with phosphate buffered saline and warmed to room temperature.

2.5.2. *Preparation of protein standards*

Bovine serum albumin (BSA) was used as a protein standard in this experiment. Twenty mg of BSA was dissolved gradually in PBS to a final volume of 20 ml (1 mg/ml) stock. The stock solution was filtered using a 0.45 μm membrane and aliquots of the solutions were stored at -20° C until use.

2.5.3. *Microprotein assay*

Standard solutions were prepared by thawing the stock solution and diluted serially from 25 to 200 μg/ml in phosphate buffered saline. From these solutions, 100 μl of each was pipetted into 12 x 100 mm dried, cleaned test tubes. The reagent blank was prepared by pipetting PBS (100 μl) into a similar test tube. Into each tube, diluted protein reagent (one ml) was added and mixed gently by vortexing. Between 5 and 30 minutes after vortexing, the absorbance at 595 nm was measured using a spectrophotometer (Shimadzu, Japan) and one millilitre quartz cuvettes read against the reagent blank. BSA or insulin standard curves were constructed (Figure 2.5) and used to determine the high (BSA) or low (insulin) molecular weight protein respectively, contained in the testicular extracts or the other solutions used in relation with this study.

2.6. Threshold toxicity assays for trypsin, proteinase K or 4-[2-aminoethyl] benzene sulfonyl fluoride

To allow studies to be undertaken on the effect on testicular extracts (TE) of treatment with trypsin and proteinase K, the proteases and their inhibitor, proteinase K inhibitor 4-[2-aminoethyl] benzene sulfonyl fluoride (AEBSF), were first tested to ascertain their threshold toxicity or "safety" level. This is

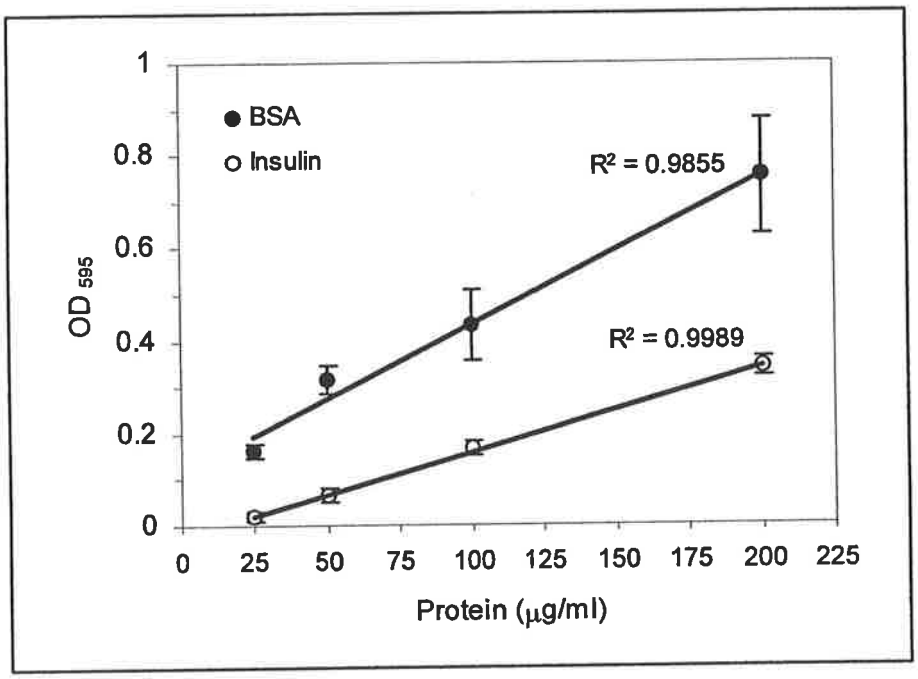


Figure 2.5 Standard curve for the protein estimation based on the method developed by Bradford. (Values are Mean ± SEM; n=3).

the level where the protease or their inhibitors give no cytotoxic effects on the lymphocyte cultures (≥ 50% Con A only response, see Figure 2.6) but retain their proteolytic/inhibitory activities.

For this purpose, trypsin was dissolved in RPMI 1640 medium without serum to a concentration of 125 μg/ml. The solution was then serially diluted with the same medium to 8 μg/ml of trypsin. After that 50 μl of each trypsin concentration was pipetted into wells of a U-bottomed culture plate (Falcon No. 3077). Proteinase K or AEBSF was also dissolved in RPMI 1640 without serum to a concentration of 200 μg/ml. This solution was then serially diluted to 6.25 μg/ml, after which 50 μl of each diluted solution of proteinase K or AEBSF was pipetted into wells of a U-bottomed culture plate.

Into the dilutions of trypsin, proteinase K, AEBSF or medium alone as control, 50 μl of a solution containing $5x10^6$ splenic T cells/ml in RPMI 1640 complete medium were added. Next, Concanavalin A (50 μl, 2.5 μg/ml) in RPMI 1640 without serum was added to the wells. Finally, the volume was made up to 200 μl/well by adding 50 μl of RPMI 1640 medium containing 10% FCS (see 2.4.7).

The ID_{50} values were accounted as safe for assays and were still effective for proteolytic treatments (see Stellrecht et al., 1997, Sun et al., 1998). It was found that ID_{50} of trypsin, proteinase K, and AEBSF were in the concentrations of 20 μg/ml, 10 μg/ml, and 50 μg/ml respectively (Figure 2.6). These concentration are comparable to those of commonly used by other groups (Kirkpatrick et al., 1985, Sung et al., 1989, and Stellrecht et al., 1997).

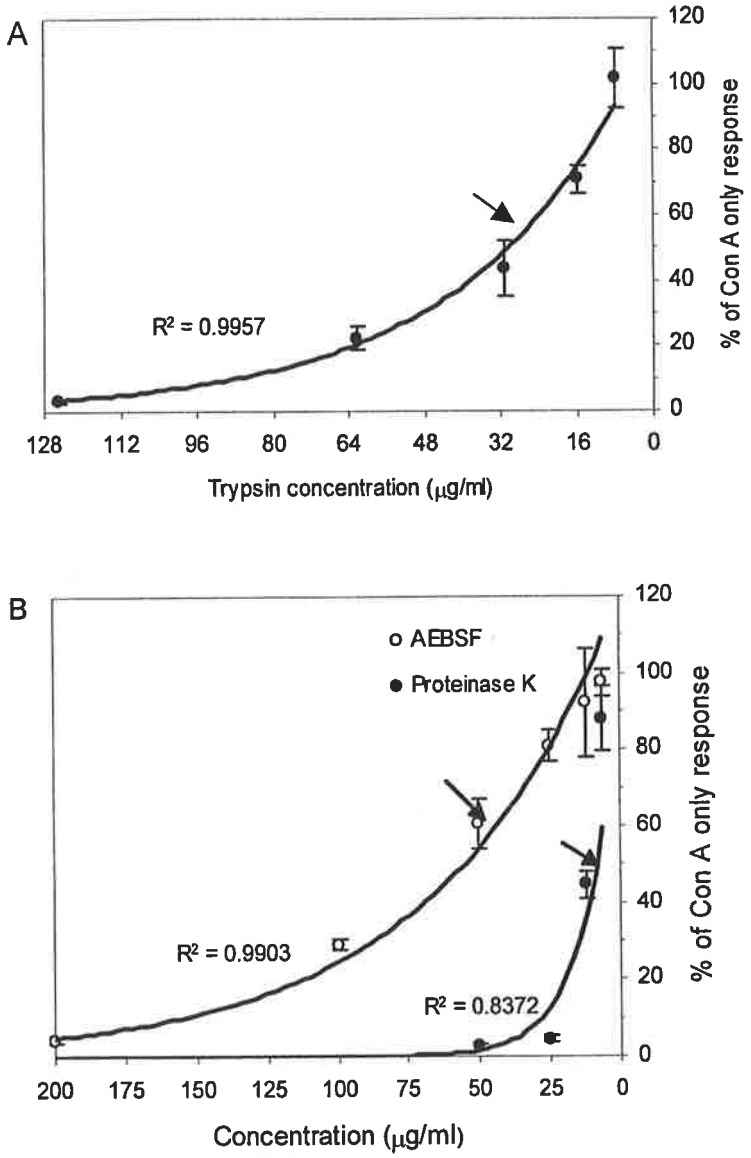


Figure 2.6 Standard curves for the estimation of maximal non-toxic level of (a) trypsin or (b) proteinase K and AEBSF in the lymphocyte proliferation assays. The non-toxic level of trypsin (20 μg/ml), proteinase K (10 μg/ml), and AEBSF (50μg/ml) chosen in this studies is indicated with an arrow. (Values are Mean ± SEM; n = 2).

2.7. Cytotoxicity assay based on Colorimetric MTT assay

In conjunction with the lymphocyte proliferation assays, a cytotoxicity assay was conducted using Rat-2 fibroblast cells to examine the potential cytotoxicity of the testicular extracts. As Rat-2 fibroblasts lack appreciable levels of nuclear thymidine kinase, measurement of the proliferation of the cells using [^{3}H] thymidine incorporation is impractical. For this reason a quantitative colorimetric assay based on the reduction of the tetrazolium salt (MTT) as developed by Mosmann (1983) was utilised.

Rat-2 fibroblast cells were cultured in several different concentrations (5×10^6 to 6.25×10^4 cells/ml) in the wells of a 96-well flat-bottomed plate (Falcon). Starting at 5×10^6 Rat-2 cells/ml fibroblasts were plated out in serial dilutions in 50 µl RPMI 1640 complete medium. The final volume of each well was made up to 200 µl by adding 50 µl of RPMI 1640 complete medium and 100 µl RPMI 1640 without serum into each well. The cells were cultured for 72 hours at 37° C in a 5% CO_2 in air atmosphere. Each culture was undertaken in triplicate and the assays were repeated four times.

Four hours before harvesting the cells, 20µl of stock MTT solution (5 mg/ml; sterilised by filtering the solution through 0.22 µm porous filter) was added to each well.

Four hours after incubation, the plates were centrifuged at 1000 x g for five minutes and the supernatants from each well were discarded. Acid-propanol (propanol containing 0.04 N HCl, 100 µl) was then added to each well. The solution in each well was mixed with a multichannel pipetter to uniformly

dissolve the blue formazan crystals and the plates were read on a Titertek Multiscan microplate reader (Flow Laboratories, North Ryde, Australia) to determine the absorbance for each well at 570 nm.

From Figure 2.7 it can be seen that after 72 h of culture, the optimum proliferation of Rat-2 fibroblast cells in this assay was achieved when the concentration of the cells was 5 x 10^6 cells/ml. For the purpose of the cytotoxicity assay in this thesis, the concentration of Rat-2 cells used was 2.5 x 10^6 cell/ml which was about 50% of the optimum proliferation.

2.8. Statistical Analysis

Data from all experiments were analysed by analysis of variance (ANOVA) by employing a computer program *SuperANOVA* developed by Abacus Concepts, Inc., Berkeley, California. Student's *t* test was used for single pairwise comparisons, and where appropriate, Duncan's New Multiple-Range test was employed for multiple comparisons. Values of $P>0.05$ were not considered significant.

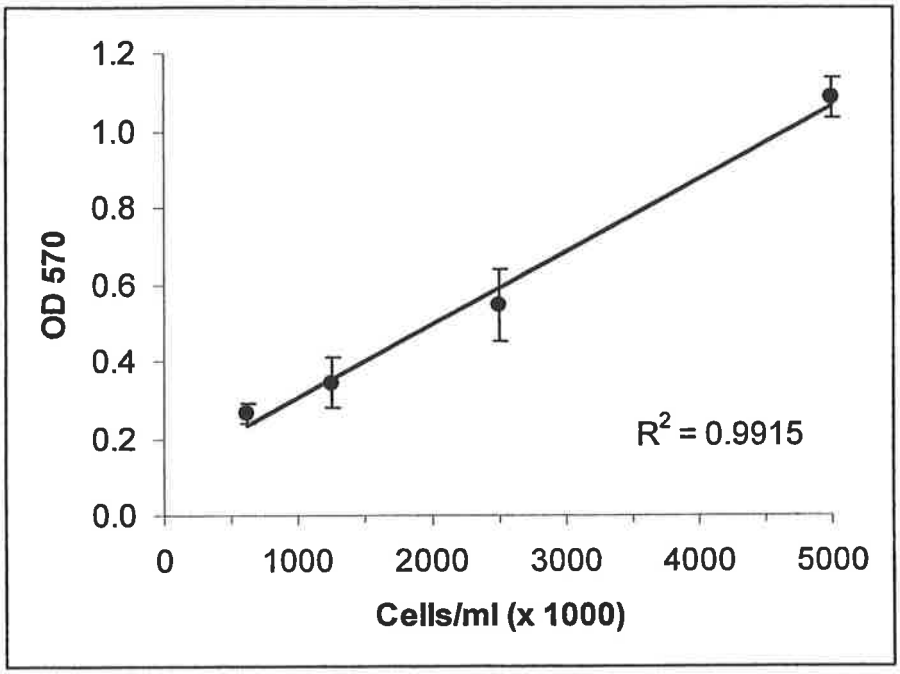


Figure 2.7 MTT cleavage by several different concentrations of Rat-2 fibroblast cells after 72 h incubation at 37° C as determined by absorbance measured at 570 nm. (Values are Mean ± SEM; n = 4).

3. Partial Characterisation of Immunosuppressive Activity in the Crude Extracts of the Rat Testis

3.1. Introduction

As reviewed in chapter one, the testicular interstitial tissue has long been recognised as an immunologically privileged site (Maddocks and Setchell, 1990). In this site, auto-, allo-, and even xenografts of various endocrine tissues survive for extended periods, and some times indefinitely unless the host has been pre-sensitised to the antigen elsewhere in the body (Ferguson and Scothorne, 1977). The mechanism of transplant survival in this area is still obscure, but a number of studies have indicated that substances present in the male reproductive tract can inhibit mitogen-induced lymphocyte proliferation *in vitro* (Hurtenbach et al., 1980, 1982; Head and Billingham, 1985; Pöllänen et al. 1988). The local secretion of immunosuppressive products in the testis has been reviewed extensively by Pöllänen et al., (1990). In addition, Maddocks and Setchell (1990) suggested that such substances could play an important role in the immune privilege of the testis.

Based on the studies undertaken to date it has been suggested that immunosuppressive activities are present in the rodent testis, in both the interstitial fluid and homogenate of rat testis (Pöllänen et al., 1988, 1989a, b, 1992, and Sainio-Pöllänen et al., 1991), and in the interstitial fluid, seminal vesicle fluid, and the extract of mouse testis (Anderson and Tarter, 1982; Emoto et al., 1989, 1990a,b, and 1991). Boar seminal plasma has also been

reported to contain immunosuppressive activity both *in vitro* and *in vivo* (Stanek et al., 1985; Dostal et al., 1995, and Veselský et al., 2000 and 2001).

It is clear that components of the testis, especially the rodent testis produce immunosuppressive factors. Immunosuppressive molecules have also been reported to be produced by isolated mouse (De Cesaris et al., 1992), and rat (Wyatt et al., 1988; Martinova et al., 1993) Sertoli cells in culture. Expression of Fas ligand (FasL) by testicular tissue has been suggested to be responsible for protection of mouse testicular (Bellgrau et al., 1995) and islet (Takeda et al., 1998) allografts from rejection. Suarez-Pinzon et al., (2000) have more recently reported that TGF-β1, not FasL, produced by mouse Sertoli cells, protects islet β-cells from destruction. Taken together, these reports suggest that the nature and characteristics of the immunosuppressive factors, and the mechanisms involved in their immunosuppressive actions are still somewhat obscure. Studies on the immunosuppressive activity in crude rat testicular extract, have therefore been conducted as a first step to characterise the crude immunosuppressive activity in order to design a purification scheme to elucidate the nature of the immunosuppressive factors. In this chapter results on the partial characterisation of the immunosuppressive activity in the crude rat testicular extract are reported.

It is worth noting here that some of the experiments carried out through this chapter are similar to those reported previously by Pöllänen et al., (1992). At the time this present study was commenced this work was just beginning to be reported and some benchmarking was considered necessary and appropriate to

establish complimentarily and/or differences between two laboratories. In 1993 Pöllänen and his co-workers suggested that the immunosuppressive factor(s) in the rat testis was a TGF-β like substance, the immunosuppressive activities of which could be abrogated by an anti-TGF-β antibody. However further studies regarding this TGF-β-like factor have been limited, and as will be discussed, the present investigations pursued somewhat different interests.

3.2. Experimental procedures

3.2.1. Suppressive activity of crude testicular extract compounds

3.2.1.1. Testicular extract and splenic lymphocyte preparations

The testicular extracts (TE) used in the following experiments were prepared as described in chapter 2.3.1. The splenic T lymphocytes, as the target for the TE immunosuppressive activity studies, were prepared as described in section 2.4.

3.2.1.2. Lymphocyte proliferation assays

The lymphocyte proliferation assays were performed as follows. The prepared splenic lymphocytes were suspended at 5 x 10^6 cells/ml in RPMI 1640 complete medium. Aliquots of the cell suspension (50 µl) were pipetted into wells of a standard 96-well culture plate with U-shaped wells (Falcon No 3077). Cell proliferation was induced by addition of a sub-optimal concentration of Concanavalin A (Con A, 2.5 µg/ml, 50 µl/well) solution in RPMI 1640 without serum (see 2.4.7 and Figure 2.4.a).

The crude testicular extracts were diluted to 1:2 - 1:16 in RPMI 1640 without serum and the diluted extract then added to the wells (50 µl/well). Some wells

were left with the addition of mitogen only, without testicular extracts, and some with neither mitogen nor TE as controls. Finally, the volume of each well was made up to 200 µl by adding the complete medium (50 µl) to each well. Each culture was performed in duplicate or triplicate. After 56 hours of culture at 37° C in an atmosphere of 5% CO_2 in air, 0.25 µCi of [^{3}H] Thymidine in 25 µl RPMI 1640 without serum was added to each well. The cells were then harvested 16 hours later onto glass fibre filter discs using a PHD cell harvester (Cambridge Technology Inc., Watertown, MA, USA). Radioactivity in the filter discs was measured using standard scintillation counting on a 1215 Rackbeta II (LKB, Wallac, Finland) β-counter.

3.2.2. Effects of timing of TE addition to Con A-induced lymphocyte proliferation

Plant lectins, such as Con A and PHA have the ability to stimulate T cell proliferation. Crabtree (1989) suggested that during stimulation, the T cell undergoes morphologic changes (blastogenesis) at about 12 h, and divides by 24 to 48 h. To observe at which stage the TE inhibits the lymphocyte proliferation after Con A stimulation, the effect of delayed addition of TE on Con A-stimulated splenic T lymphocytes in culture was conducted. Diluted TE (1:4, 50 µl/well) was added to cultures of splenic T cells at various times (0, 12, 24, 36 and 48 hour) after Con-A addition. Cells were cultured for a total period of 72 h, and proliferation then assessed as in 3.2.1.2.

3.2.3. Effects of preincubation of splenic T cells with TE

Anderson and Tarter (1982) suggested that suppression of mitogen-induced T cell proliferation by mouse seminal plasma (SP) was due to the blocking of

mitogen activity, either by direct binding of SP components to mitogens, or blocking of mitogen binding on the lymphocyte surface. In addition to the observations conducted in 3.2.2, and in order to investigate whether the mechanism of TE immunosuppressive activity was as a direct effect on the T cell, the splenic T cells were pre treated with TE for 60 minutes, before the Con A stimulation.

Splenic T cells (500 µl, 5 x 10^6 cells/ml) in RPMI 1640 complete medium were incubated with crude TE (1:4 dilution in PBS, 500 µl) or with PBS alone (500 µl, as control) for 60 minutes at 37° C. Cells were then washed twice with one millilitre of RPMI 1640 without serum and adjusted to 5 x 10^6 cells/ml in RPMI 1640 complete medium. The cells then plated out into the U-shaped wells of a 96-well culture plate (50 µl/well) and cultured to assess lymphocyte proliferation as above (3.2.1.2). Assays were carried out in triplicate cultures.

3.2.4. Effects of Temperature and pH on the Crude TE Immunosuppressive activity

There are strong indications that immunosuppressive activity in the rat testis is a protein moiety (Pöllänen et al., 1992). In general, proteins can be denatured by heat and by treatment with extreme pH (acidic or alkaline), which usually changes the biological activity of the proteins (Wilson, 1994). To determine whether the immunosuppressive activity of TE was affected by temperature and pH changes, the TE was treated with several different temperatures and pH exposures (see below).

Aliquots of crude TE were heated for 10 minutes in a water bath at the temperature of 25°, 56°, 80° or 100° C, and then cooled at 4° C for 15 minutes. Each fraction was then centrifuged at 1000 x g for 10 minutes. Additional aliquots had their pH lowered to 1.9 using 1 M HCl or raised to 11.2 using 1 N NaOH, or kept at pH 7.2 with equivalent amounts of Milli-Q water added for controls. These aliquots were incubated at room temperature for 60 minutes, and then centrifuged at 1000 x g, 4° C for 10 minutes. The supernatant of each portion was then collected and neutralised to pH 7.2 using 1 N NaOH or 1 M HCl as appropriate. pH neutral controls were again treated with comparable volume of Milli-Q water.

Samples were then filtered using 0.22 μm filter membranes. Total protein concentrations were measured using the Bradford method (see 2.5), and immunosuppressive activities were tested (see 3.2.1.2).

3.2.5. Charcoal treated TE

In order to eliminate the possibility that the suppression of Con A-induced splenic T cells proliferation was due to steroids present in the TE, the TE was treated with dextran-coated charcoal to remove the steroids. The following procedure modified from Pöllänen et al. (1988), was applied.

The following ingredients were dissolved in 100 ml Milli-Q water and stirred on ice:

Dextran T-20	0.028 g
Activated charcoal	0.100 g
Tris-HCl	2.000 g
EDTA	0.300 g
Milli-Q water (on ice)	1000.000 ml

While the solution was being stirred continuously, a 10 ml aliquot was transferred into fresh Milli-Q water (90 ml) to a final dilution of 1:10 (v/v) and stirred continuously on ice. While this solution was being stirred, a one millilitre aliquot was removed and centrifuged at 1500 x g for 20 minutes at 4° C. The supernatant was then removed and the pellet mixed carefully with TE (2 ml). This solution was then incubated at 20° C for 60 minutes with continuous shaking. Following incubation, the solution was centrifuged at 1500 x g for five minutes at 4° C, and the supernatant collected. The whole procedure was repeated twice in an attempt to remove as much steroid as possible (Pöllänen et al., 1988). The supernatant was then filtered sterile with 0.2 μm filter, aliquoted and stored for further assay or treatments.

The efficiency of the removal process was assessed by measuring the testosterone concentration in the TE before and after charcoal treatments using a Diagnostic Systems Laboratories (DSL, Webster, TX) double-antibody ^{125}I radioimmunoassay (RIA) test kit (DSL-4100). The assay followed the manufacturer's recommended protocol for *in vitro* diagnostic use. All samples were measured in one assay in triplicates with internal variation of 7% and the assay sensitivity was 0.4 nmol/ml. The immunosuppressive activity of treated and untreated TE was measured using the procedure outlined in 3.2.1.2.

3.2.6. Species dependence of TE immunosuppressive activities

In order to determine whether the rat TE immunosuppressive activity was species specific, the rat testicular immunosuppressive activity was tested in vitro against lymphocytes of cows, sheep, and pigs, isolated from freshly drawn venous blood by density centrifugation of blood on Ficoll-Paque (Pharmacia, Biotech, Uppsala, Sweden); and lymphocytes isolated from the mouse spleen as in 3.2.1.2. Isolation of lymphocytes on Ficoll-Paque was carried out from ten milliliters of heparanized blood obtained from each animal and diluted with an equal volume of sterile PBS. Diluted bloods were then layered onto 10 ml aliquots Ficoll-Paque in 50-ml conical tubes and centrifuged at 400xg for 20 minutes at 20°C. Lymphocytes were then gently removed from the plasma/Ficoll interface, transferred to sterile tubes and washed twice with 10 ml sterile PBS before being aliquoted out for culture with TE as in 3.2.1.2.

3.2.7. Proteolytic (Trypsin and proteinase K) treatment of TE

Trypsin is a protease usually used in protein structure elucidation and sequence analysis. This serine endopeptidase specifically hydrolyses proteins and peptides at the C-terminal side of lysine and arginine residues. The activity of this protease can be terminated or inhibited by trypsin inhibitors. One of the inhibitors is known to be present in foetal bovine serum (Davis et al., 1986), therefore the effects of trypsin in culture can be inactivated when the culture medium contains serum.

Proteinase K is another protease usually used for total degradation of proteins during the isolation of DNA or RNA. It is also used for the specific modification of proteins and glycoproteins on cell surfaces. It has broad specificity cleaving peptide bonds primarily after the carboxyl group of N-substituted, hydrophobic amino acids. Proteinase K can be inactivated by protease inhibitors such as diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), or 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF). AEBSF is the least toxic yet has comparable inhibitory activity (Boehringer Manheim, 1996).

To assess the nature of the immunosuppressive activity in the crude TE, the TE was treated with both proteases. Medium containing FBS (10%) and AEBSF were used to inhibit trypsin and proteinase K activity respectively.

Neat TE was aliquoted (500 µl) into a 2.5 ml Eppendorf tube and trypsin (20 µg/ml, 500 µl) or proteinase K (20 µg/ml, 500 µl) was added, shaken gently then incubated for 60 minutes at 37° C in an atmosphere of 5% CO_2 in air. The reaction was terminated by adding RPMI 1640 containing FBS (10%, 500 µl; trypsin-treated cultures) or AEBSF (50 µg/ml, 500 µl; proteinase K-treated cultures) in RPMI 1640 without FBS. After a brief shaking, the solutions were incubated for another 10 minutes, and the volume of each solution was then made up to 2 ml by adding RPMI 1640 medium without FBS (500 µl). The final concentration of each sample or protease or inhibitor was a 1:4 dilution of the starting concentration, which was known to be 'safe' for the lymphocyte cultures (see 2.6 and Figure 2.6.a and b). The solutions were then centrifuged

for 30 seconds at 1000 x g using a Micro Centaur (SANYO) centrifuge, and cooled to 4° C.

For controls, medium without FBS and containing no sample, or containing trypsin with- or without medium supplemented with FBS (10%), or containing proteinase K with-or without AEBSF, or containing AEBSF alone were also included in the experiments.

The supernatants of each solution including controls were tested for their immunosuppressive activity. From each supernatant, 50µl of the solution was pipetted into each well of a U-shaped culture plate, which contained splenic T cells for the lymphocyte proliferation assays as described above (3.2.1.2). The assays were repeated from three different cultures of triplicate wells unless otherwise stated.

3.2.8. Ammonium sulphate precipitation

Ammonium sulphate is commonly used to differentially precipitate proteins in solution. Harlow and Lane (1988) explain briefly the mechanisms of protein precipitation by ammonium sulphate. In solution, proteins form hydrogen bonds with water through their exposed polar and ionic groups. The water molecules can be removed from the protein in the presence of high concentrations of small, highly charged ions such as ammonium sulphate. This decreases the protein solubility, resulting in precipitation. This precipitate can be then readily redissolved and in general, retain its native protein conformation and activity. The concentration at which proteins will precipitate depends on the structure of the protein.

In this study crude testicular extracts (15 ml) were treated with four different final concentrations of ammonium sulphate (10, 20, 30 and 40 per cent, w/v). The TE was brought primarily to 10% (w/v) saturated ammonium sulphate by adding the solid salt (5.6 g/100 ml) gradually, stirred for 30 minutes on ice, and centrifuged at 3000 x g for 30 minutes at 4° C and the first supernatant was then carefully recovered. With a similar procedure, the secondary precipitations of 20, 30 or 40% (w/v) saturated ammonium sulphate were obtained from the resultant supernatants by adding 5.7, 5.9, or 6.2 g solid salt/100 ml respectively.

The resultant precipitates were then dissolved in one third of the starting volume using phosphate-buffered saline (PBS, 5 ml, pH 7.3) then transferred to dialysis tubing with molecular weight cut off <12 kDa and dialysed against four changes of PBS overnight at 4° C. In conjunction with this, crude TE (5 ml) was also dialysed in a similar manner to the treated TE as a control.

After dialysis, the solutions were removed from the tubing and clarified by centrifugation at 3000 x g for 30 minutes at 4° C. Next, the supernatants were collected and filtered using 0.45 μm filter membranes and the protein concentration of each fraction was determined using the method of Bradford (see 2.5). Finally, the treated fractions and controls were stored at -20° C until their immunosuppressive activity could be assessed (see the procedure in 3.2.1.2).

3.2.9. Suppressive activity in normal rat serum and tissues other than testis

In order to determine whether the immunosuppressive activity was present in serum or in tissues other than the testis, peripheral blood serum and extracts of rat brain, kidney and liver (see 2.3.2 and 2.3.3) were tested for their suppressive activity of lymphocyte proliferation. The assay procedure applied for this purpose was similar to the assay described for the testicular extract (see 3.2.1.2), and for every assay, TE was also included for comparison.

3.2.10. Assay for IL-2 activity

Proliferation of T cells following activation, such as by dendritic cells, antigens or mitogens, is critically dependent upon IL-2. This cytokine, which is produced at the time of T cell activation, acts only on cells that express high affinity IL-2 receptors, not on resting T cells. The IL-2 receptor is also expressed by activated T-blast cells in order to allow them to further respond to IL-2 produced by the cell itself or by another T-cell subset, which in turn stimulates T cell proliferation resulting in clonal expansion of the specific T cells (reviewed from Austyn and Wood, 1993, and Roitt, 1994, see also Quemeneur et al., 2002 and Fabbri et al., 2003). According to Gearing and Bird (1987), optimum IL-2 production *in vitro*, can be achieved 40-48 h after the activation of T cells with mitogens. The IL-2 production can then be tested using a bioassay by measuring the increase in proliferation of an IL-2-dependent cell line such as CTLL, or of a short term-lymphoblast (Gearing and Bird, 1987).

To investigate whether the immunosuppressive activity of TE in suppressing Con A-induced splenic T cell proliferation was effected through the suppression of IL-2 production, the following experiment was conducted.

Supernatants from inactivated or activated (2.5 μg/ml of Con A) splenic T cells cultured for 48 h with or without TE (1:2 dilution) were collected. The supernatants were tested for IL-2 activity by their ability to support the growth of the IL-2-dependent cell line CTLL, in a proliferative assay as previously described by Chen et al., (1993). The CTLL cells were cultured in DMEM containing FBS (10%), glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml), and β-mercaptoethanol (5×10^{-5} M), designated as DME-10, with 20 U/ml IL-2.

Prior to assay, CTLL were washed three times in PBS and resuspended at a density of 3.3×10^4 cells/ml in DME-10 without IL-2. The supernatants to be tested (50 μl each) were added into 96 well culture plates and the CTLL suspension (150 μl, 5000 CTLL/well) was added to the supernatants. As controls, CTLL cells were also cultured with, or without the addition of IL-2 (50 μl/well, 20U/ml). After 18-24 h of co-culture, the CTLL were pulsed with ^{3}H-Thymidine (1 μCi/well) and incubated for a further 6 h. The CTLL cells were then harvested and the ^{3}H-thymidine incorporation of the cells was measured as in 3.2.1.2. Assays were done in triplicate cultures.

3.2.11. Neutralisation of IL-2 activity using anti recombinant rat IL-2 antibody

In addition to the IL-2 activity assay, the effects of neutralizing IL-2 by anti recombinant rat IL-2 antibody were also investigated. Isolated T cells were

firstly induced with 2.5 μg/ml Con A overnight, then washed three times with PBS before treating with recombinant rat IL-2 (20 ng/ml) and diluted (1:4) TE. In conjunction with this experiment, anti rrIL-2 antibody was also applied to the cultures at a concentration of 5 μg/ml; a concentration established in preliminary investigations as providing 50% inhibition of T cells proliferation in response to 50 ng/ml IL-2. Cell proliferations were assessed as described in 3.2.1.2.

3.3. Results

3.3.1. Suppressive activity of the crude testicular extract

Crude testicular extracts significantly suppressed either Con A (2.5 μg/ml) or PHA (5 μg/ml) induced splenic T lymphocyte proliferation in a dose dependent manner (Figure 3.1 a and b). This suppressive activity was not due to the presence of cytotoxic agents in the TE, since the cell viability was not affected ($p>0.05$) by varying concentrations of TE after 24 h of culture (see Table 3.1).

3.3.2. Effects of delayed addition of TE to Con A-induced lymphocyte proliferation

The effects of the addition of TE at various times after initiation of splenic T cells activation with Con A, are presented in Figure 3.2. Optimal suppression could be achieved when the TE was added at the initial time of stimulation. Delay of TE addition reduced the suppressive activity. Furthermore, preincubation of the lymphocytes with TE for one hour followed by washing, adjustment of the cell concentrations, and culture in the presence of Con A maintained a state of suppressed immune responsiveness (Figure 3.3). These

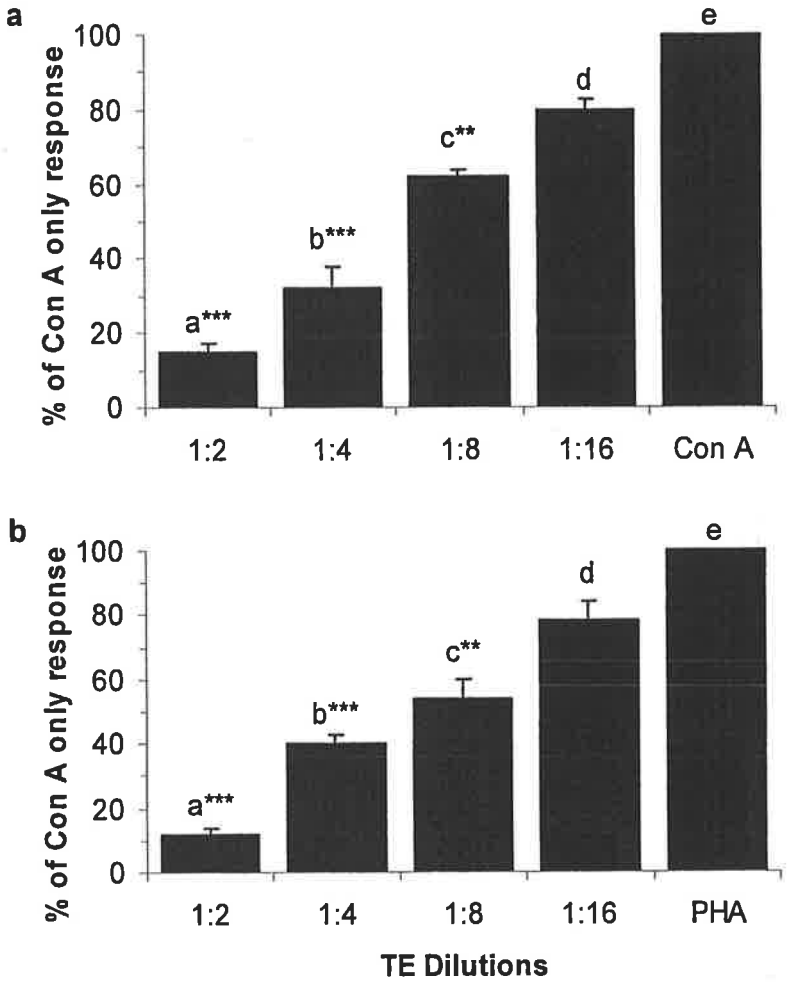


Figure 3.1 Effects of crude rat testicular extracts on the proliferative response of splenic T cells to (A) Con A or (B) PHA.

Splenic T cells (5x106/ml) were cultured with Con A (2.5 μg/ml) or PHA (5 μg/ml) for 72 h. Bars are the mean ± SEM of (A) 5 or (B) 4 separate cultures. Values with different superscripts show significant differences at $P<0.05$ between doses of TE or significant differences at **: $P<0.01$; ***: $P<0.001$ when compared with the mitogen only response (100%) tested with Duncan's new multiple-range test.

Table 3.1 Viability, as measured by 0.4% (v/v) trypan blue exclusion, of TE-treated splenic T lymphocytes cultured for 24 h.

Culture condition	Cell viability (%)
Cell only	93.11±1.20
Cell + Con A	95.22±1.35
Cell + TE (1:2) + Con A	93.79±1.02
Cell + TE (1:4) + Con A	94.57±0.36
Cell + TE (1:8) + Con A	92.48±1.04
Cell + TE (1:16) + Con A	93.15±1.07

Data were derived from three separate cultures of duplicate wells. Values are Mean ± SEM.

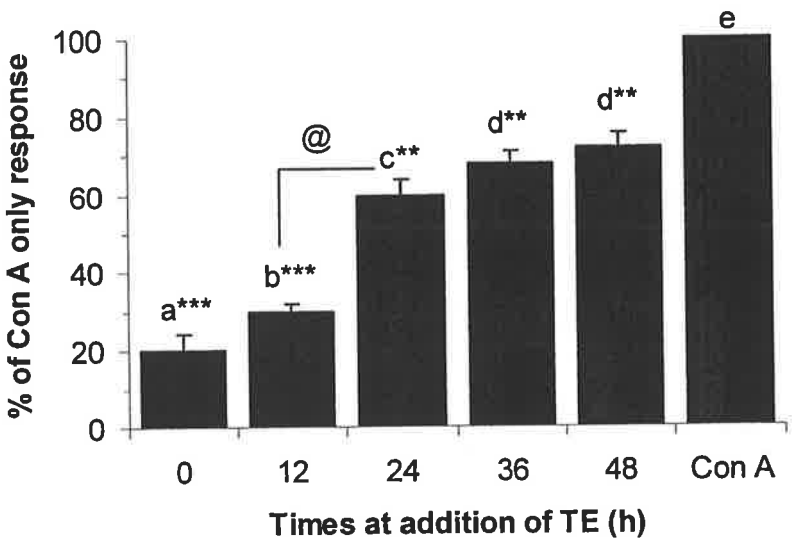


Figure 3.2 Effect of delayed addition of TE on the proliferative response of Con A-stimulated splenic T cells in culture.

TE (1:4 dilution) was added at the initiation of culture at 0 h, or after 12, 24, 36, or 48 h of culturing of Con A-stimulated splenic T cells. Bars are the mean ± SEM of 5 separate cultures, expressed as a percentage of the response of splenic T cells to Con A in the absence of TE. Values with different superscripts are significantly different at $P<0.05$ or @: $P<0.01$ between treatments. **: $P<0.01$, ***: $P< 0.001$ when compared with Con A only response (100%) tested with Duncan's new multiple-range test.

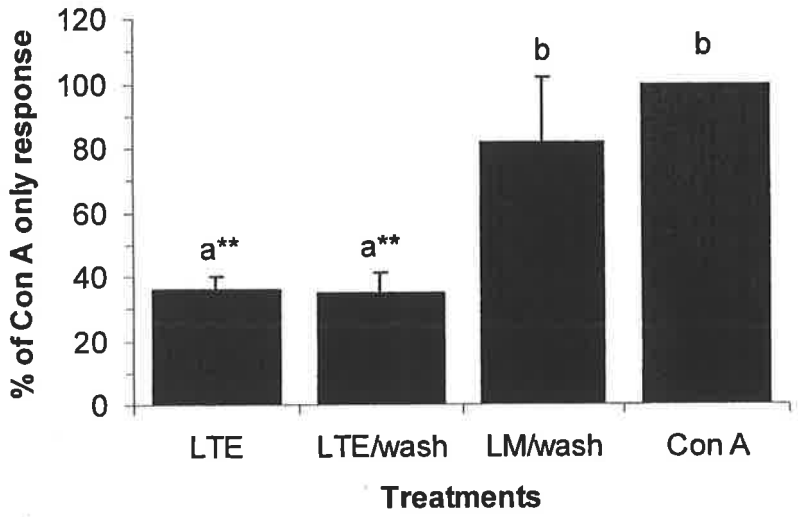


Figure 3.3 Effects of preincubation of splenic T cells (lymphocytes, Lø) with TE followed by washing, on their proliferation in culture in the presence of Con A.

LTE, Lø cultured with TE; LTE/wash, Lø incubated with TE (1:4) for 60 minutes followed by double washing, cell concentration adjustment and culture in the presence of Con A; LM/wash, Lø incubated with medium alone for 60 minutes followed by double washing, cell concentration adjustment and culture in the presence of Con A; Con A, Lø (neat) cultured in the presence of Con A alone. Values are mean ± SEM of triplicate cultures, and different superscripts show significant differences at $P<0.05$ between treatments. **:$P<0.01$ when compared with Con A only response (100%); Duncan's new multiple-range test.

results show both an immediate and permanent immunosuppressive effect of TE on T cells. The results also indicate that the inhibition of T cell proliferation is not due to a direct interaction with Con A, but rather as a direct effect of components of the TE on T cells.

3.3.3. Effect of Temperature and pH on Crude TE Immunosuppressive activity

Preincubating TE at various temperatures and pH values as shown in Figures Figure 3.4 and Figure 3.5 did not abrogate the suppression of Con A-induced splenic T lymphocyte proliferation. From these results it can be seen that the immunosuppressive activity of TE was slightly but not significantly increased after heat treatment, and was relatively pH stable. The results indicate that the immunosuppression was not likely to be attributable to a large protein, although many such proteins can refold following denaturation with treatments such as RNase A (Mui et al., 1985; Torella et al., 1994; Ruppolo et al., 1996; and Saito et al., 2001).

Changes in the protein concentration after treatment are shown in Table 3.2. The decreasing protein concentrations reported in Table 3.2 are most likely due to protein precipitation since it was observed that there was a precipitate after treatment. The precipitate contained no immunosuppressive activity (data not shown).

3.3.4. Effect of steroid removal

To determine whether the suppression of Con A-induced splenic T cell proliferation is due to steroid effects, the TE was charcoal-treated in an attempt to remove or significantly reduce steroid concentrations. Measurements of

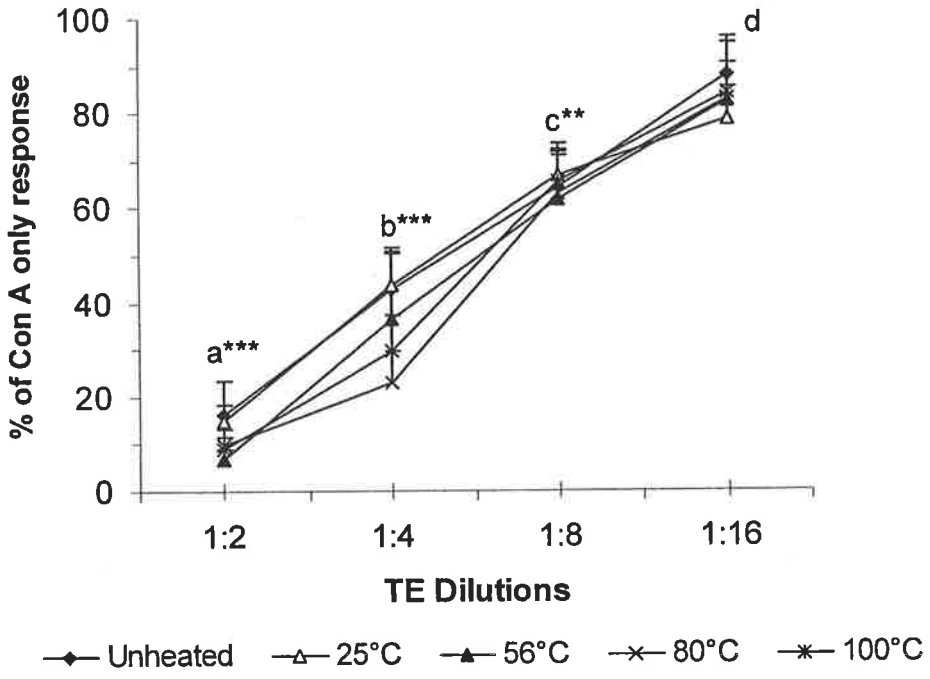


Figure 3.4 Effects of heat treatment on the immunosuppressive activities of crude TE.

Comparisons are between the respective unheated TE and each heated group (heated at 25°, 56°, 80° or 100° C for 10 minutes) at each dilution. The results are expressed as percentage of the response of splenic T cells to Con A in the absence of TE. Values are mean ± SEM of 4 different cultures. No significant difference ($P>0.05$) was observed between treatments compared with unheated TE at the appropriate dilution. Different superscripts show significant differences at $P<0.05$ between dilutions with all treatments or controls. **: $P<0.01$, ***: $P<0.001$ when compared with Con A only response (100%); Student *t* test.

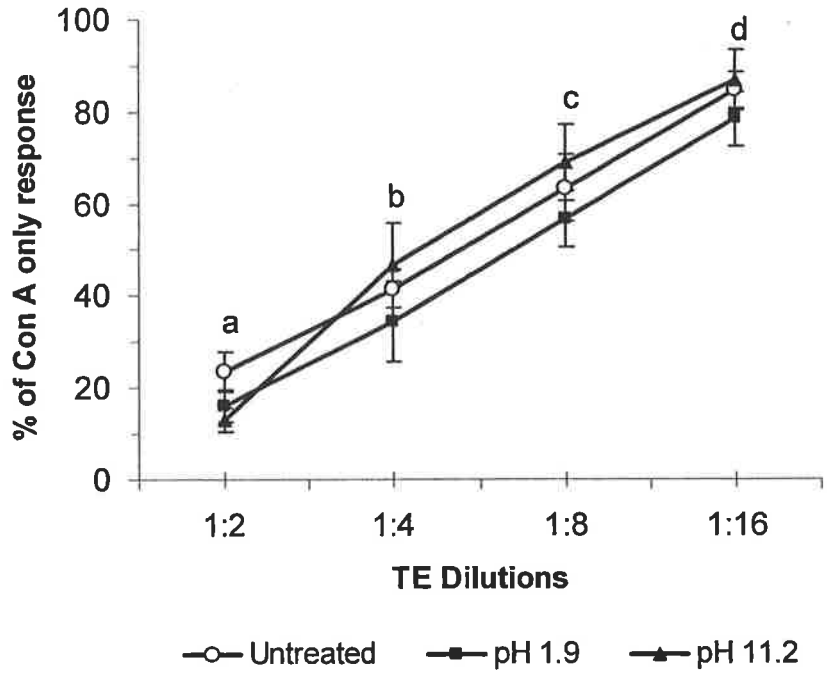


Figure 3.5 Effects of pH changes on the immunosuppressive activities of crude TE.

Comparisons are between the respective untreated TE and each treated group (pH 1.9 or pH 11.2 for 60 minutes) at each dilution. No significant difference ($P>0.05$) was observed between treatments compared with untreated TE at the appropriate dilution. Values are mean ± SEM of four different cultures. Different superscripts show significant differences at $P<0.01$ between dilution with all treatments; Student's *t* test.

Table 3.2 Protein concentration of rat testicular extracts after heat and pH treatment.

Treatment	Protein (mg/ml)
A. Heated	
1. Control	12.98 ± 0.22 [a]
2. 25° C	10.00 ± 2.20 [a]
3. 56° C	6.00 ± 0.20 [b]
4. 80° C	0.85 ± 0.05 [c]
5. 100° C	0.65 ± 0.05 [c]
B. pH	
1. Control	12.35 ± 0.25 [a]
2. pH 1.9	4.00 ± 0.25 [b]
3. pH 11.2	2.50 ± 0.30 [b]

Values with different superscripts show significant differences at $P<0.01$ compared with control within a given treatment (Student's *t* test). Data are represented by mean ± SEM; n=2. See 3.2.4 for experimental procedure.

steroids (represented by testosterone and progesterone) before and after charcoal treatments of TE showed that progesterone concentration was very low (< 1 nmol/L) both before and after treatment, while testosterone concentration in TE (Figure 3.6) was significantly reduced from 56.56 ± 1.41 nmol/L to 13.16 ± 1.10 nmol/L after charcoal treatment.

The results presented in Figure 3.7 indicate that the significant reduction in steroid concentrations in TE following dextran-coated charcoal treatment did not impair the suppression of Con A-induced splenic T cell proliferation by the testicular extracts.

3.3.5. Species dependence of TE immunosuppressive activities

Rat TE was able to suppress the proliferation of mouse, sheep, and cow lymphocytes in a dose dependent manner but had little effect on pig lymphocytes (Figure 3.8). There was no effect of the immunosuppressive factors on the pig lymphocytes possibly due to the different or lack of receptor on the swine lymphocytes. However further study isneeded to clarify this phenomenon.

3.3.6. Effects of proteolytic treatment

In order to determine whether the immunosuppressive activity in the TE was affected by proteolytic digestion, the TE was treated with trypsin and proteinase K. The results revealed that the suppressive activity of the TE was affected by proteolysis (Figure 3.9). Interestingly, while the suppressive activity was reduced significantly ($P<0.01$) by trypsin (Figure 3.9.a),

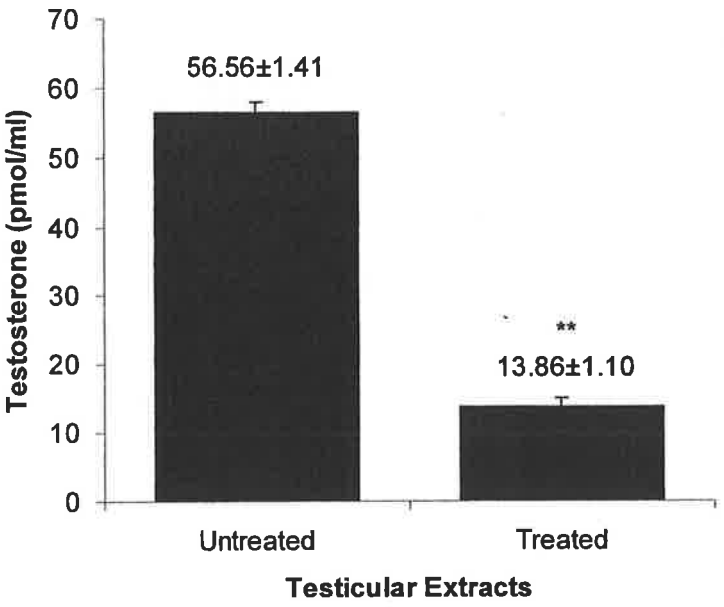


Figure 3.6 Testosterone concentration in crude testicular extract before (untreated) or after treatment with dextran-coated charcoal (treated).

Values are mean ± SEM of 3 experiments. ** = P <0.01; Student *t* test.

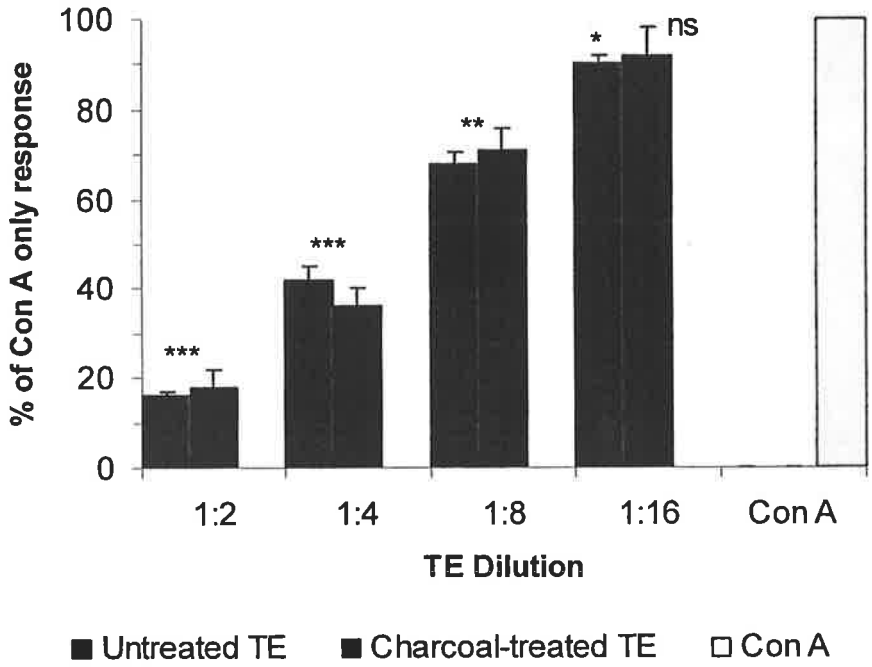


Figure 3.7 Suppressive activity of charcoal-treated TE.

No significant difference (P>0.05) was observed between the respective treated and untreated TE at any dilution. Significant differences (P<0.01) were observed between the Con-A treatment and the TE (charcoal-treated or untreated) for most TE dilutions. Only the charcoal-treated TE at a 1:16 dilution was not significantly (ns) different to the Con A treatment alone. Values are mean ± SEM of three different cultures. *: P<0.05. **: P<0.01, ***: P<0.001, ns: no significant compared with Con A only response (100%); Student's t test.

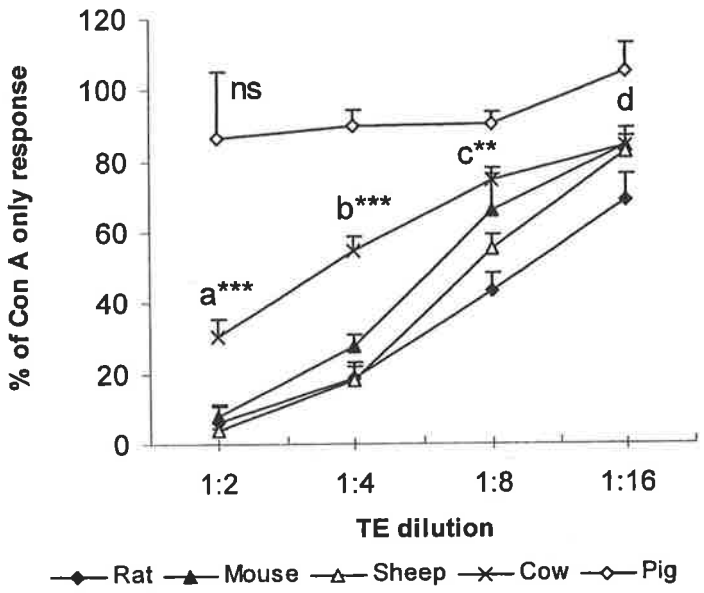


Figure 3.8 Effect of rat TE on Con A-induced proliferation of lymphocytes isolated from the blood of sheep, cow and pig and from the spleen of rat and mouse.

Values are mean ± SEM of triplicate cultures. Different superscripts show significant differences at $P < 0.05$ between dilutions. **: $P<0.01$, ***: $P<0.001$, ns: nonsignificant when compared with Con A only response (100%); Student's *t* test.

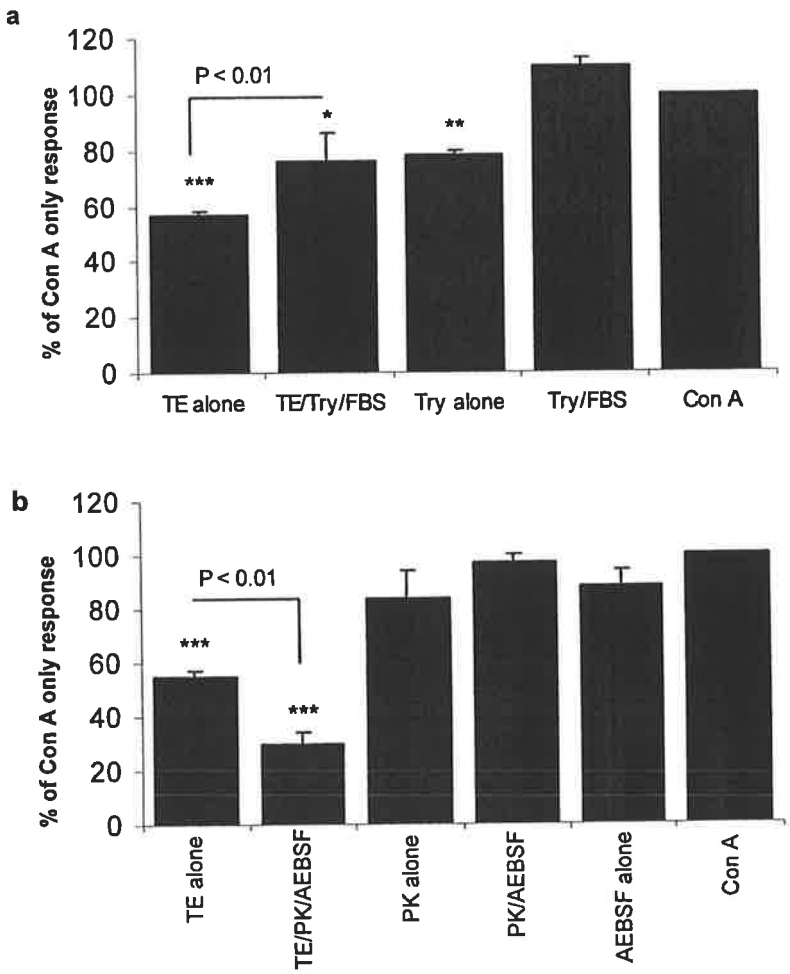


Figure 3.9 The effect of proteolytic treatments of the rat testicular extracts on immunosuppressive activity.

TE (1:4) was treated with either (a) trypsin or (b) proteinase K for 60 minutes. Reactions were terminated by addition of (a) FBS (10%) or (b) AEBSF respectively. The effect of the treated TE was then tested on Con A-induced splenic T cell proliferation. Values are mean ± SEM of three different cultures. *: P<0.05, **: P<0.01, ***: P<0.001 when compared with the Con A-only response (100%); Student's *t* test.

the activity was enhanced after proteinase K treatment (Figure 3.9.b). This was not due to cytotoxic effects of proteinase K or its inhibitor, AEBSF, as culture of splenic T cells in the presence of proteinase K alone, proteinase K and AEBSF, or AEBSF alone did not affect the Concanavalin A -induced splenic T cell proliferation (Figure 3.9.b).

Trypsin specifically hydrolyses proteins and peptides at the C terminal side of lysine and arginine residues. The significant reduction of the immunosuppressive activities of TE following treatment with trypsin in this study suggests that the immunosuppressive activities are proteinaceaous, and contain a number of basic amino acids residues (lysine and arginine).

3.3.7. Ammonium sulphate precipitation

To ascertain whether the immunosuppressive factor(s) in the TE could be precipitated by ammonium sulphate, investigations on the effects of several concentrations of ammonium sulphate on the immunosuppressive activity of TE were undertaken.

Four different concentrations of ammonium sulphate (10, 20, 30 and 40%) were used to precipitate proteins in the TE. Protein precipitated with 30% ammonium sulphate gave the strongest immunosuppressive activity (Figure 3.10 and Table 3.3). These results, in conjunction with the results of proteolytic digestion, reinforce the suggestion that the immunosuppressive activity in TE is associated with proteinaceous material.

In addition, from Figure 3.10 it can also be seen that immunosuppressive activity of crude TE was reduced significantly after dialysis (using dialysis tube with a 12 kDa molecular weight cut off). This suggests that factors of low MW contribute significantly to the immunosuppressive activity in TE, a point also indicated in section 3.3.3.

3.3.8. Suppressive activity in rat serum and tissues other than testis

In order to ascertain whether a suppressive activity similar to that reported in TE is also present in the serum or tissues other than testis, investigations on rat serum, and extracts of rat brain, kidney and liver were undertaken.

Figure 3.11 shows that suppression of Con A-induced splenic T cell proliferation was also achieved using rat serum and extracts of rat brain and kidney without affecting cell viability, except at the highest (325 μg/ml) concentration of the kidney extract (Table 3.4). In contrast to brain and TE, the kidney extracts and serum showed significant immunosuppression at a concentration >81 μg/ml. Furthermore, heat (56° C) treatment abolished immunosuppressive activity of brain but not the testis extracts; this treatment caused the serum and the kidney extracts to clot (forming a gelatin-like substance). From these results it can be seen clearly that the immunosuppressive activity found in the TE is different from that found in rat serum or the kidney. The suppressive activity found in the liver extracts appeared to be due largely to its inherent cytoxicity with less than 5% of cells remaining viable after 24 h of culture (Table 3.4), as assessed by trypan blue exclusion.

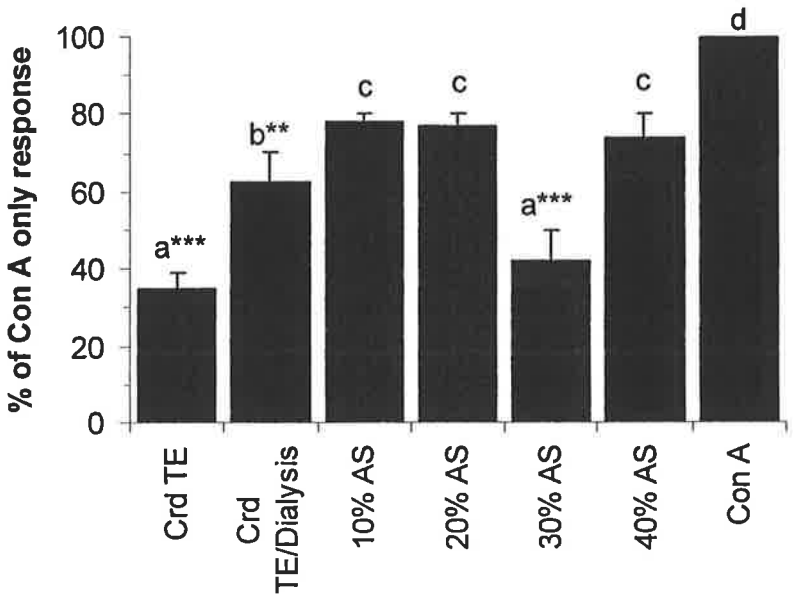


Figure 3.10 Precipitation of TE immunosuppressive activity with different concentrations of ammonium sulphate.

Crd TE: Crude TE without dialysis, Crd TE/Dialysis: Crude TE dialysed in conjunction with the crude TE treated with 10, 20, 30 and 40% of ammonium sulphate (AS). Bars are the mean ± SEM of 3 separate cultures. For the assays, samples were diluted 1:4. Values with different superscripts show significant differences at $P<0.05$. **: $P<0.01$, ***:$P<0.001$ when compared with the Con A-only response (100%); Student's *t* test.

Table 3.3 Suppressive activity of aliquoted TE precipitated with different concentrations of ammonium sulphate.

Sample	Vol. (ml)	Suppressive activity (%)	Value
Crude TE	5	64	1.00
Crude TE/dialyse.	5	35	0.55
10% AS	5	12	0.20
20% AS	5	20	0.30
30% AS	5	56	0.90
40% AS	5	24	0.38

Suppressive activity (%) was calculated based on the suppression of Con A-induced splenic T cell proliferation by crude TE (1:4 dilutions). In this experiment the suppressive activity of crude TE (1:4 dilutions) as the control was 64% with a conversion value of one. Suppressive activity of treated crude TE was calculated against the control value (eg. Sample of 10% AS: 12/64 = 0.20). Data were from three separate cultures. See 3.2.8 for experimental procedure.

Furthermore, based on the immunosuppressive activity per microgram of protein, it can be seen in Figure 3.11 that the strongest immunosuppressive activity in this study was expressed by testicular extract.

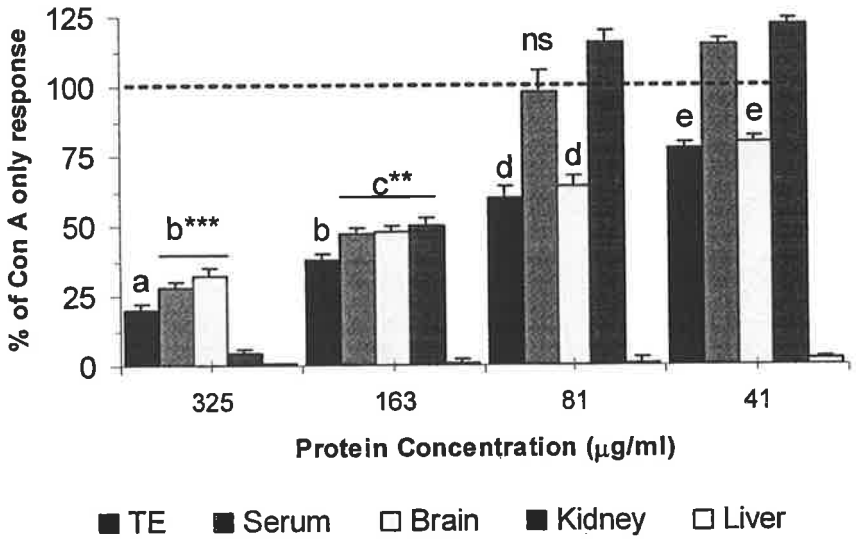


Figure 3.11 Effect of rat TE, serum, brain, kidney and liver extracts on the proliferation of splenic T lymphocytes.

Values are the mean ± SEM of 3 separate cultures. Different superscripts show significant differences at $P<0.05$ between samples. **: $P<0.01$; ***: $P<0.001$ and ns: non significant compared with Con A only response (100%, broken line); Student's *t* test. Data from liver extract were excluded from analysis due to a tendency for cytotoxic effects.

Table 3.4 Viability of splenic-T lymphocytes cultured for 24 h with several concentrations of rat serum and extracts of rat testis, brain, kidney and liver.

Samples	Cell viability (%)			
	Protein concentration (μg/ml)			
	325	163	81	41
Testis extract	94.30 ± 2.26	95.79 ± 1.12	93.54 ± 0.30	90.10 ± 4.93
Serum	91.30 ± 2.83	88.88 ± 4.13	91.17 ± 0.81	93.93 ± 6.19
Brain extract	91.20 ± 2.97	96.60 ± 1.13	95.78 ±1.10	95.40 ± 0.85
Kidney extract	24.80 ± 2.33	96.10 ± 1.84	96.20 ± 1.41	96.75 ± 0.71
Liver extract	4.18 ± 1.20	4.25 ± 1.63	6.84 ± 3.06	4.77 ± 1.20

Values are mean ± SEM of duplicate cultures.

3.3.9. Assay for IL-2 activity

As mentioned previously, activated T cells produce IL-2, which in turn induces proliferation of other T cells through IL-2-receptor interaction. Optimal IL-2 production can be achieved 40-48 h after the activation of T cells with mitogens *in vitro* (Gearing and Bird, 1987).

It is possible that the TE-induced inhibition of Con A-induced splenic T cell proliferation is due to inhibition of IL-2 production. Results presented in Figure 3.12 show clearly that supernatant collected from activated splenic T cells cultured for 48 h in the presence of TE provided significantly ($P<0.01$) less support for the proliferation of the IL-2-dependent cell line, CTLL, than control culture supernatants. Optimal proliferation was achieved in the presence of IL-2 and was also achieved in the presence of supernatant from activated splenic T cells without TE. These results clearly indicate that the immunosuppression demonstrated by TE also results in a reduction in IL-2 concentrations.

Similarly, IL-2 induced T cell proliferation was also suppressed by crude TE; this effect becomes profound in the presence of anti-rrIL-2 antibody (Figure 3.13) providing further evidence that TE inhibits the IL-2 activation pathway in T cells but that this may involve other mechanisms that the IL-2 receptor.

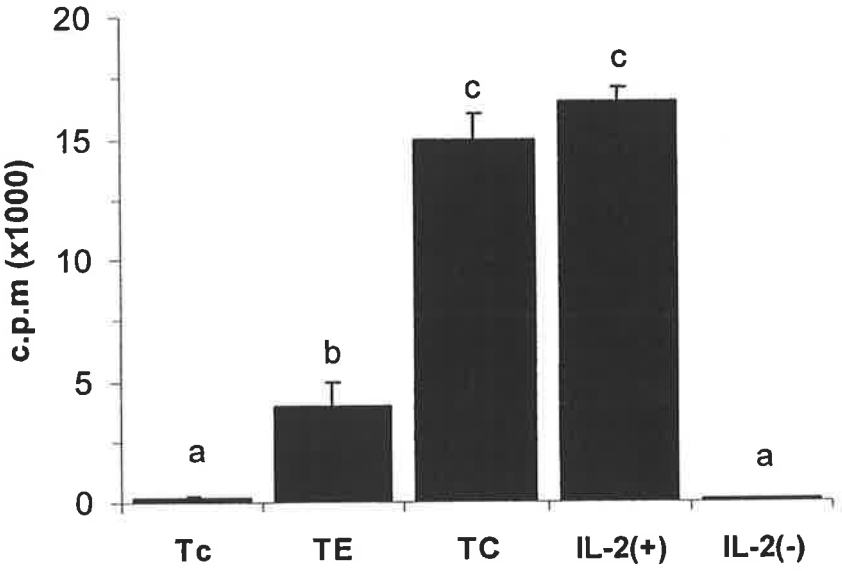


Figure 3.12 Effects of TE (1:4) on the proliferation of IL-2 dependant CTLL cells.

The IL-2-dependent CTLL cell line was cultured for 48 h in the presence of culture supernatants of T cells cultured alone (Tc), or with TE and Con A (TE), or with Con A only (TC). As controls, CTLL cells were cultured with- [IL-2 (+)] or without [IL-2(-)] the addition of IL-2. Values are the mean ± SEM for triplicate cultures. Values with different superscripts are significantly different at $P<0.01$; Duncan's new multiple-range test.

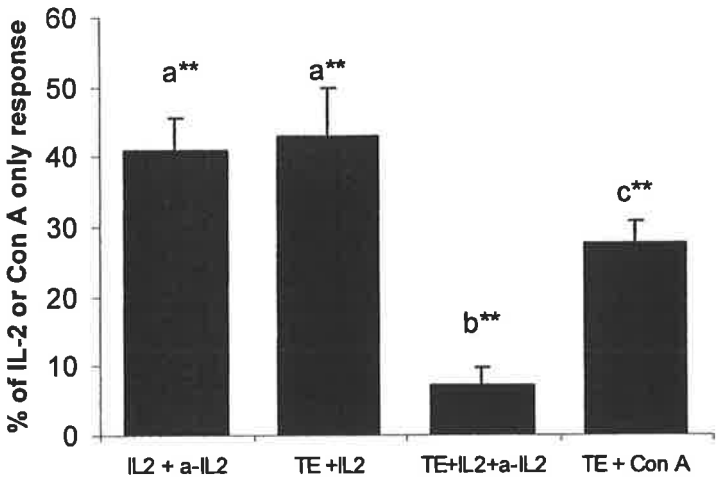


Figure 3.13 Inhibition of recombinant rat IL-2-induced T cell proliferation by crude TE (1:4).

Splenic T cells were firstly induced with 2.5 μg/ml Con A overnight, then washed three times with PBS before treating with recombinant rat IL-2 (20 ng/ml) and crude TE (1:4). Anti rrIL-2 antibody (a-IL2) was also applied to the cultures at a concentration of 5 μg/ml. Cell proliferations were assessed as described in 3.2.1.2 in the presence of rrIL-2 and anti-rrIL-2 antibody (IL2+a-IL2), testicular extracts and rrIL-2 (TE+IL2), testicular extract + rrIL-2 + anti rrIL-2 (TE+IL2+a-IL2), or testicular extract + Con A without rrIL-2 (TE+Con A). Values (Mean ± SEM) with different superscripts show significant differences at $P<0.01$ between treatments, or at $P< 0.001$ (**) when compared with the IL-2 or Con A only response (100%). Student *t* test (n=3).

3.4. Discussion

The results of the investigations reported in this chapter show that a crude extract of the rat testis (TE) suppressed the proliferation of both Con A and PHA-induced splenic T cell cultures in a dose-dependent manner (Figure 3.1). Proliferation of recombinant rat IL-2-induced splenic T cells was also inhibited by the crude TE. The suppressive activity was not due to cytotoxicity of the TE since cells remained viable following co-culture for 24 hours (Table 3.1). These results are comparable to those of previous investigations with interstitial fluid and homogenate of rat testis as reported by Pöllänen et al., (1988, 1989a, b, 1992), and Sainio-Pöllänen et al., (1991), and with interstitial fluid, seminal vesicle fluid, and extracts of mouse/murine testis (Anderson and Tarter, 1982; Emoto et al., 1989, 1990a, b, and 1991).

Immunosuppressive activity was also observed in rat serum, brain, and kidney extracts. However inhibition by serum and kidney extracts may be due to an artifact since heat treatment (56° C) of these two substances resulted in the formation of clots of gelatin-like material. Because of this, comparative studies between TE, serum, and kidney extracts were excluded due to the difficulty in interpreting the data. In addition, the immunosuppressive activity of the serum and kidney extracts occurred at only high concentrations (Figure 3.11). Although brain extracts gave a similar immunosuppressive activity to TE, this activity was abrogated by heat treatment while this did not happen for the TE. Therefore it can be concluded that the nature of the immunosuppressive factor(s) in the testicular extracts appears to be different from the factor(s) in the brain extract.

The action of TE in suppressing splenic T cell proliferation is rapid with optimal suppression achieved when TE was added within 12h of lymphocyte mitogenic (Con A) stimulation (Figure 3.2). This suggests that optimal immunosuppression occurs when T cells undergo morphologic changes or blastogenesis, a process in which T cells considerably increase in size early during antigen-induced activation (see Crabtree, 1982). This result is consistent with the results reported previously showing that proteins secreted by rat- (Wyatt et al., 1988) or mouse- (De Cesaris et al., 1992) Sertoli cells, and mouse seminal vesicle fluid (Emoto et al., 1990b), or mouse testicular extract (Emoto et al., 1991), suppressed lymphocyte proliferation early in the mitogen stimulation process.

This present study also indicates that the immunosuppressive activity is abrogated by trypsin, and could be precipitated by ammonium sulphate (30%), suggesting that the factor is a protein. Furthermore the immunosuppressive activity was found to be heat and pH stable, which suggests that it is a small protein or peptide. These results are consistent with the reduction of immunosuppressive activity following dialysis with a 12 kDa cut off dialysis tube (Figure 3.10 and Table 3.3), and suggest that the most potent immunosuppressive factor(s) has a molecular weight less than 12 kDa which is in contrast to those previously reported (Pöllänen et al., 1992). However, further study is needed to clarify this (see Chapter 4). The immunosuppressive factor(s) do not appear to be steroids since removal of most, albeit not all, steroids by charcoal extraction did not abolish the immunosuppressive activity of TE (Figure 3.7). Similar results have been reported by Pöllänen et al.,

(1988). Even though low concentrations of testosterone could be detected after charcoal treatments (about 13 nmol/L; Figure 3.6), this concentration is much lower than those found from veins at the proximal end of the rat spermatic cord (123.06 ± 24.75 nmol/L, Maddocks and Sharpe, 1989) but is about 2.5 fold higher than testosterone in the rat peripheral venous blood (4.55 ± 0.55 nmol/L, Maddocks and Sharpe, 1989). Kanda et al., (1996) have reported that human testosterone concentrations of up to 1000 nmol/L did not affect T cell viability. It is concluded from these preliminary investigations that the immunosuppressive activity of rat testicular extract in this study is unlikely to be due to steroid hormones.

The mechanism of suppression may reside in the inhibition of IL-2 production. IL-2 is known to be produced by activated T-cells (Quemeneur et al., 2002, Nelson, 2004), which in turn stimulates propagation of T cells through interaction of IL-2 and its receptors on the surface of activated T cells. *In vitro* studies have shown that the optimal production of IL-2 can be achieved 40-48 h after the activation of T cells with mitogens (Gearing and Bird, 1987).

In the present study, supernatants from Con A-induced splenic T-cells cultured in the presence of TE (1:4 dilution) for 48 hours, contained significantly ($P<0.01$) less IL-2 (Figure 3.12) than T cells cultured without TE. These results are consistent with the previous suggestion that suppression of T cell proliferation by TE is not due to the binding of TE components to Con A but by reducing IL-2 production. This is supported by the results showing that TE also suppressed recombinant IL-2-induced splenic T cells proliferation, and the

immunosuppression seen in the presence of TE was increased in the presence of an anti-IL-2 antibody (Figure 3.13). Similar results were also reported by Pöllänen et al., (1990) who found that rat testicular fluid suppressed recombinant IL-2-induced rat lymphocyte proliferation. De Cesaris et al., (1992) also reported that IL-2 secretions were reduced drastically by mouse Sertoli-secreted proteins. And more recently Veselský et al., (2003) reported that production of IL-2 and interferon-γ (IFN-γ) by Con A-stimulated mouse spleen cells was lowered by a boar seminal immunosuppressive fraction.

A limitation in the consistency of other published work concerning the nature of the immunosuppressive substances present in the male genital tract has been highlighted in the introduction to this thesis. Most works have been focused on the high molecular weight factors such as TGFβ, while the low molecular weight was neglected since it was thought that the low molecular weight correlated with non-specific cytotoxic effects. However as mentioned previously, in this chapter it was revealed that preincubating TE at various temperatures and pH did not abrogate TE immunosuppressive activity. A change in the protein concentration after these treatments showed that large proteins were precipitated during the treatments, yet the immunosuppressive activity was retained. Furthermore, removing the low molecular weight substances using a 12 kDa cut off dialysis tube resulted in a reduction in the immunosuppressive activity. Taken together, these results suggest that the most potent immunosuppressive factor(s) has molecular weight less than 12 kDa. Interestingly, the TE in this present study expresses immunosuppressive activity without indication of cytotoxic effects. These results are quite

different with those previously reported (for review, see Pöllänen et al., 1990). There is an urgent need therefore to identify specifically the nature and identity of the immunosuppressive activity, especially the most potential involvement of the low molecular weight substances. These needs are pursued in the studies reported in the next chapter of this thesis.

4. Separation of Rat Testicular Extracts: Potential Immunosuppression of Low Molecular Weight Compounds

4.1. Introduction

During the last decade, several studies have investigated immunosuppressive factors in the testis in order to elucidate the mechanisms responsible for providing immune privilege in this organ (reviewed by Hedger and Meinhardt, 2003). Attempts to characterise the biochemistry of these immunosuppressive factors have been disappointing (see Chapter 1.6). It has been demonstrated that the immunosuppressive factors in the testis consist of both high and low molecular weight proteins. Less attention has been paid to the low molecular weight substances because their immunosuppressive activity has been reported to be correlated with cytotoxic effects (reviewed by Pöllänen et al., 1990). However, a number of recent reports have indicated that some low molecular weight substances contribute immunosuppressive activity without expressing cytotoxic effects (Dostal et al., 1995; Krishnan et al., 1995; Djian et al., 1996). A small molecular mass inhibitor (<10 kDa) of interleukin-1 (IL-1) bioactivity secreted in vitro by testicular macrophages has also been reported (Hayes et al., 1996). The results of the present study (as described in Chapter 3) also indicate that a potent immunosuppressive activity in the rat TE is associated with a substance of molecular weight of less than 12 kDa. Importantly this immunosuppressive activity was not cytotoxic, and demonstrated sensitivity to

the specific serine protease trypsin, indicative of a polypeptide immunosuppressive factor.

This chapter describes various attempts to further isolate and characterise the immunosuppressive factors in the rat testis, with particular attention to the low molecular weight components.

PART ONE

Separation of Crude TE using Sephadex G-25

4.2. Separation of Crude TE

4.2.1. Aims

Initial separation of interstitial fluid (Pöllänen et al., 1988) or homogenate of rat testis (Sainio-Pöllänen et al., 1991) using a Sephadex G-25 column has been reported, with three major fractions of molecular weight (Mr) of >5 kDa, 1-5 kDa, and < 1 kDa prepared. From these fractions, fractions with Mr >1 kDa were reported to exhibit immunosuppressive activity. In order to further investigate which of these molecular weight fractions contained the strongest immunosuppressive activity, the crude TE was separated by column chromatography.

4.2.2. Methods

In the present study, charcoal treated-crude TE (prepared as described in 2.3.1) was separated in the first instance, using a Sephadex G-25, PD-10 column (1.5 x 5 cm, Pharmacia, Uppsala, Sweden). The column was equilibrated with phosphate buffered saline (PBS, pH 7.2), and the crude TE (2.5 ml; 13 mg/ml)

in PBS was applied and eluted with PBS at pH 7.2. Fractions (0.5 ml each) were collected and absorbance was monitored at 280 nm. Each fraction was then tested for its ability to suppress Con A-induced splenic T lymphocyte proliferation (see 3.2.1.2). Fractions, which contained potent immunosuppressive activity, were pooled and stored at -20° C for further investigation.

In addition, pooled HMW-TE (Mr > 5 kDa) and LMW-TE (Mr 1-5 kDa) active fractions were tested for their testosterone concentration by RIA as presented in Chapter 3.

4.2.3. Results and Outcomes

Twenty fractions were collected from the separation of crude TE using a G-25, column. Fractionation resulted in a consistent elution profile over several separations. A representative profile is shown in Fig. 4.1.a. and consists of two distinct peaks.

Based on the manufacturer's (Pharmacia, Uppsala, Sweden) specifications, a high (Mr >5 kDa), medium (1-5 kDa), and low molecular weight (< 1 kDa) fraction are achieved when crude protein is separated by PD-10 column chromatography. Following the manufacture's specified protocol, fraction 6-12 were pooled as Mr > 5 kDa, fractions 13-16 as Mr 1-5 kDa, and fractions 17-20 as Mr < 1 kDa (Figure 4.1). The immunosuppressive activity of each fraction is presented in Figure 4.1.b. The proliferation of splenic T cells was significantly ($P<0.001$) suppressed by fractions 13-16 (Mr 1-5 kDa), and weaker immunosuppressive activity was observed for the higher molecular

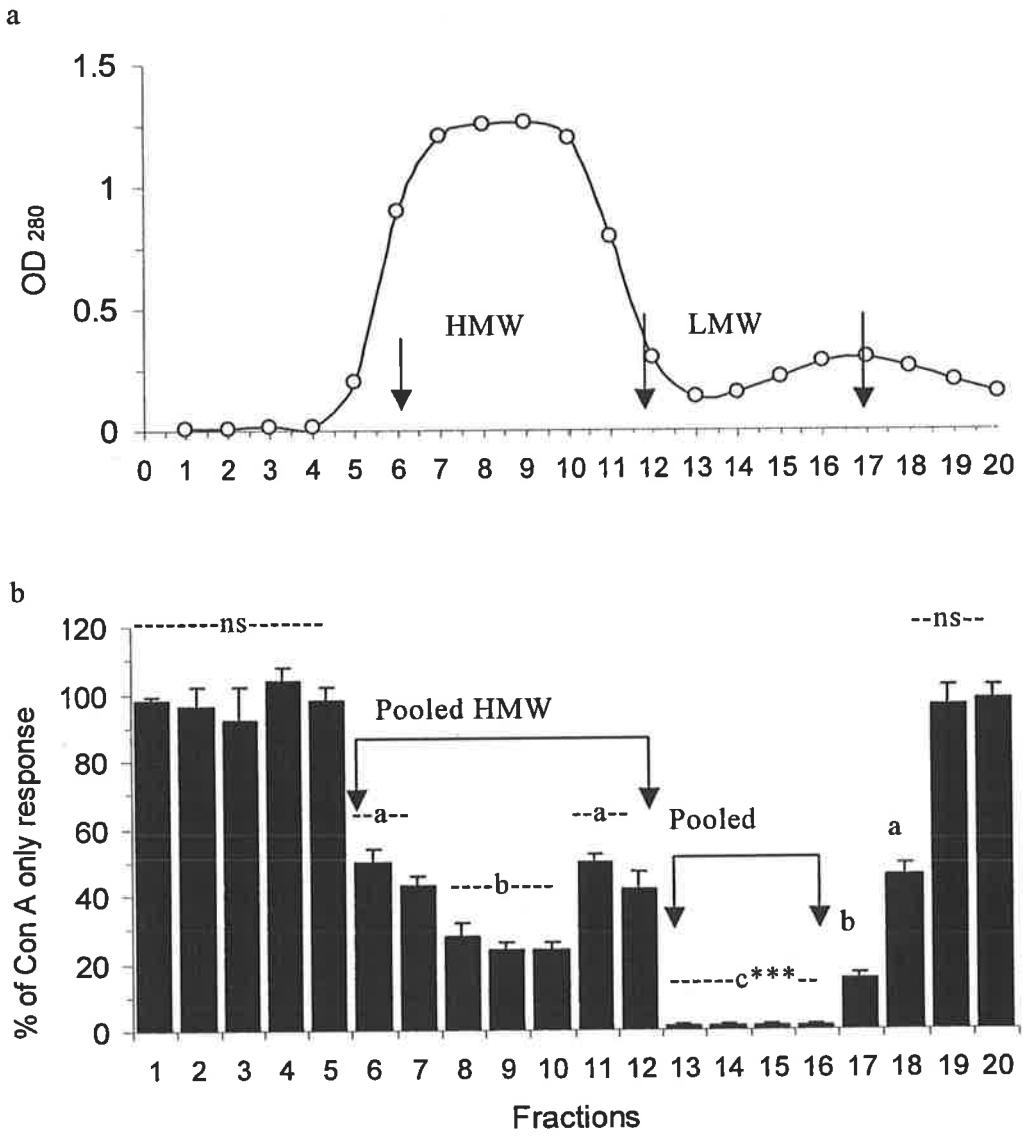


Figure 4.1 (a) Representative chromatographic pattern obtained from fractionation of crude TE on a PD-10 column.

The column was equilibrated with PBS (pH 7.2); crude TE (2.5 ml) was then applied and eluted with PBS. Fractions were collected (0.5 ml each) by gravity feed.

(b) Effects of each fraction on Con A-induced T cell proliferation.

Values shown are Mean ± SEM of triplicate cultures. Values with different superscripts show significant differences at $P<0.01$. ***: $P<0.001$; ns: non significant when compared with Con A only response (100%) tested with Duncan's new multiple-range test. Pooled fractions are indicated.

weight fractions, i.e. fractions 6-12 (Mr >5 kDa), whilst no suppressive activity was noted for the fractions 19 and 20 (Mr <1 kDa).

The fractions were pooled into three groups based on the estimation of their molecular weight and their corresponding immunosuppressive activity. The immunosuppressive activity of the three pooled fractions was then established over a range of dilutions. Results of the lymphocyte proliferation assays for these three pooled fractions are presented in Figure 4.2. It can be seen that fractions with Mr > 1 kDa suppressed the Con A-induced splenic T cells proliferation in a dose dependent manner. Furthermore, fractions with Mr 1-5 kDa (in this study, designated as low molecular weight, LMW-TE), gave the highest ($P<0.001$) immunosuppressive activity compared with the crude TE or the high molecular weight fraction (Mr > 5 kDa). The immunosuppressive activity for this LMW-TE was 2.5 fold greater than the suppressive activity of the crude TE or of the HMW-TE.

The results of testosterone assays (Figure 4.3) show that the testosterone concentrations of both high- and low molecular weight pooled fractions were below the detection limit (0.4 pmol/ml with 7% internal variation) of the testosterone assay used (Figure 4.3).

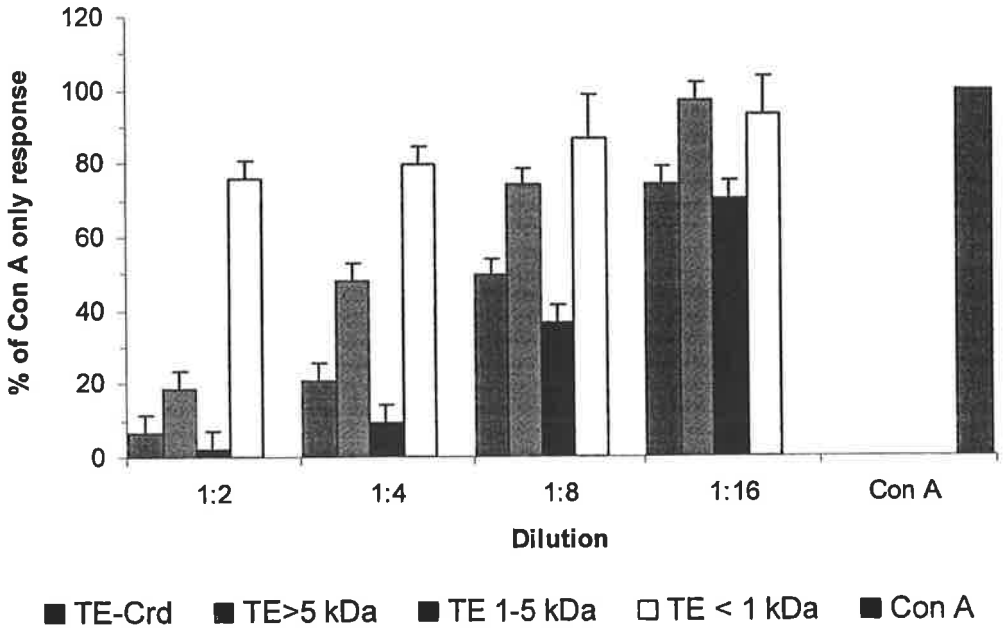


Figure 4.2 Immunosuppressive effects of crude TE (pre column) and three pooled TE fractions (TE > 5 kDa, 1-5 kDa and less than 1 kDa, separated using a PD-10 column, see experimental procedures) on the Con A-induced proliferation of splenic T cells.

Results are expressed as a percentage of the T cell proliferation observed in the Con A-stimulated control (100%). Values are the Mean ± SEM; n=4. All fractions greater than 1 kDa were immunosuppressive, TE>5 kDa was less efficient than crude TE ($P<0.01$), while TE 1-5 kDa was more efficient ($P<0.001$). Data were analysed by analysis of variance and the differences were tested with Duncan's new multiple-range test.

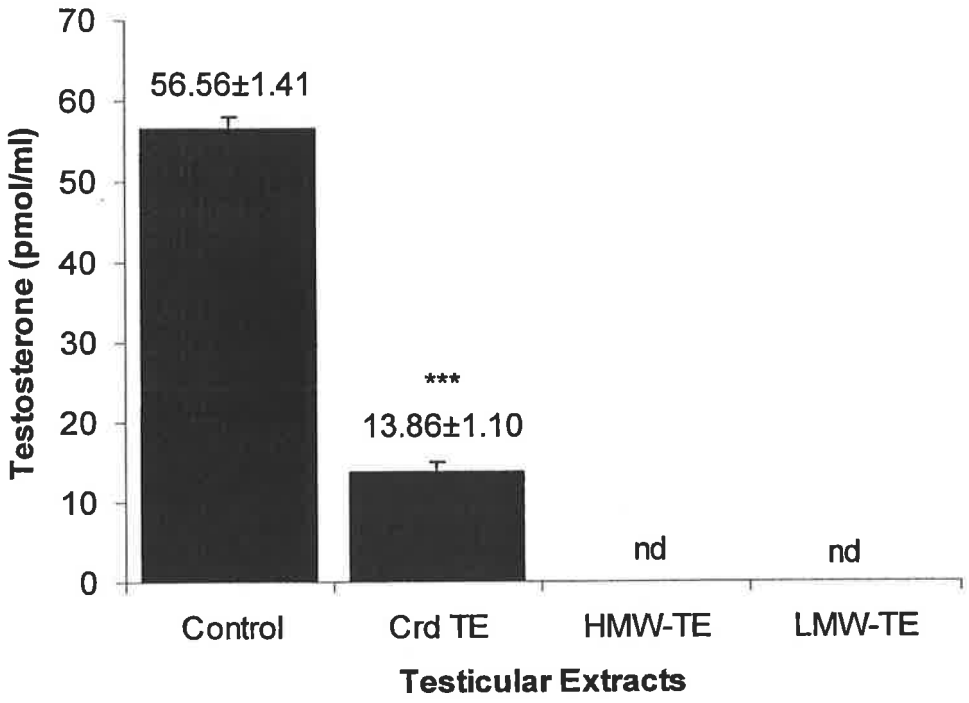


Figure 4.3 Concentration of testosterone in the crude (Crd-TE), high- (HMW-TE), and low- (LMW-TE) molecular weight of testicular extracts after charcoal treatments.

nd: not detectable, below the assay sensitivity of 0.4 pmol/ml; ***: $P < 0.001$ significantly different when compared with untreated (Control) samples (Student's t-test); n=3.

4.3. Size exclusion chromatography using a Sephadex G-25

4.3.1. Aims

The chromatographic resolution of PD-10 column is very poor and that gel filtration of small molecules is often unreliable for determining molecular weight. Other interactions between the molecule and gel, such as hydrophobicity and charge interactions, can override the filtration efficiency. For these reasons, the crude TE was also separated using a Sephadex G-25, C26/70 (26 mm i.d. x 70 cm height) gel filtration column (Pharmacia Biotech, Uppsala). This was used with a Gradifrac low pressure chromatography system (Pharmacia, Uppsala). This separation was undertaken in order to more accurately estimate the relative molecular weight (Mr) of the active fractions of the TE.

4.3.2. Methods

Crude TE (one ml) was applied to the column and eluted with PBS pH 7.2 at a flow rate of one ml/min. Absorbance was monitored at 280 nm and the eluate was collected in 5 ml fractions. Fractions were concentrated using a vacuum evaporator (Vacuspin "Virtis", NY, USA). The resultant pellets were subsequently dissolved in PBS pH 7.2 (one ml each), and their immunosuppressive activities were tested using a lymphocyte proliferation assay as described in 3.2.1.2.

Molecular weight (Mr) standards of bovine serum albumin (BSA, 66.2 kDa), trypsin inhibitor (20.1 kDa), cytochrome c (12.5 kDa), and aprotinin (6.5 kDa),

were used to clarify the elution profile. The standards (one ml each) were applied to the column and eluted with PBS pH 7.2 at a flow rate of one ml/min in a similar manner to the TE as described above.

4.3.3. Results and Outcomes

The relative molecular weight standard curve was drawn on the basis of the elution volume (retention time) of each standard (drawn on the linear scale) plotted against the corresponding molecular weight values (on a logarithmic scale, see Figure 4.4). This standard curve was used to estimate (by interpolation) the relative molecular weight of TE active fractions based on the retention times of the fraction which contained immunosuppressive activity.

Gel filtration chromatography of the crude TE on a sephadex G-25 column operated using a Gradifrac low pressure system resulted in a protein profile consisting of five peaks (Figure 4.5) over 33 fractions. From the lymphocyte proliferation assays, it was found that fractions 6, 10 and 11 were able to suppress Con A-induced splenic T cell proliferation (also in Figure 4.5). Estimation of the relative molecular weight of the active fractions, on the basis of the standard curve presented in Figure 4.4, suggested that fraction 6 corresponds to a relative molecular weight of 6.5 kDa, while fractions 10 and 11 were of a lower relative molecular weight. Nominally, these appear to be around 1.6 and 1.0 kDa, but more sensitive analysis is required to confirm this, as these samples fall outside the range of standards run in this protocol (see 4.10).

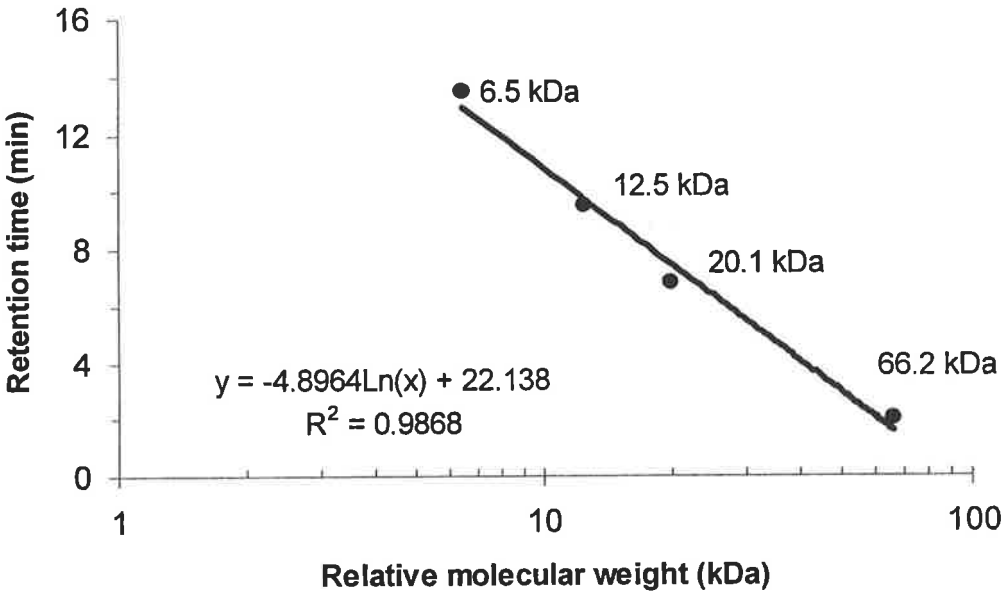


Figure 4.4 Standard curve for the estimation of the relative molecular weight (Mr) of fractionated TE using a Sephadex G-25 Sephadex C 26/70 column operated with a Gradifrac system.

Standards used were aprotinin (6.5 kDa), cytochrome c (12.5 kDa), trypsin inhibitor (20.1 kDa), and BSA (66.2 kDa).

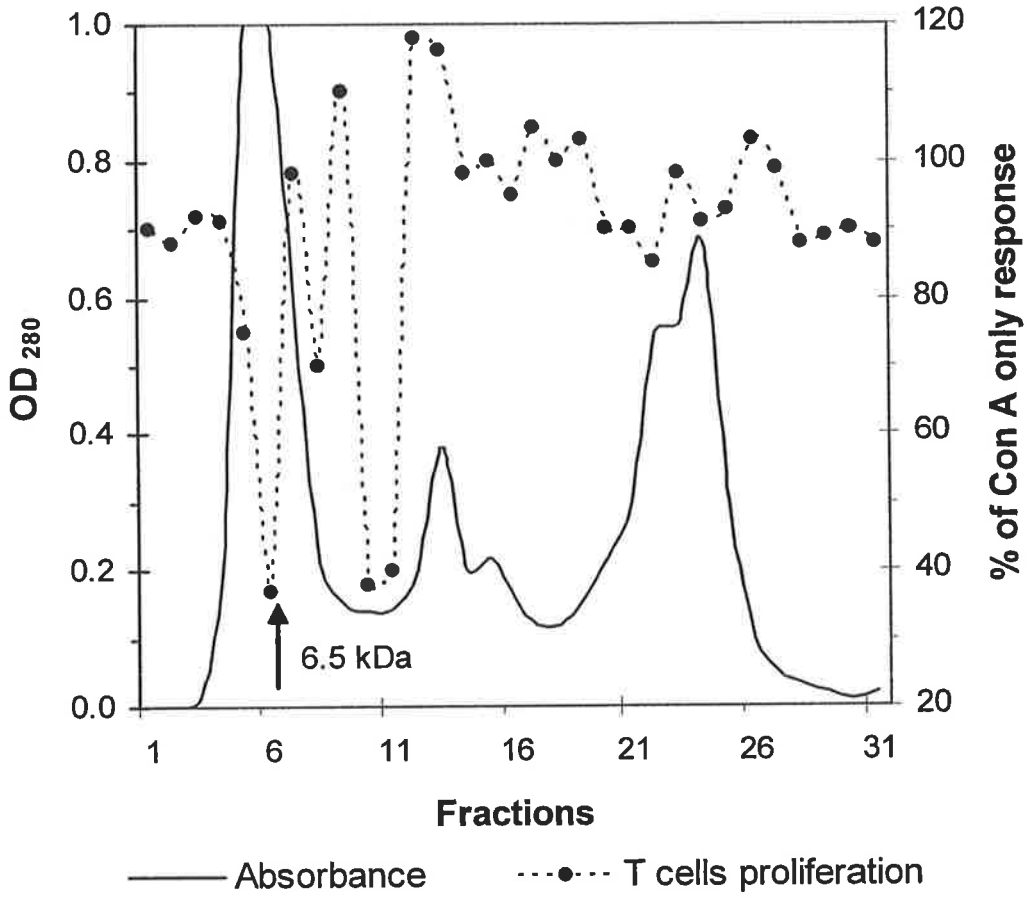


Figure 4.5 Representative chromatographic profile obtained from the fractionation of crude testicular extracts on a Sephadex G-25 column operated using a Gradifrac low pressure system.

A significant inhibition of T cell proliferation was achieved with fractions 6, 10, and 11 with the approximate molecular weight of the active fractions of less than 7.2 and nominally 1.6 and 1.0 kDa respectively (see Fig. 4.4). These results are in complete agreement with the results found in 4.2.3.

4.4. Effects of temperature, pH, and proteolytic on the immunosuppressive activity of low molecular weight TE

4.4.1. Aims

Since the low molecular weight fraction of testicular extract (LMW-TE) gave the highest ($P<0.001$) immunosuppressive activity compared with the crude and high molecular weight testicular extracts (see 4.2.3; Figure 4.1), the particular characteristics of this fraction were examined further. This included studies of the effects of temperature and pH changes, and the effect of proteolytic treatment.

4.4.2. Methods

The methods applied for the studies of temperature and pH lability, and the effect of proteolytic treatment were as described in the experimental procedures of Sections 3.2.4 and 3.2.7 respectively.

4.4.3. Results and Outcomes

4.4.3.1. Temperature and pH lability of LMW-TE immunosuppressive activity

The suppression of Con A-induced splenic T cell proliferation by LMW-TE was not abrogated by heat or pH as presented in Fig. 4.6. and Fig. 4.7. Although the suppressive activity was slightly increased after treatments, this was not significant ($P>0.05$). These results support the hypothesis that the immunosuppressive species are small peptides. Small peptides generally refold readily after heat or pH treatment to reconstitute their activity.

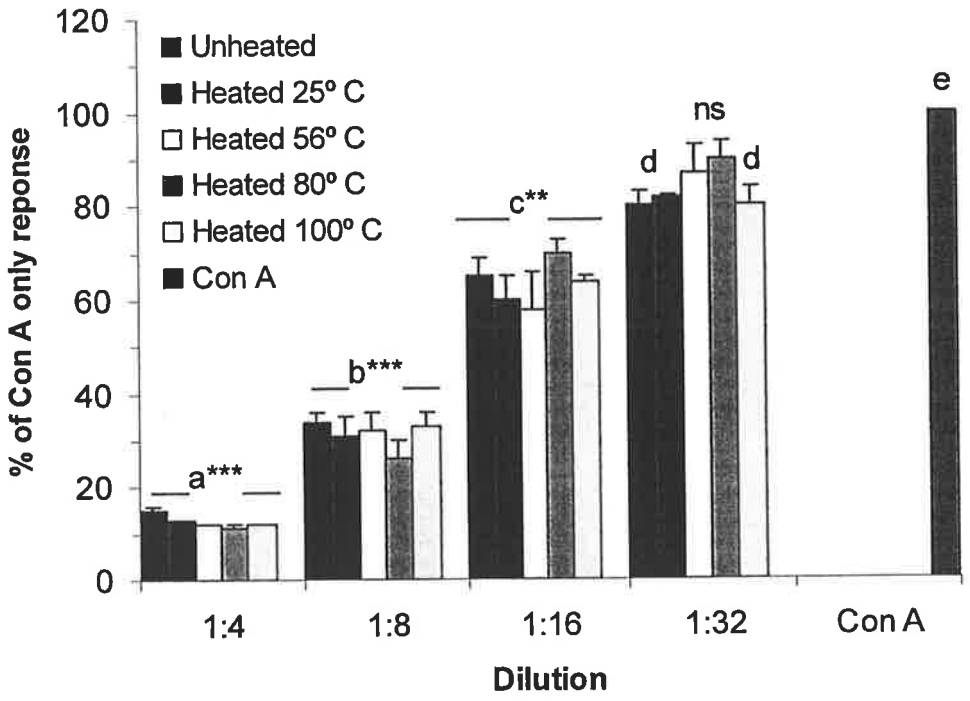


Figure 4.6 Effect of heat treatment on the immunosuppressive activities of low molecular weight TE (LMW-TE).

The results are expressed as a percentage of the response of splenic T cells to Con A only in the absence of LMW-TE. Values are Mean ± SEM of three different cultures. Values with different superscripts show significant differences at $P<0.05$. **: $P<0.01$; ***: $P<0.001$; ns = non significant when compared with Con A only response. Data were analysed by analysis of variance and the differences were tested with Duncan's new multiple-range test.

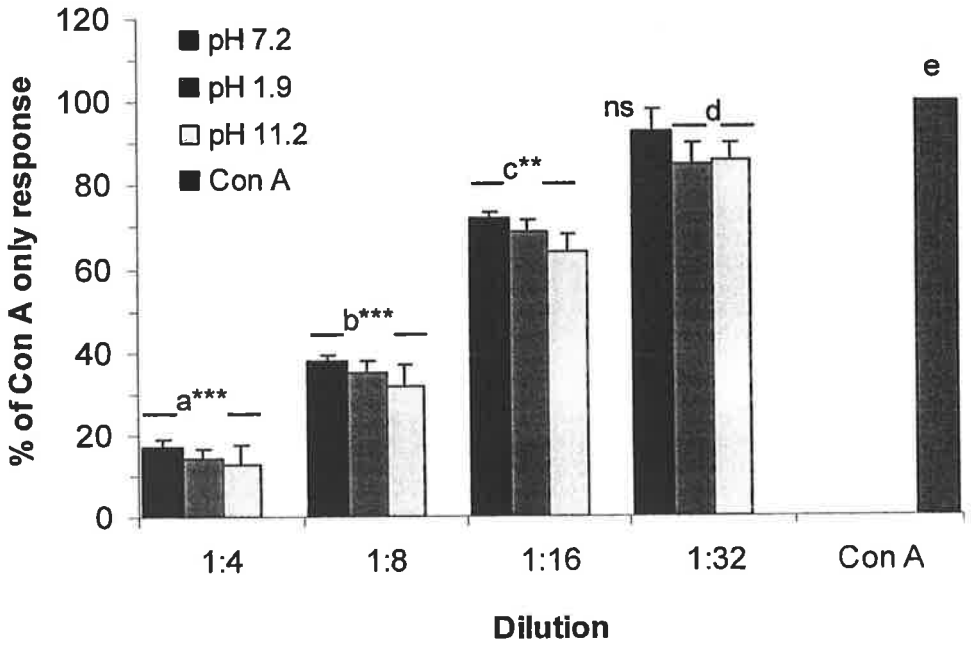


Figure 4.7 Effect of pH on the immunosuppressive activities of low molecular weight TE (LMW-TE).

Comparisons are between the respective untreated LMW-TE and each treated group at each dilution. No significant difference ($P>0.05$) was observed between treatments compared with untreated LMW-TE at the appropriate dilution. Values are Mean ± SEM of three different cultures.

Values with different superscripts show significant differences at $P<0.05$; **: $P<0.01$; **: $P<0.001$; ns = non significant when compared with Con A only response. Data were analysed by analysis of variance and the differences were tested with Duncan's new multiple-range test.

These results corroborate the results presented in Section 4.2.3, Figure 4.2 that fractions with high molecular weight gave much less immunosuppressive activity. Similarly, in Section 3.2.4 the finding that temperature and pH changes do not abrogate the immunosuppressive activity of crude TE may be explained by the presence of low molecular weight compounds in the crude TE.

4.4.3.2. Proteolytic stability of LMW-TE immunosuppressive activity

In order to determine whether the suppressive activity of LMW-TE is affected by proteolytic digestion, the LMW-TE was treated with trypsin (a serine protease which cleaves peptide bonds at the c-terminal side of arginine and lysine) and proteinase K (a serine protease cleaving peptide bonds at the C-terminal side of N-substituted, hydrophobic amino acids).

The results (Figure 4.8) revealed that the suppressive activity of the LMW-TE was affected partially by trypsin but not by proteinase K. This is consistent with studies presented with crude TE in Section 3.2.7 and indicates that the active species are peptidic in nature and contain basic amino acids (arginine and lysine) but not hydrophobic amino acids.

Addition of trypsin followed by addition of foetal bovine serum (FBS), to terminate the action of trypsin, reduced the activity of LMW-TE. However, addition of proteinase K followed by AEBSF did not alter the activity of LMW-TE. As demonstrated previously (Section 3.2.7) neither trypsin nor proteinase K exhibit inhibitory effects on Con A stimulated lymphocytes (see Figure 4.8.a and b).

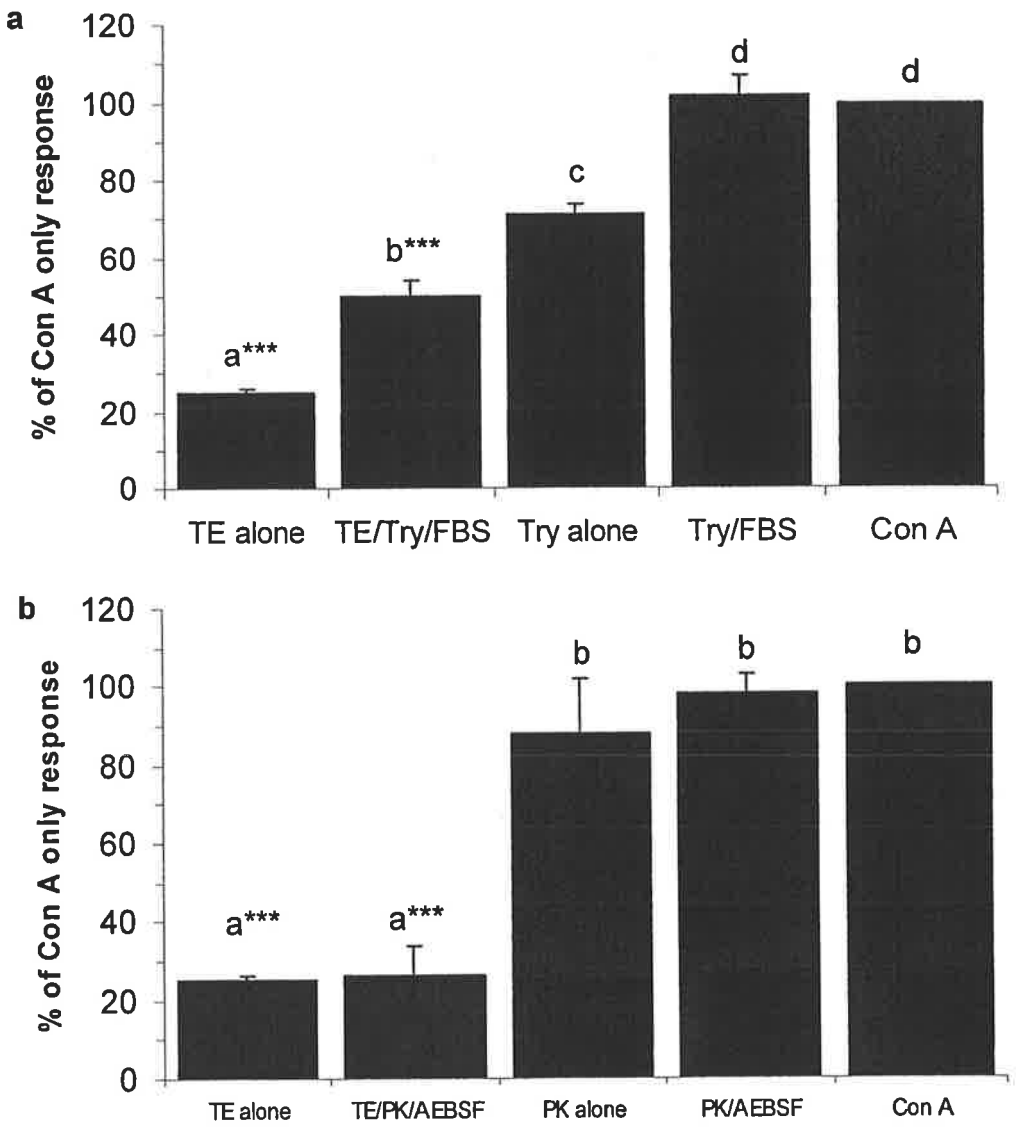


Figure 4.8 (a) Proteolytic treatments of the rat low molecular weight testicular extracts (LMW-TE). The LMW-TE (1:4) was treated with trypsin or (b) proteinase K for 60 minutes.

Reactions were terminated by 10% foetal bovine serum (FBS) or AEBSF respectively (see experimental procedures, section 3.2.6). Values are Mean ± SEM of three different cultures. Different superscripts indicate significant differences at $P<0.01$; ***$P<0.001$ when compared to the Con A only response (Student's *t* test).

4.5. Effects of timing of addition the LMW-TE on Con A-induced lymphocyte proliferation

4.5.1. Aims

In order to investigate at which stage the LMW-TE inhibits the lymphocyte proliferation after Con A stimulation, the effect of delayed addition of LMW-TE to activated lymphocyte cultures was investigated.

4.5.2. Methods

Diluted LMW-TE (1:4, 50 µl/well) was added to cultures of splenic T cells at various times (0, 12, 24, 36 and 48 h) after Con A addition. The cells were cultured for a total period of 72 h, and lymphocyte proliferation then assessed as in 3.2.1.2.

4.5.3. Results and Outcomes

Delaying the addition of the low molecular weight TE (LMW-TE) resulted in a reduction of the suppressive effects on the proliferation of splenic T cells (Figure 4.9). Although suppression was effective when LMW-TE was added at the start of culture, LMW-TE was also able to inhibit the proliferation of splenic T cells ($P<0.01$) up to 48 h after Con A stimulation. These results suggest that the immunosuppressive action is predominantly effected at an early stage of stimulation, and it continues to affect many cells, even if added late in culture. These results with LMW-TE are similar to those obtained with crude TE as presented in Figure 3.2.

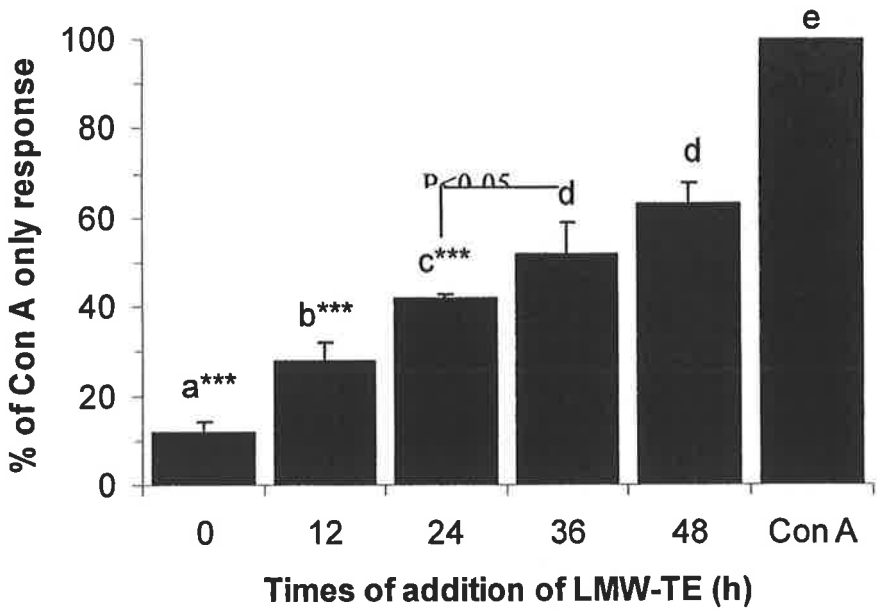


Figure 4.9 Effect of delayed addition of low molecular weight TE (LMW-TE, 1-5 kDa) on the Con A proliferative response of splenic T cells in culture.

LMW-TE (1:4 dilutions) was added at the initiation of culture at 0 h, or after 12, 24, 36, or 48 h of culture of Con A-stimulated splenic T cells. Values are the Mean ± SEM of three separate cultures, expressed as a percentage of the response of splenic T cells to Con A in the absence of LMW-TE (100%). Values with different superscripts are significantly different at $P<0.01$; ***$P<0.001$ when compared with Con A only response (Student's *t* test).

4.6. Effects of LMW-TE on the proliferation of Rat-2 cells

4.6.1. Aims

To determine whether the LMW-TE affected the proliferation of cells other than lymphocytes, Rat-2 fibroblast cells were used in comparison to splenic T cells. Short-term incubation studies were also undertaken to investigate whether the suppressive effect of the LMW-TE was due to inhibition of proliferation or due to a general cytotoxic effect.

4.6.2. Methods

LMW-TE (1:2-1:16 dilutions) was pipetted into wells of a 96-well flat-bottomed culture plate (50 µl/well). Rat-2 fibroblast cells (2.5 x 10^4 cells/ml, see 2.7) were then added (50 µl/well). In parallel splenic T cells (5 x 10^6 cells/ml) were also cultured with Con A (2.5 µg/ml, 50 µl/well). The volume of the wells was then made up to 200 µl by adding 50 µl of RPMI 1640 containing serum (10% foetal bovine serum) and 50 µl of RPMI 1640 without serum to each well of each treatment. The cells were then cultured for a total period of 72 hours at 37° C, in an atmosphere of 5% CO_2 in air. The controls for Rat-2 cells were cultures of Rat-2 cells alone (without LMW-TE), whereas the controls for the splenic T cells were the cultures of splenic T cells with Con A only. Due to the lack of appreciable levels of nuclear thymidine kinase in the Rat-2 cell line (see 2.7), cell proliferation in both cell types was measured by a colorimetric assay developed by Mossman (1983), as described in section 2.7.

Four hours before harvesting the cells, 20 µl of stock MTT solution (5 mg/ml, see 2.7) was added to each well, and the cells were preincubated for another four hours. The plate was then centrifuged at 1000 x g for five minutes at 20°C and the supernatants from each well were carefully discarded. Following this, acid-propanol (propanol containing 0.04 N HCl, i.e. 4 ml of 1 N HCl for 96 ml of propanol; 100 µl) was added to each well. Finally, the solution in each well was mixed with a multichannel pipetter to uniformly dissolve the residual blue formazan crystals and the plates were read on a Titertek Multiscan microplate reader (Flow Laboratories, North Ryde, Australia), with the absorbance at 570 nm. The cultures were performed in triplicates and the assays were repeated three times.

4.6.3. Results and Outcomes

The results of the colorimetric assays are presented in Figure 4.10. It was found that the LMW-TE did not affect the proliferation of Rat-2 cells even at high concentration (1:2 dilutions), while lymphocyte proliferation was strongly ($P<0.01$) affected even at a low (1:16 dilutions) concentration. These results suggest that the immunosuppressive activity of LMW-TE is not a general phenomenon and may be specific to lymphocytes. However since in this experiment only one cell line was used, the specific effect of LMW-TE needs to be further tested using other cell types.

Although the proliferation of Rat-2 cells was not affected by culturing with LMW-TE for 72 h, there is a concern that this cell line is particularly resilient. Therefore, the effect of LMW-TE was also investigated over a short period of

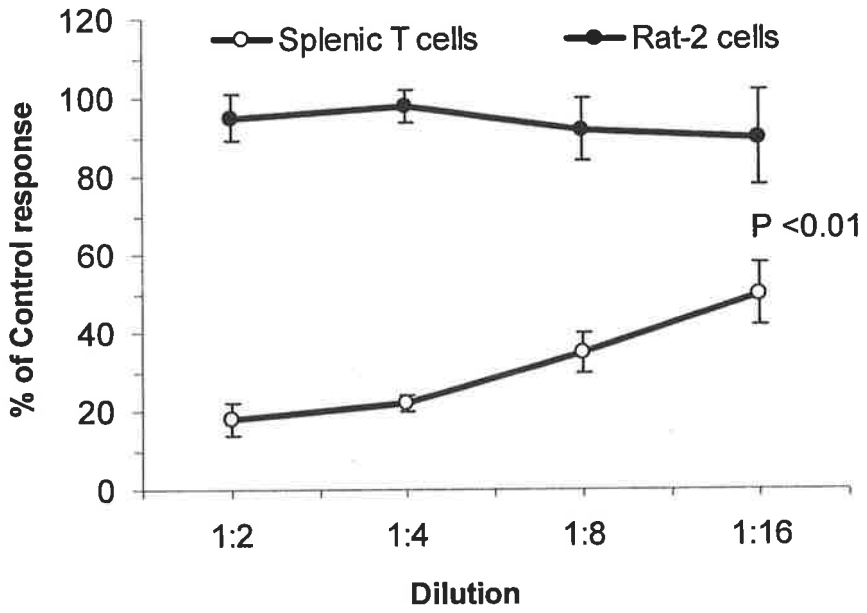


Figure 4.10 Effects of low molecular weight TE (LMW-TE) on the proliferative response of splenic T cells to Con A and of Rat-2 fibroblast cells.

The proliferations were measured with MTT colorimetric assays. The results are expressed as percentage of response of splenic T cells to Con A, or response of Rat-2 cells in the absence of LMW-TE. Values are the Mean SEM; n =3 for splenic T cell-, and n = 4 for Rat-2 cell cultures. LMW-TE did not suppress the proliferation of Rat-2 fibroblast cells. $P<0.01$ (Student's *t*-test).

culture. The results are presented in Figure 4.11. Even though the proliferation of Rat-2 cells changed over the period of culture, the proliferation was not affected by the presence of the LMW-TE, even with a short time (24 h) of culture, at all concentrations of the LMW-TE compared to the control (cells only). These results suggest that the LMW-TE is not cytotoxic, and that the effects reported in Figure 4.10 are not simply due to the robustness of the Rat-2 fibroblast cell line.

4.7. Effects on IL-2 production

4.7.1. Aims

In chapter 3 it was proposed that the mechanism of action of the immunosuppressive activity in crude TE was through the inhibition of IL-2 production. In the present study we observed that semi-purified LMW-TE immunosuppressive factors in the rat testis gave the strongest immunosuppressive activity. It was therefore of interest to investigate whether this activity had a similar mechanism of suppressing activated T cells as the crude TE. A similar question was also posed for the HMW-TE. For these reasons, IL-2 production from Con A-activated T cells cultured in the presence of LMW-TE or HMW-TE was investigated.

4.7.2. Methods

The investigation of IL-2 production (through the response or proliferation of CTLL cell cultures) was undertaken using the same procedures as described previously for use with the crude TE (Section 3.2.103.2.10).

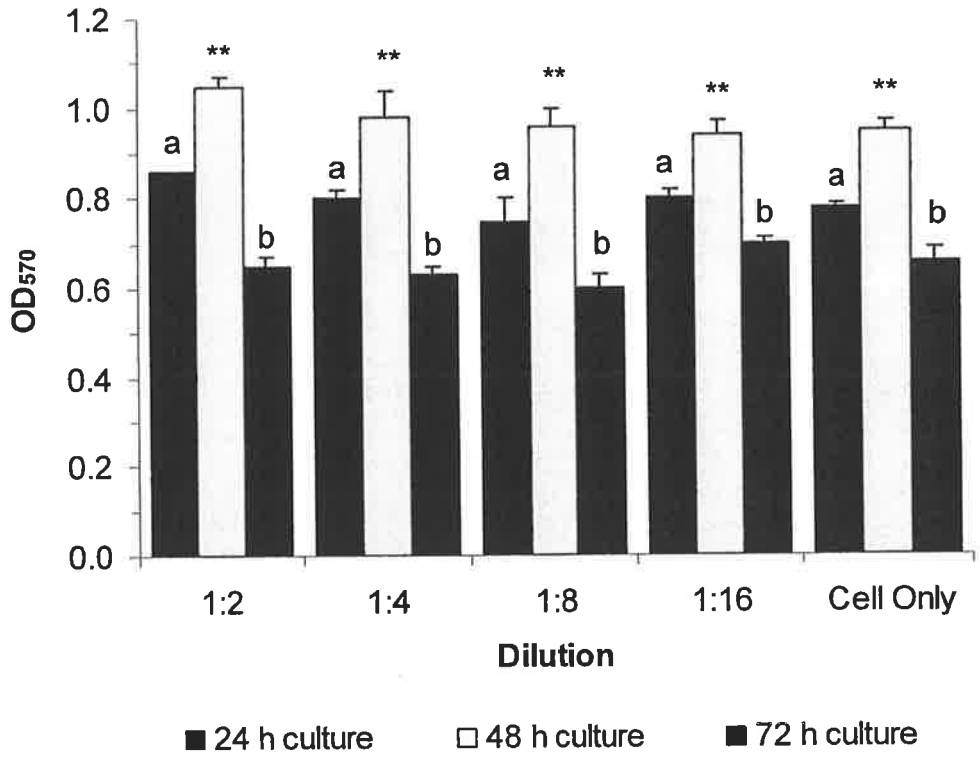


Figure 4.11 Effects of low molecular weight TE (LMW-TE) on the proliferative response of Rat-2 fibroblast cells at different times of cultures.

The proliferative responses were measured with MTT colorimetric assays in three different cultures. Values with different superscripts show significant differences at $P<0.05$; $^{**}P<0.01$ compared with24 and 72 h of cultures. No significant difference ($P>0.05$) was observed between dilutions compared with the Rat-2 cells cultured without the presence of LMW-TE at the appropriate time of culture. Data were analysed by analysis of variance and the differences were tested with Duncan's new multiple-range test.

Supernatants from inactivated or activated (2.5 μg/ml of Con A) splenic T cells cultured for 48 h with or without LMW-TE or HMW-TE (1:4 dilutions) were collected. The supernatants were tested for IL-2 activity by their ability to support the growth of the IL-2-dependent cell line CTLL, in a proliferative assay as outlined in Chapter 3.2.10. Assays were done on triplicate cultures.

In addition, the effects of TE immunosuppressive factors (1:4 dilutions) on the proliferation of splenic T cells induced with recombinant rat IL2 (rrIL2) were also investigated with the procedure described in 3.2.11.

4.7.3. Results and Outcomes

Results of the investigation of IL-2 production by activated T cells cultured in the presence of LMW or HMW testicular extracts using the CTLL or splenic T cell proliferation assay are presented in Fig. 4.12. It is clear from these results that LMW-TE, unlike crude- or HMW-TE, did not affect T-cell production of IL-2 (Figure 4.12.b).

Figure 4.13 shows the immunosuppressive activities of rat testicular extracts on the rr IL2-induced T cells proliferation. In general, it can be seen that all fractions were able to suppress IL-2 induced T cell proliferation with the effects becoming profound in the presence of ant-rrIL-2 antibody.

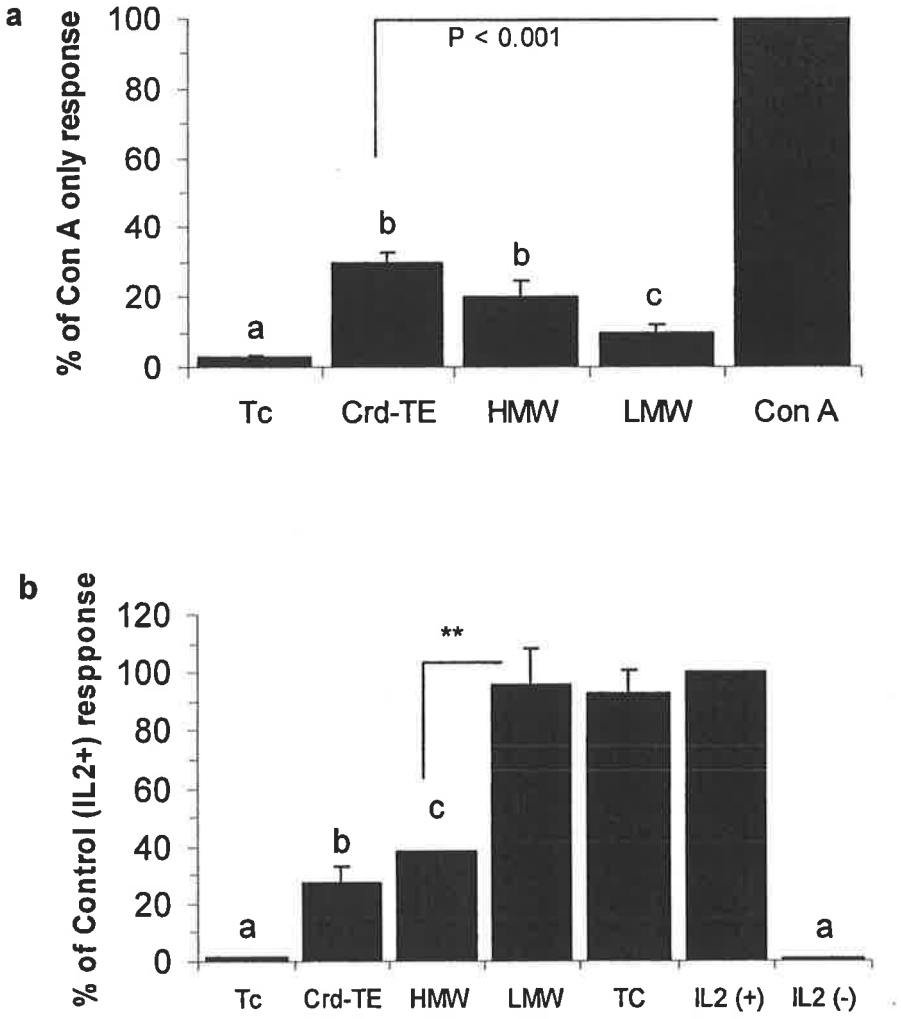


Figure 4.12 (a) Proliferation of Con A-induced splenic T cells cultured with crude- (Crd), high- (HMW), or low- (LMW) molecular weight testicular extracts.

The proliferation is expressed as a % of the Con A only response. Experimental procedure was as outlined in 3.2.1.2.

(b) Proliferation of IL-2-dependant-CTLL cells cultured with the supernatants (50 µl each) of splenic T-cells cultured for 48 h, alone (Tc) or with: testicular extracts (Crd, HMW, or LMW) and Con A, or Con A alone (TC).

As controls, CTLL were cultured with- (IL2+) or without (IL2-) interleukin 2. The proliferation is expressed as a % of control (CTLL with IL2) response. Experimental procedure was as outlined in 3.2.10. Values are Mean ± SEM of triplicate cultures. Different superscripts show significantly differences at $P < 0.05$; **: $P < 0.01$.

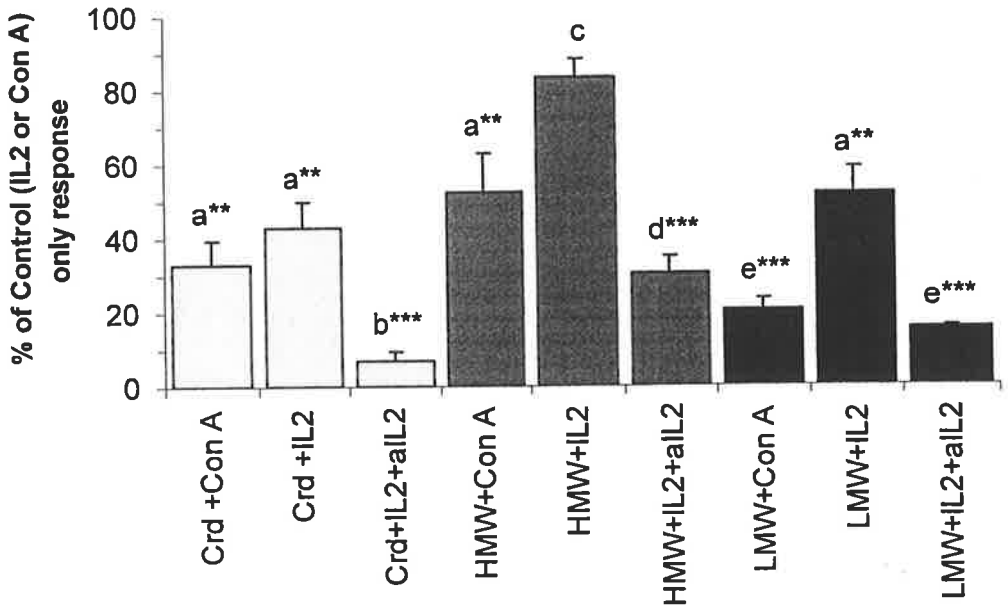


Figure 4.13 Effect of TE (1:4) on IL2 induced T cells proliferation in the presence of anti IL2.

T cells were cultured in the presence of: (1) Crude TE and Con A (Crd+ConA), or IL2 (Crd+IL2), or IL2+ anti-rrIL2 antibody (Crd+IL2+aIL2); similarly for (2) high molecular weigh TE + Con A (HMW+ConA), or HMW-TE + IL2 (HMW+IL2), HMW+IL2+anti-rrIL2 antibody (HMW+IL2+aIL2); and (3) low molecular weigh TE + Con A (LMW+ConA), or LMW-TE + IL2 (LMW+IL2), LMW+IL2+anti-rrIL2 antibody (LMW+IL2+aIL2

Values are Mean ± SEM of triplicates cultures. Different superscripts indicate significant differences at $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared to the control (**IL-2** or Con A, 100%) **only response (Student's *t* test).**

4.8. Apoptosis

4.8.1. Aims

In the present studies it has been demonstrated that crude-, high molecular weight-, or low molecular weight rat testicular extracts were able to suppress Con A induced T cell proliferation in a dose dependent manner as presented in Chapter 3. In this section we analysed further whether the immunosuppressive activity could be related to the mechanisms of programmed cell death (apoptosis).

4.8.2. Methods

The apoptosis assays were performed using the Vybrant™ Apoptosis Assay Kit #2 (Molecular Probe, The Netherlands) according to the manufacturer's protocol. Briefly splenic T cells alone, or Con-A induced T cells were cultured for 48 h in the presence of diluted (2 to 8 fold dilutions) crude-, HMW-, and LMW-TE as outlined in 3.2.1.2. Cells were then harvested and washed firstly in cold PBS then in 1X Annexin-Binding Buffer (ABB, 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Treated and untreated cells (T cells only, or T cells with Con A but without TE) were then harvested, suspended and adjusted to 1 X 10^6 per ml in 100 µl ABB. Into each 100 µl of cell suspension 5 µl of 100 µg/ml Alexa Fluor 488 annexin V and 1 µl of 100 µg/ml propidium iodide working solution was added and mixed gently. The cells were subsequently incubated in the dark at room temperature for 15 minutes. Following this incubation period, 400 µl of ABB was added, mixed gently and the samples kept on ice for analysis by flow cytometry (FCM, BD FACS

Canto™, BD Biosciences Immunocytometry System, USA) at room temperature by differentiation of at least 20,000 cells. Dot plots were evaluated quantitatively using BD FACSDiva™ Flow Cytometry Acquisition and Analysis Software (BD Biosciences Immunocytometry System, USA). The numbers of apoptotic cells were recorded as a percentage of the treated cells, corrected for the basal level of apoptosis seen in the control cultures (i.e. cells without TE). Assays were carried out in triplicates.

4.8.3. Results and Outcomes

Figure 4.14 a, b, c, and d are representations of FCM analysis of the differentiation of Con A-induced T cells without TE (control), or with the presence of diluted (1:4) crude-, HMW-, and LMW-TE respectively. In each panel, the lower left quadrant (P1) corresponds to intact-viable T cells, the lower right quadrant (P4) corresponds to apoptotic T cells, while the necrotic cells are shown in the upper right quadrant (P5). The histogram in Figure 4.15 shows the effects of rat TE on the Con-A induced T cells for (a) apoptosis and (b) proliferation. The results show that the suppression of Con A induced T cell proliferation by crude-, HMW-, and LMW-TE all involve the process of some programmed cell death or apoptosis mechanisms. This is most pronounced in the LMW-TE fraction (and the crude TE).

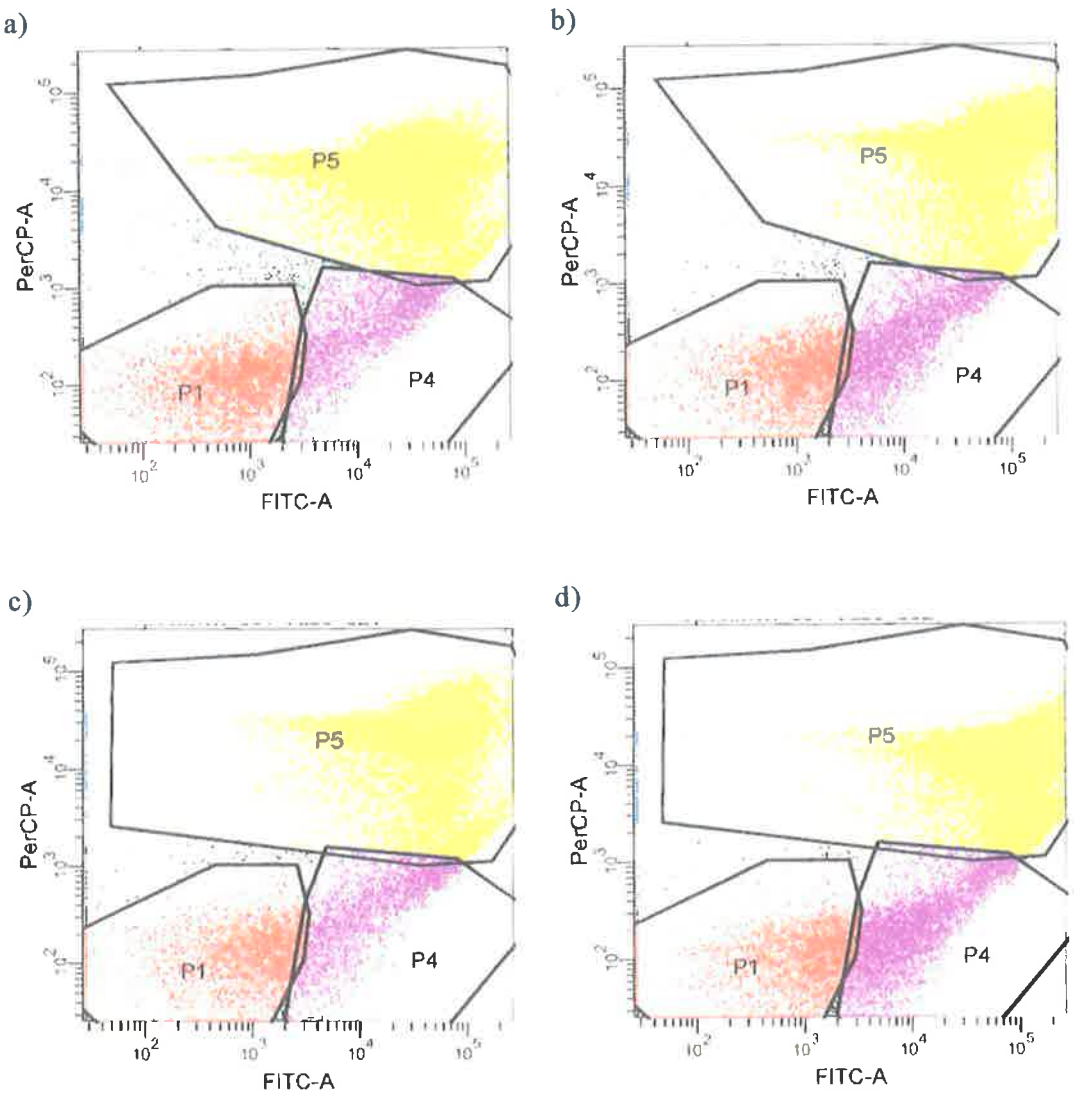


Figure 4.14 Representative flow cytometric analysis (FCM) of apoptotic cells.

The apoptotic cells were assayed with Vybrant™ Apoptosis Kit (Annexin, FITC-A/Propidium iodide, PerCP-A) in (a) untreated (as control) T cells, and cells treated with 1:4 dilutions of (b) crude-, (c) HMW-, and (d) LMW-TE. For each panel, the lower left quadrant corresponds to intact-viable T cells (P1), the lower right quadrant corresponds to apoptotic T cells (P4), while the necrotic T cells are shown at the upper right quadrant (P5).

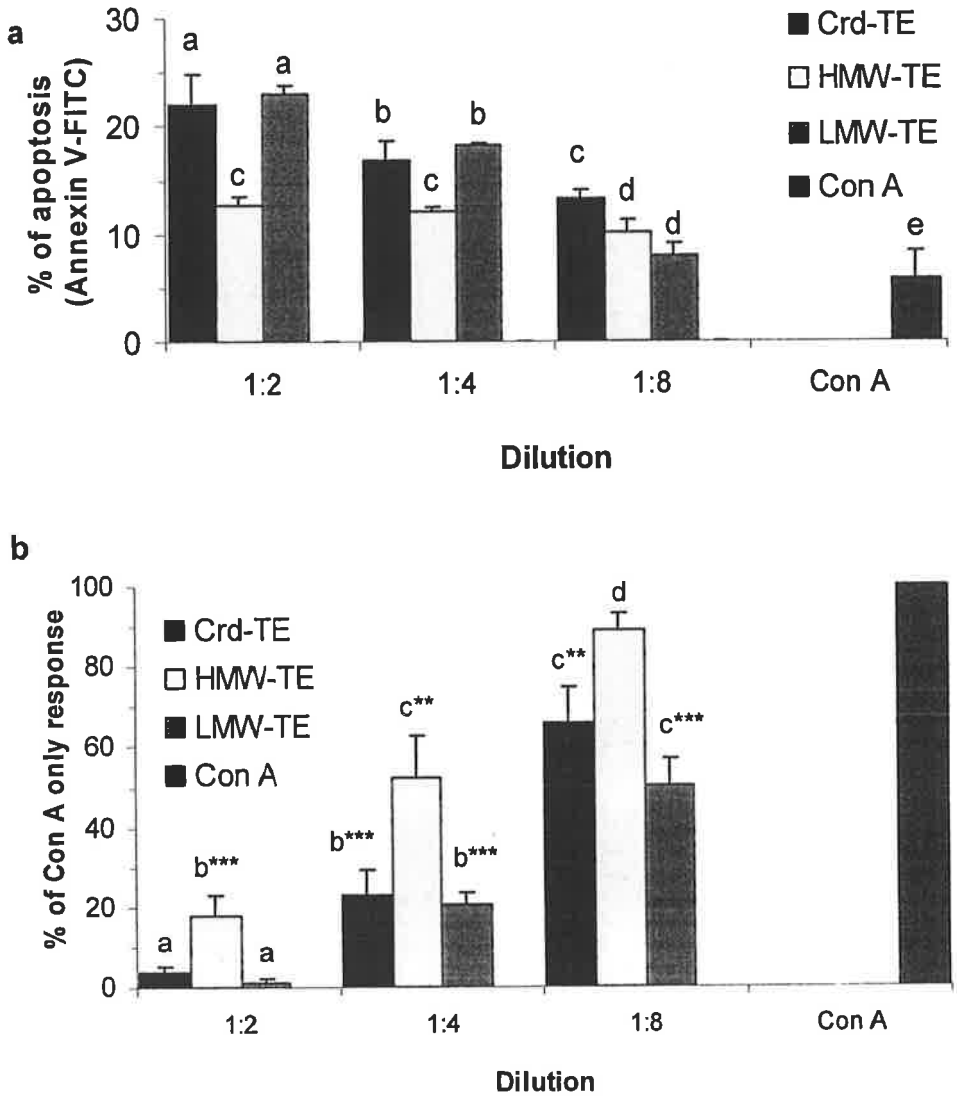


Figure 4.15 Effects of testicular extracts on (a) Con A-induced T cell apoptosis, and (b) the corresponding Con A-induced T cell proliferations.

Values are Mean ± SEM of triplicate cultures. Values with different superscripts are significantly different at $P<0.05$; **: $P<0.01$; ***: $P<0.001$ when compared with Con A only response (Student's *t* test).

4.9. Effects of immunosuppressive activity on CD4 and CD8 T cells

4.9.1. Aims

Many studies have shown that depletion of $CD4^+$ T cells can reduce the rejection of allografts by the host (Pearson et al., 1993). This indicates that $CD4^+$ T cells which recognize antigen-presenting cells via MHC class II molecules may play an important, if not compulsory, role in allograft rejection. Interestingly, studies on the T cell subsets in the testis shown that $CD8^+$ T cells are more frequent observed compared to $CD4^+$ T cells (Hedger et al., 1998b), which is quite different to blood or other connective tissues in which $CD4^+$ T cells are more commonly observed (Westermann and Pabst, 1992; see also Section 2.4.6). Whether the lack of $CD4^+$ T cells in the testis is related to the immune privilege status of the testis is still not clear. It has been suggested that the accumulation of $CD8^+$ T cells in the testis may possibly be due to the presence of testicular immunomodulatory cytokines (see Tompkins et al., 1998, and Hedger et al., 1998b).

In this section investigation on the effects of the TE immunosuppressive peptides on $CD4^+$ and $CD8^+$ T cells is reported.

4.9.2. Methods

$CD4^+$ and $CD8^+$ T cell populations were prepared by negative selection with the "StemSep™ Rat T cell Separation" (Stemcell Technologies, Vancouver, Canada) procedure according to the manufacturer's protocol. Briefly, two aliquots of splenic T cells isolated as outlined in 2.4.4, were prepared (5-10 x

10^7 cells/ml each); one for $CD4^+$ and one for $CD8^+$ T cell preparations. The cells were resuspended in PBS with 2% (v/v) foetal bovine serum. Into each one ml suspension, 100 µl of the specific antibody cocktail (Rat $CD4^+$ T #3L10142 or Rat $CD8^+$ T #2H156226 antibodies) was added and the suspensions then incubated on ice for 30 minutes. Following incubation, 60 µl of magnetic colloid (# 04F11840) per ml cells was added and mixed well. The suspensions were incubated on ice for 15 minutes then passed through a magnetic separation apparatus. The flow through was collected and washed with PBS. The isolation process was repeated three times. The isolated $CD4^+$ and $CD8^+$ T cells were then cultured with or without testicular extracts to investigate the effects of immunosuppressive activity in the testicular extracts on the isolated T cell subsets as described in Section 3.2.1.2.

4.9.3. Results and Outcomes

Both the high and low molecular weight testicular extracts inhibited significantly ($P<0.001$) Con A induced $CD4^+$ and $CD8^+$ T cell proliferation at 2- and 4-fold dilutions (Figure 4.16). Interestingly, the LMW-TE had a more significant suppressive effect on both $CD4^+$ and $CD8^+$ T cells than did the HMW-TE and continued to suppress $CD8^+$ proliferation at greater dilutions (8 and 16 fold) than did the HMW-TE.

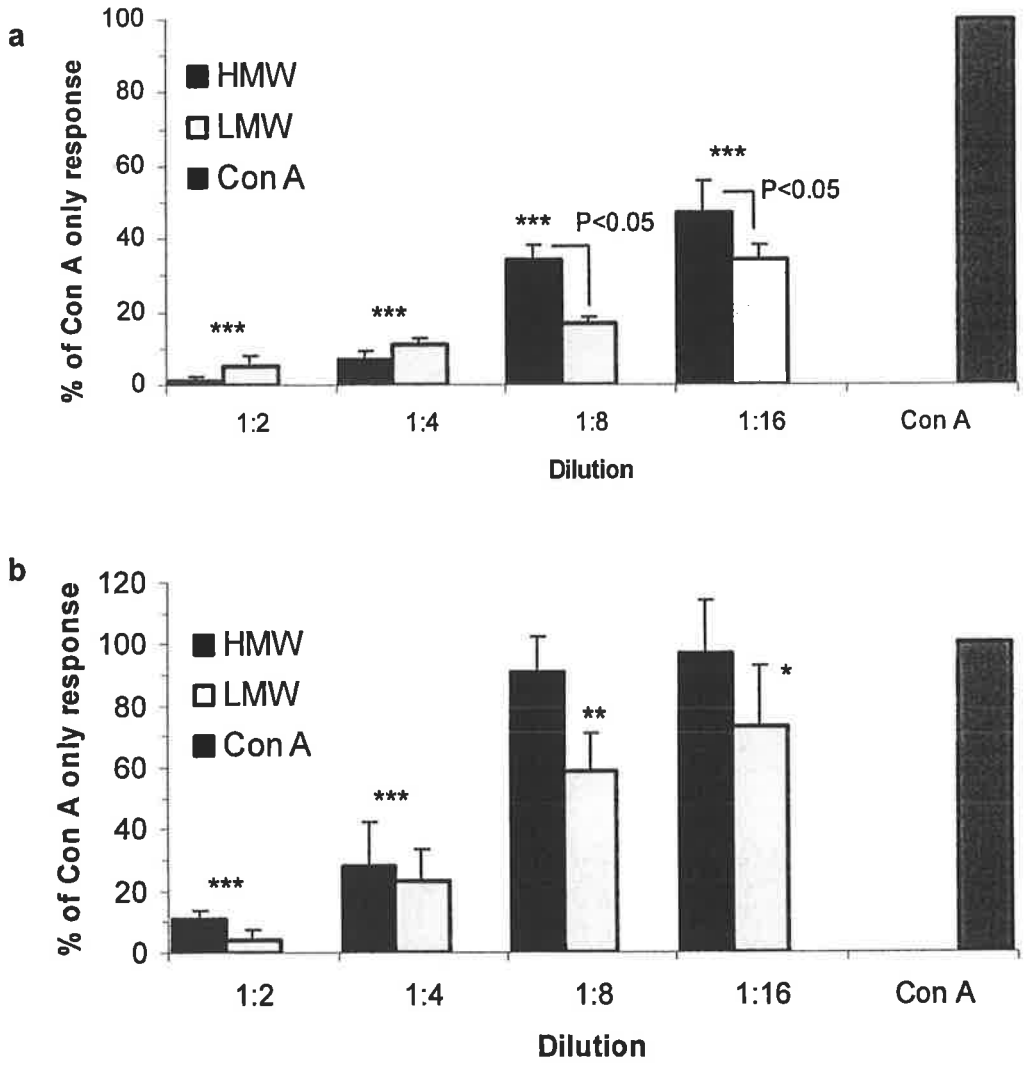


Figure 4.16 Effects of high- (HMW-TE) and low- (LMW-TE) molecular weight testicular extracts on Con A induced (a) CD4+ or (b) CD8+ T cell proliferation.

Values are Mean ± SEM of triplicate cultures. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ when compared to the Con A only response (Student's *t* test).

PART TWO

Purification of the immunosuppressive factors

In the following experiments, the chromatographic separations were performed on a SMART System HPLC (Pharmacia Biotech, Uppsala, Sweden).

Because the more potent immunosuppressive activity was observed in the fractions with relative molecular weight around 6.5 kDa or less (see 4.2.3), purification was focused mainly on the LMW-TE. For this purpose, the LMW-TE was prepared using Centricon-10 concentrators (Amicon, Beverley, MA, USA, Mr cut off 10 kDa). This method was chosen because, firstly, the yield obtained was relatively consistent in volume and in molecular weight cut off (10 kDa), and secondly because the filtrate produced was suitable for injection into the instruments (such as HPLC columns) used in the purification process.

Aliquots of crude TE (2 ml each, prepared as in 2.3.1) were applied to a Centricon filter unit and centrifuged at 2000 x g for 120 minutes at 4° C. The filtrates (Mr < 10 kDa; 1.5 ml from 2 ml crude TE) were pooled, filter sterilised (0.22 μm filter), aliquoted (one ml), and stored at -20° C until used for the purification process. In some cases, TE < 10 kDa was concentrated using a vacuum evaporator (Vacuspin "Virtis", NY, USA) and stored at - 20°C until purified.

4.10. Estimation of molecular weight of the immunosuppressive activity of LMW-TE

4.10.1. Aims

Studies presented earlier in this thesis suggest that the immunosuppressive activity in the rat testis is a small peptide (see 4.2.3). In order to investigate more accurately the relative molecular weight of this putative peptide, the TE Mr <10 kDa fraction was further separated using Superdex Peptide PC 3.2/30, a prepacked column for high performance size exclusion chromatography of peptides.

4.10.2. Methods

TE < 10 kDa (25 ul) was loaded on to a pre-equilibrated Superdex peptide column and run in PBS (pH 7.2) at a flow rate of 100 µl/min. Fractions (50 µl each) were assessed for their immunosuppressive activity as in 3.2.1.2. The molecular weight of the active fractions was estimated by comparison to the retention times of standard molecular weight proteins (Figure 4.17). These molecular weight standards were Glycine-6 (0.36 kDa), Substance P (1.35 kDa), Gastrin I (2.13 kDa), Aprotinin (6.5 kDa), and Cytochrome C (12.5 kDa).

4.10.3. Results and Outcomes

Results of interpolating data showed that the molecular weight of immuno suppressive activity in TE < 10 kDa was estimated at between 1 and 3.5 kDa (Figure 4.18, Figure 4.19, and Figure 4.19).

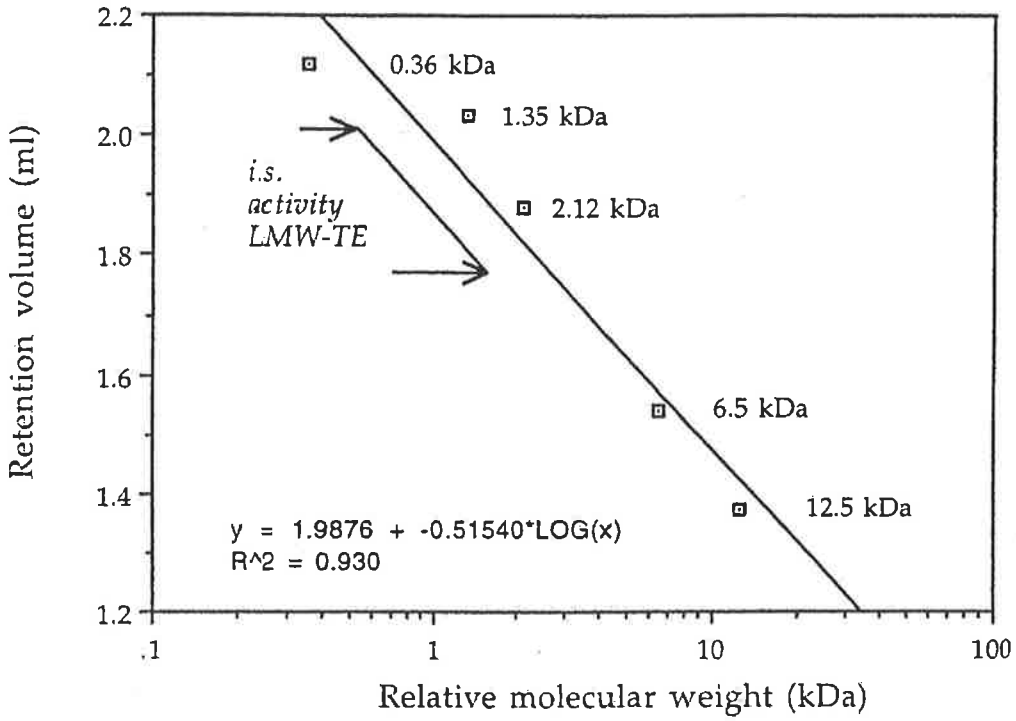


Figure 4.17 Standard curve for the estimation of relative molecular weight (Mr) of LMW-TE using Superdex Peptide PC 3.2/30.

Samples (25 µl each) were eluted by linear salt gradients (NaCl, 0-1 M) with a flow rate of 100 µl/min, for 60 minutes. Standards used (kDa) were $(Gly)_6$ (0.36), Substance P (1.35), Gastrin I (2.12), Aprotinin (6.5), and Cytochrom C (12.5). Immunosuppressive (i.s) activity was found in Fig. 4.11 to correspond to the molecular weight range indicated.

The interpretation of the TE molecular weight using chromatographic separation was carried out in physiological buffers. In such condition it should be noticed that marker proteins used for the size calculations may not at all apply to the separated sample proteins or molecularweight of the biomolecules. The use of classical methods to determine the molecular size of proteins and peptides include chromatographic separations under denaturating conditions, most frequently sodium dodecyl sulphate poliacrylamide gel electrophoresis (SDS-PAGE), is more reliable. For instance, a hydrophobic and/or fibrillar structure would frequently result in retardation on gel permeation chromatography compared to a hydrophilic and/or globular protein of the sam molecular size, when separated under non-denaturing conditions. However, separation under denaturing condition was incompatible with the bioassay methods used for detection of the TE immunosuppressive activity in the present study.

Figure 4.18. Molecular weight determination of LMW-TE using size exclusion chromatography with a Superdex PC 3.2/30 column.

LMW-TE (25 µl; Mr < 10 kDa) in PBS was injected onto the column and then eluted using PBS for 60 min with a flow rate of 100 µl/min. Potentially important peaks related with immunosuppressive activity are found in fractions 28-30, 32-37, and 38-40 (see also Figure 4.19).

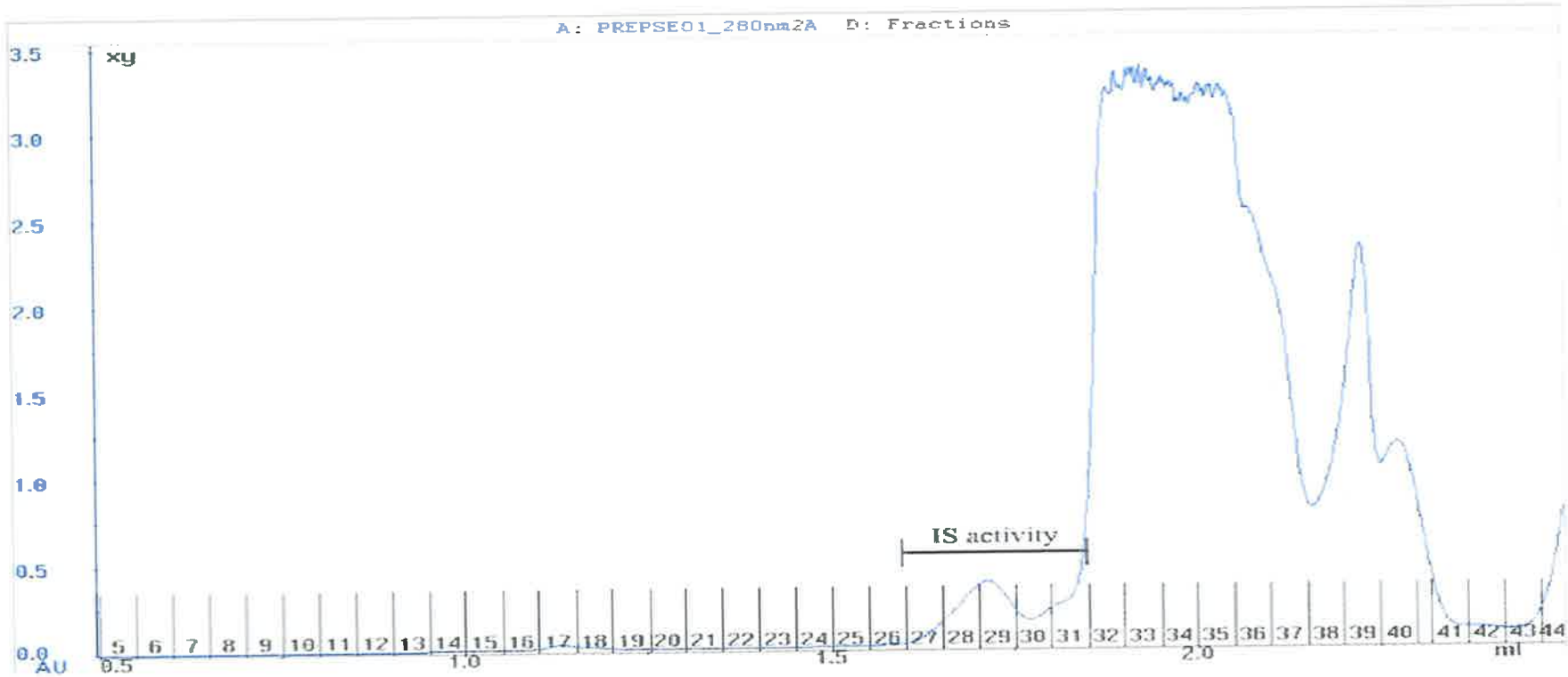


Figure 4.18 Molecular weight determination of LMW-TE using size exclusion chromatography with a Superdex PC 3.2/30 column.

Figure 4.19. Proliferation of splenic T cells induced by Con A in the presence of LMW-TE fractions fractionated using Superdex Peptide PC 3.2/30 column.

A significant inhibition was achieved with fractions 28-30 which formed a significant protein peak in the elution profile (see Figure 4.18) and which correlates with a molecular weight of approximately between 1 and 3.5 kDa (see Figure 4.17). Values are the mean of duplicate cultures expressed as a percentage of the Con A only response (100%).

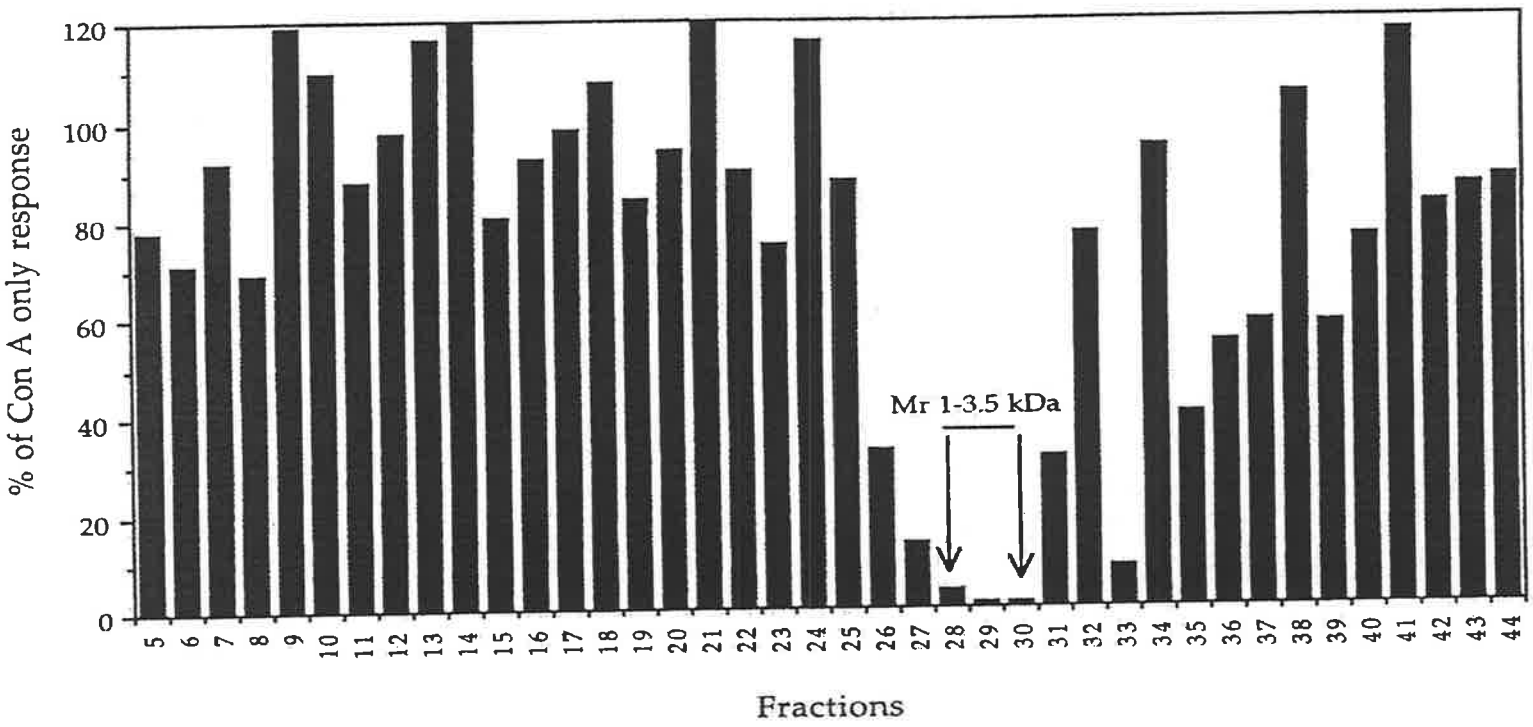


Figure 4.19 Proliferation of splenic T cells induced by Con A in the presence of LMW-TE fractions fractionated using Superdex Peptide PC 3.2/30 column.

4.11. Exploration of potential matrices for the purification of the size fractionated LMW-TE

Purification of size-fractionated LMW-TE proved immensely difficult, and a number of potential matrices and techniques were investigated for this purpose.

4.11.1. High performance ion exchange chromatography

4.11.1.1. Methods

Initial attempts to purify the immunosuppressive activity present in the LMW fraction of testicular extract (LMW-TE) were conducted using the method of high performance ion exchange (HP-IEX) chromatography with both cation (Mono S) and anion (Mono Q) exchange resins. Mono S, PC 1.6/S (0.10 ml) and Mono Q, PC 1.6/S (0.10 ml) (Pharmacia Biotech, Uppsala, Sweden) are designed for fast, sensitive and high-resolution cation and anion (respectively) exchange separations of proteins or peptides in the micro preparative scale. Three pH buffered systems were used in these separations: (a) acidic (formic acid buffered system, pH 4), (b) neutral (phosphate buffered, pH 7.2), and (c) basic (tris buffered, pH 9,0). (Refer to Section 2.8, for the buffer preparation).

4.11.1.2. Results and Outcomes

Results of the HP-IEX chromatography indicated that the immunosuppressive activity bound weakly to the cation exchange (Mono S) resin column in acidic (Figure 4.20 and Figure 4.21), but not in neutral or basic buffered systems. No

Figure 4.20. Elution profile of LMW-TE (blue line, A_{280} nm) preparation by Cation-exchange chromatography on a Mono S, PC 1.6/5 (0.10 ml) column.

LMW-TE (100 µg; 200 µl in formic acid, 50 mM) was injected to the column. The column was equilibrated with formic acid (50 mM, pH 4). Linear gradient elution was performed using a 0 – 1 M NaCl linear gradient (red line) for 60 min with flow rate of 100 µl/min. Fractions 1-25 were tested for immunosuppressive activity as per Figure 4.21.

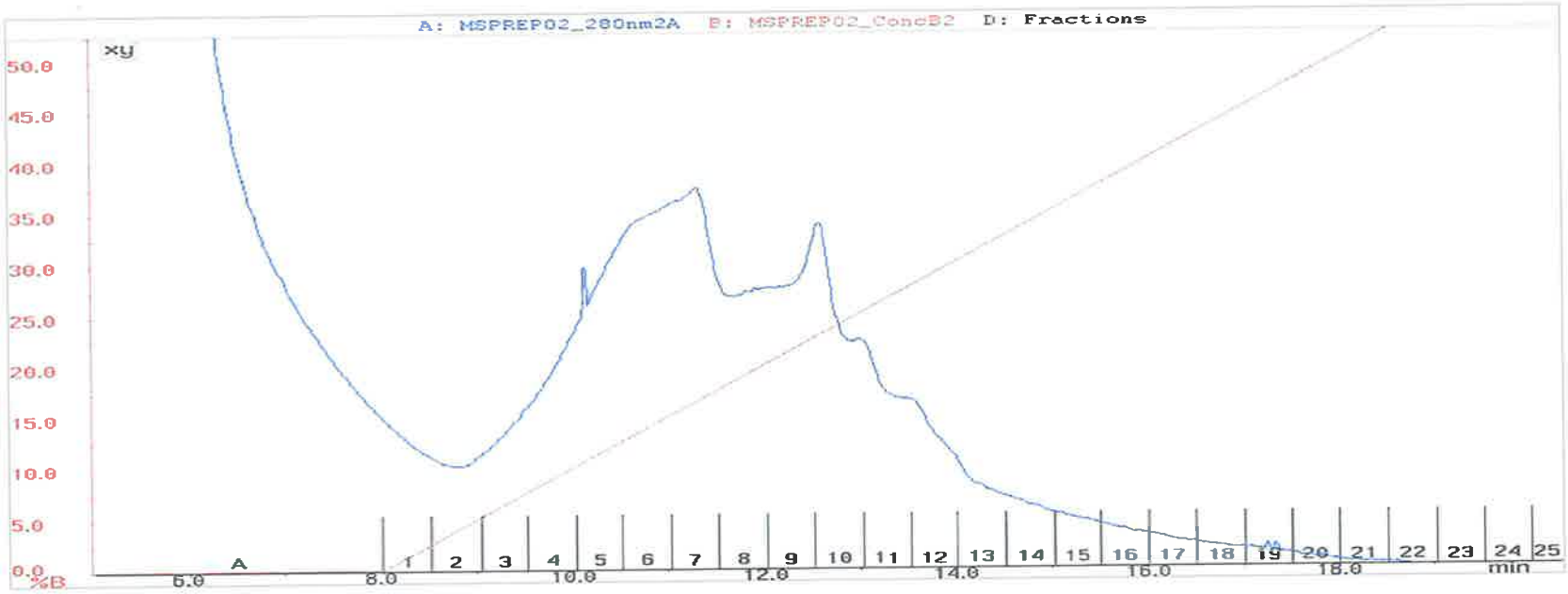


Figure 4.20 Elution profile of LMW-TE (blue line, A_{280} nm) preparation by Cation-exchange chromatography on a Mono S, PC 1.6/5 (0.10 ml) column.

Figure 4.21. Proliferation of splenic T cells induced by Con A in the presence of LMW-TE fractions separated using a Mono S, PC 1.6/5 column (see Figure 4.20).

A significant inhibition of lymphocyte proliferation was achieved with fractions 10 and 12. Values are the means obtained from duplicate cultures presented as a percentage of the Con A only value (100%). Ctrl. = control, i.e. LMW-TE before separation.

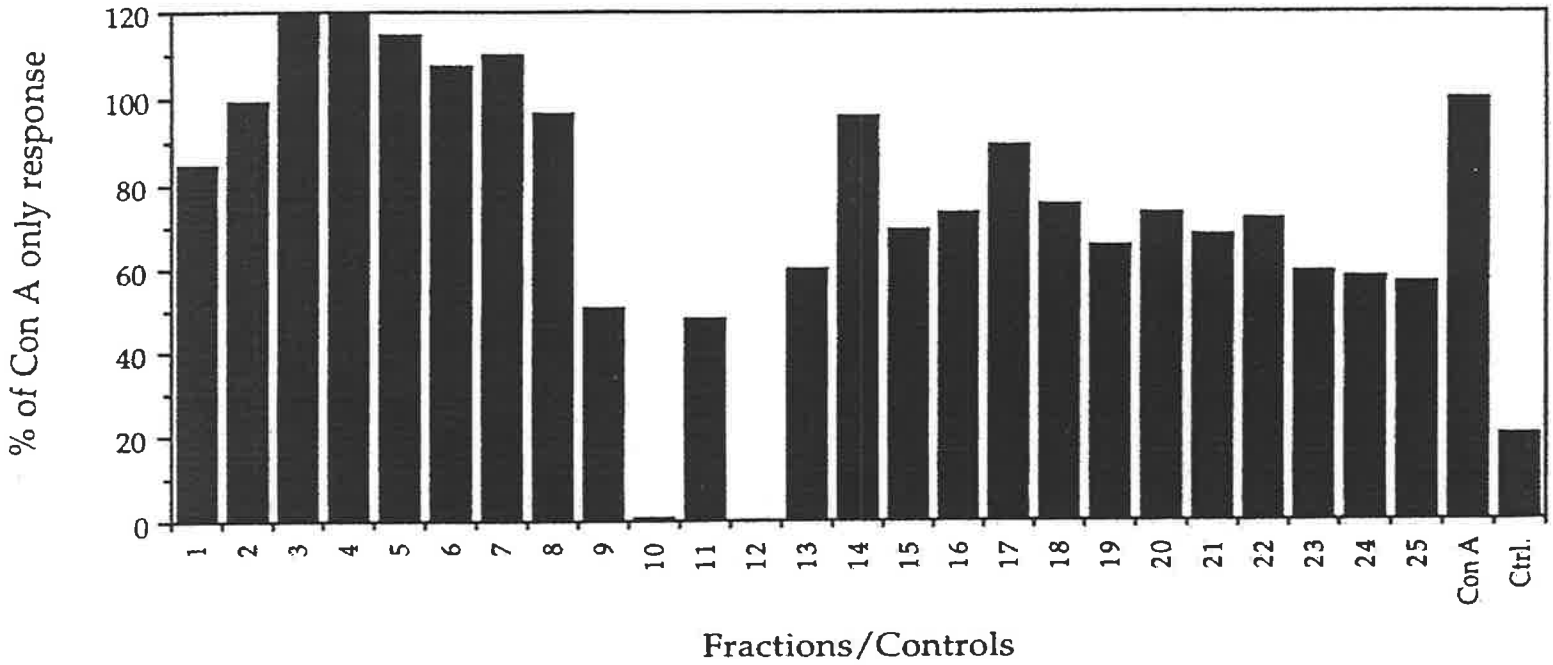


Figure 4.21 Proliferation of splenic T cells induced by Con A in the presence of LMW-TE fractions separated using a Mono S, PC 1.6/5 column (see Figure 4.20).

binding was observed to the anion exchange (Mono Q) resin in any of the three buffer systems.

4.11.2. Reversed-phase HPLC

4.11.2.1. Methods

Since most small peptides do not necessarily bind well to IEX resins, we tried reverse phase high performance liquid chromatography (RP-HPLC), a technique more amenable to peptide separations (Purcell et al., 1992, 1993, 1995, and Aguilar et al., 1993). A stainless steel column SC 2.1/10 (0.35 ml) prepacked with μRPC C2/C18 silica particles (Pharmacia Biotech, Uppsala, Sweden) was used. This column is designed for fast, sensitive, and high-resolution reverse-phase separations of proteins or peptides in the micropreparative scale.

The preliminary studies using an acidic buffer of trichloroacetic acid (TFA, pH 2.1) as an ion-pairing agent showed that in this system the immunosuppressive activity bound weakly to the μRPC column. Thus a second ion-pairing agent, heptafluorobutyric acid (HFBA) was employed. This buffer is often used in determination of amino acid sequences in proteins (Ohguro and Palczewski, 1995) and it has been demonstrated that HFBA is not only an appropriate substitute for TFA, but on many occasions may improve resolution or enhance selectivity of peptide separations (Ohguro and Palczewski, 1995; Pearson and McCroskey, 1997).

Concentrated TE < 10 kDa (100 µg) was dissolved in 1 ml of 0.1% (v/v) heptafluorobutyric acid (HFBA) in Milli-Q water (buffer A). Linear gradient elution was performed using buffer A (0.1%, v/v HFBA) as the ion pairing agent and acetonitrile (0-80%, v/v) as the organic modifier over a gradient time of 60 minutes with a flow rate of 200 µl/min. Fractions collected were concentrated using a vacuum evaporator (Vacuspin "Virtis", NY, USA), and then redissolved in RPMI-1640 without serum (100 µl for each fraction). The immunosuppressive activity of each individual fraction was then assessed as outlined in 3.2.1.2.

4.11.2.2. Results and Outcomes

Purification using these procedures apparently improved the binding of the immunosuppressive activity to the column (Figure 4.22 and Figure 4.23). The active fractions (Fractions 15, 16 and 20, 21) were pooled and each pool refractionated on a second chromatographic gradient (µRPC C2/C18 PC 2/10 column, 0.1% v/v HFBA, 0-80% v/v acetonitrile, 200 µl/min). However this second dimension of RP-HPLC resulted in a decrease or loss of the immunosuppressive activity (Figure 4.24 and Figure 4.25).

One could argue that detection of eluted substances after the chromatographic separations was alwys done by automatic reading of the absorbance at 280 nm. This wave length requires the presence of aromatic amino acids to show any signal. For small molecular weight peptides, as in the present study, it is not liklely that such amino acids are absent. Therefore an alternative would have been to read absorbance at 214 or 205 nm, detecting peptide bonds and most

Figure 4.22. Reverse-phase HPLC profile of LMW-TE (blue line, A_{280} nm) on a μRPC C2/C18 column (0.35 ml).

LMW-TE (100 μg; 1 ml in HFBA 0.1%, v/v) was injected onto the column.The column was equilibrated with HFBA (0.1%, v/v, in water). Linear gradient elution was performed using acetonitrile (0-20% in 0.09%, v/v, HFBA, blue line) for 60 min with flow rate of 200 μl/min. Fractions 1-29 were tested for immunosuppressive activity as per Figure 4.23.

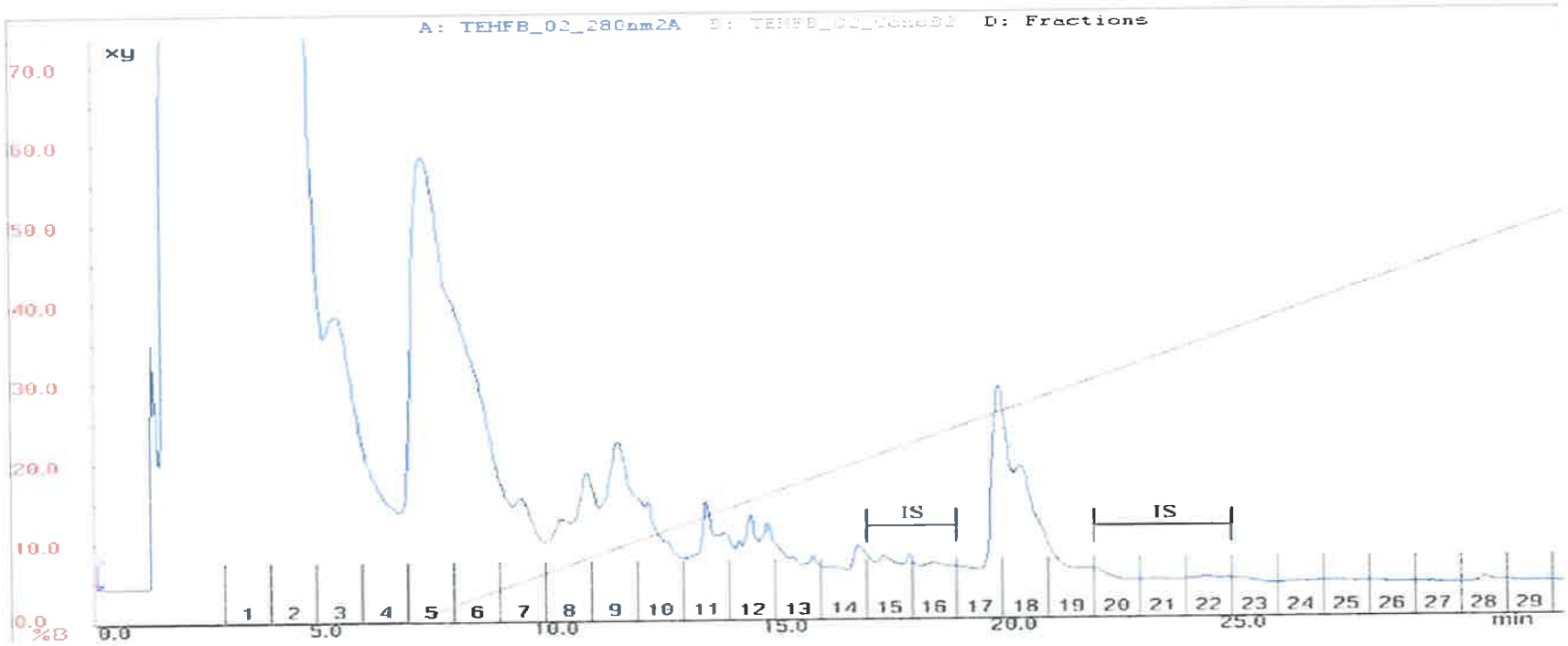


Figure 4.22 Reverse-phase HPLC profile of LMW-TE (blue line, A_{280} nm) on a µRPC C2/C18 column (0.35 ml).

Figure 4.23. Proliferation of splenic T cells induced by Con A in the presence of LMW-TE fractions separated with RP-HPLC method using a μRPC C2/C18 column (see Figure 4.22).

A significant inhibition of lymphocyte proliferation was achieved with fractions 15, 16, 20 and 21. Values are the means obtained from duplicate cultures presented as a percentage of the Con A only value (100%). Ctrl. = control, i.e. LMW-TE before separation.

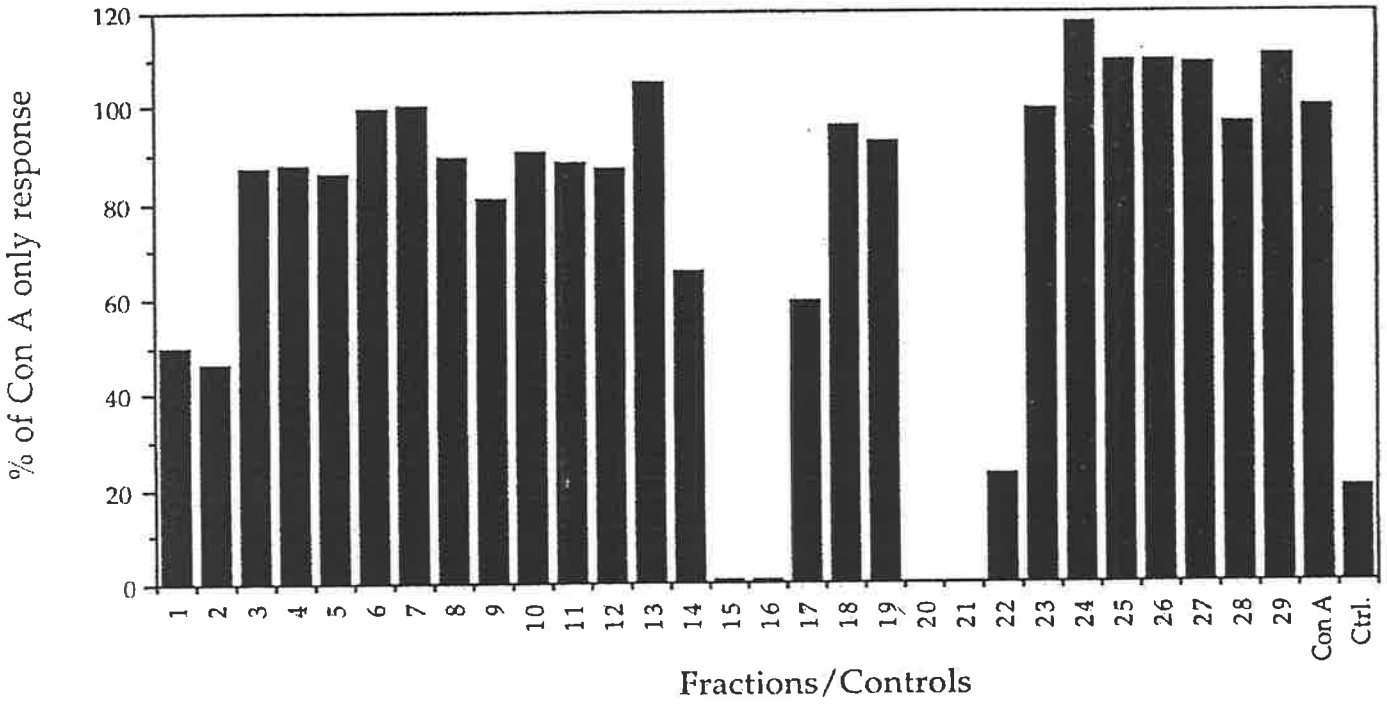


Figure 4.23 Proliferation of splenic T cells induced by Con A in the presence of LMW-TE fractions separated with RP-HPLC method using a µRPC C2/C18 column (see Figure 4.22).

Figure 4.24 Representative RP-HPLC profile (blue line A_{280} nm) of pooled active fractions 15 and 16 (Figure 4.22 and Figure 4.23) eluted from a µRPC C2/C18 column (0.35 ml).

The pooled fractions (in 200µl HFBA 0.1% v/v) were injected onto the column. The column was equilibrated with HFBA (0.1%, v/v in MQ water). Linear gradient elution (red line) was performed using acetonitrile (0-20% in 0.09%, v/v, HFBA) for 60 min with flow rate of 200 µl/min. Fractions 1-29 were tested for immunosuppressive activity as per Figure 4.25

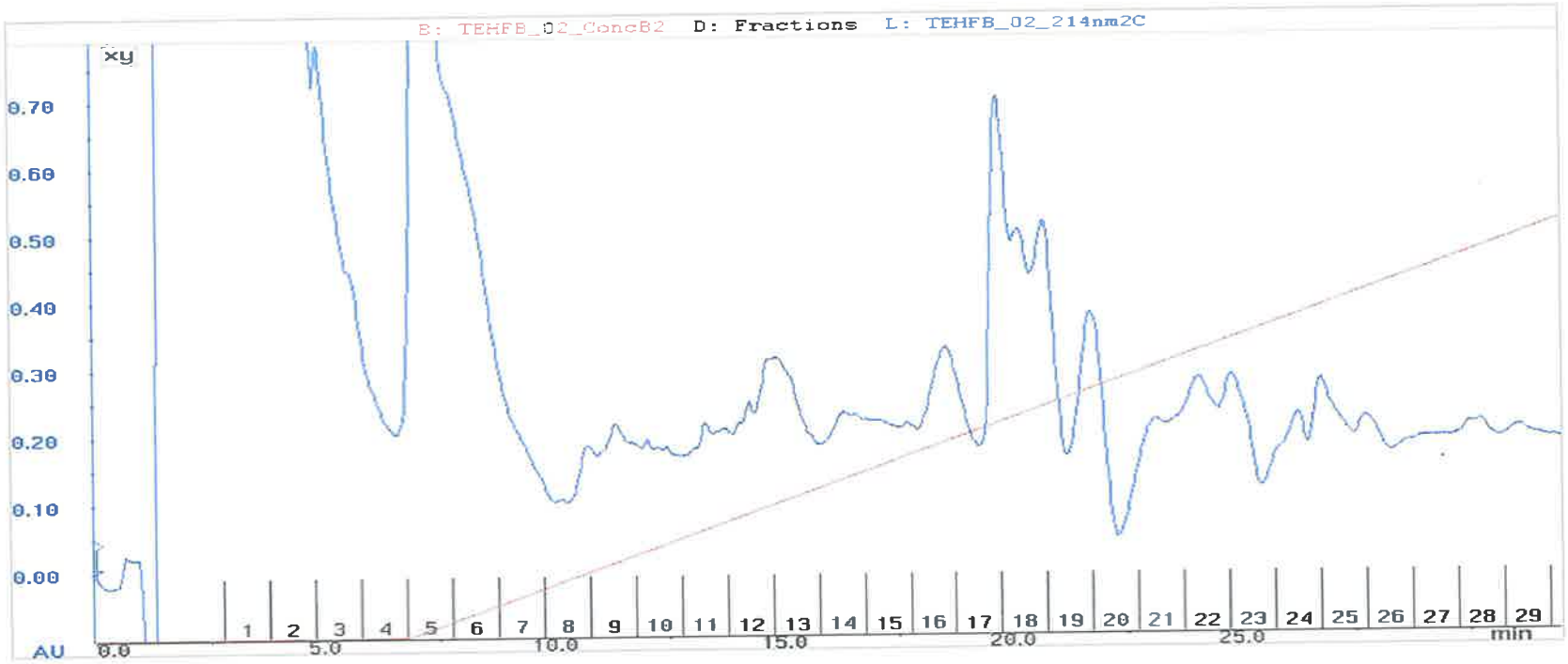


Figure 4.24 Representative RP-HPLC profile (blue line A_{280} nm) of pooled active fractions 15 and 16 (Figure 4.22 and Figure 4.23) eluted from a µRPC C2/C18 column (0.35 ml).

Figure 4.25 Proliferation of splenic T cells induced by Con A in the presence of fractions of the pooled active fractions 15 and 16 (see Figure 4.22 and Figure 4.23) purified with RP-HPLC method using μRPC C2/C18 column (see Figure 4.24).

No immunosuppressive activity was observed after this purification process. Values are the means obtained from duplicate cultures presented as a percentage of the Con A only value (100%). Ctrl. = control, i.e. LMW-TE before separation.

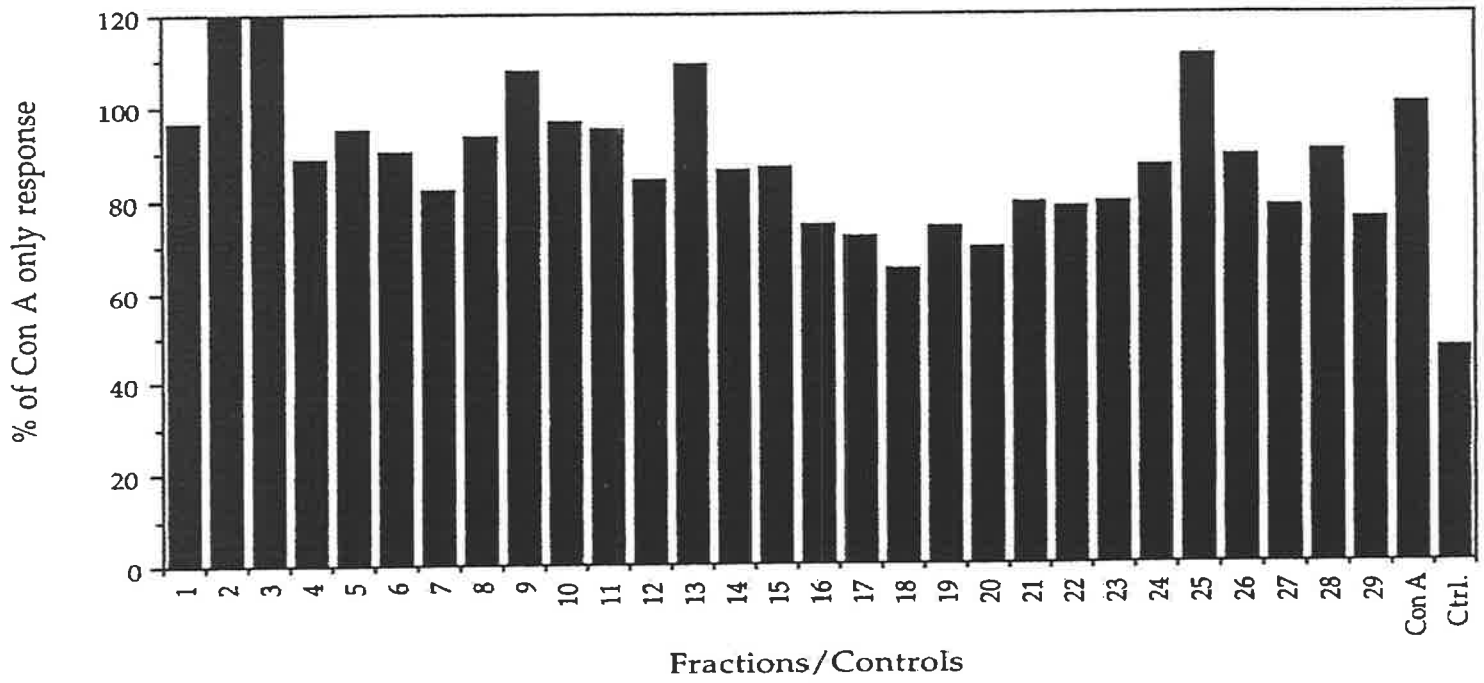


Figure 4.25 Proliferation of splenic T cells induced by Con A in the presence of fractions of the pooled active fractions 15 and 16 (see Figure 4.22 and Figure 4.23) purified with RP-HPLC method using μRPC C2/C18 column (Figure 4.24).

substances, respectively. However, since all eluted fractions were tested this does not affect the results therefore the A280 curves may not be relevant for the eluted activity.

Unfortunately, due to time constraints further experiments to purify the potential LMW-TE immunosuppressive activity could not be carried out.

5. General Discussion

The present study was undertaken in an attempt to isolate and further characterise the immunosuppressive factors in the rat testis. These factors have been thought to be important in affording the immune privileged status of this site (for recent reviews, see Maddocks and Setchell, 1990, Streilein, 1993 and 1995, Filippini et al., 2001).

Since T cells play an important role in cell-mediated immune responses, which also regulate tissue graft rejection, a bioassay for T lymphocyte proliferation was employed to assess the immunosuppressive activity in the rat testis. For this purpose, T cells were isolated from splenic lymphocytes using the method modified from Gutierrez et al., (1979). The isolated T cells tested using FACS analysis consisted of pure α/β TcR positive cells (Chapter2).

Studies using crude testicular extract revealed that mitogen (Con A or PHA), as well as IL-2 induced-splenic T-cell proliferation could be suppressed by a factor(s) in the extract. These results confirm and extend previous studies that demonstrated that rat testis produces immunosuppressive factors (Pöllänen et a1., 1988; Wyatt et al., 1988; Metafora et al., 1989; Sainio-Pöllänen et al., 1990, 1991; and Hedger et al., 1990, 1994).

Partial purification of these immunosuppressive factors revealed the heterogeneity of the immunosuppressive activity in the rat testis. There would now appear to be at least two group of molecules involved, having high- and low molecular weights. As presented in Chapter 3 the most potent

immunosuppressive factors are of low molecular weight and during dialysis (Chapter 3; Fig. 3.8), or separation using a PD-10 column (Chapter 4), the immunosuppressive activity of crude TE decreased when the low molecular weight fractions were separated out. These molecules have an apparent molecular weight in the range of 1-3.5 kDa (Chapter 4).

Characterisation of the low molecular weight immunosuppressive factors showed that the factors are heat and pH stable, but sensitive to proteolysis (trypsin) suggesting they are peptidic in nature and are not of steroidal origin. These characteristics are similar to that observed in the crude testicular extract and lend further support to previous suggestions that immunosuppressive factors in the rat testis are proteins or peptides (Chapter 3). The present study has also revealed that the immunosuppressive activity of both the crude and the low molecular weight testicular extracts is not due to a general cytotoxic effect. These was demonstrated with both T cell viability tests examined by trypan blue exclusion and by studying cytotoxicity on an independent cell line using Rat-2 fibroblast cells.

The characteristics of the immunosuppressive peptides reported in this thesis have several similarities to those described from Sertoli cell cultures. Wyatt et al., (1988) reported that immunosuppressive proteins secreted by rat Sertoli cell cultures were heat stable. Additionally, De Cesaris et al., (1992) reported immunosuppressive proteins secreted by mouse Sertoli cells that were sensitive to trypsin which could lead us to suggest that our immunosuppressive peptide(s) may be of Sertoli cell origin. However both groups have reported a

different molecular weights of their immunosuppressive proteins. Wyatt et al., (1988) claimed the molecular weight of the immunosuppressive proteins were 10-25 kDa, while De Cesaris et al., (1992) observed a molecular weight greater than 30 kDa. These molecular weights are very different from the molecular weight of peptides reported in the present study. A rat seminal plasma protein (SV-IV) has also been reported (Metafora et al., 1989) as having similar characteristics to the molecules reported on in this thesis, however its molecular weight of 9.758 kDa (Pan and Li., 1982) is also quite distinct from ours. Furthermore it is difficult if not impossible to determine whether the immunosuppressive activity present in the seminal plasma is with a testicular origin or not.

Substance P has a molecular weight of less than 5 kDa (Wilson et al., 1988) and seems to be the closest immunosuppressive factor in molecular size to the immunosuppressive peptides reported in the present study. Wilson et al., (1988) have reported that substance P can also inhibit proliferation of Con A-activated T-cells without any effect on IL-2 production. This is quite interesting since in the present study we observed the suppression of Con A-induced T-cell proliferation by the low molecular weight immunosuppressive peptides without an effect on IL-2 production (Chapter 4). However, recently it has also been reported that substance P is able to inhibit apoptosis of immune cells such as $CD4^+/CD8^+$ thymocytes in mice both in vitro and in vivo (Dimri et al., 2000), and assists in delaying apoptosis of human neutrophils (Böckmann et al., 2001). In stark contrast, our LMW testicular extracts were found to inhibit T cell proliferation by promoting apoptosis. This suggests that

our LMW testicular peptide(s) is distinct from substance P, as well as from those of other rat testicular immunosuppressive proteins reported elsewhere.

The apparent uniqueness of the immunosuppressive activity in the present study prompted attempts to purify the low molecular peptides as presented in Chapter 4. However as demonstrated in Chapter 4, the immunosuppressive activity was unable to be retained during the purification process.

The reduction or loss of immunosuppressive activities during purification may be due to the nature of the peptides. Either the peptides form complex structures such as a heterodimer or higher order oligomer, which dissociate and segregate during purification (Friedrich et al., 2004), or are relatively unstable under the purification conditions. A further possibility is that the low molecular weight immunosuppressive activity has a tendency to bind other proteins nonspecifically and requires them to act as carriers. This is supported by Pöllänen et al., (1990) who demonstrated that some low molecular weight substances e.g. α- and β-endorphin, met-encephalin, substance P, and arginine vasopressin which may be present in the testis and possess immunoregulatory activity, become active only when they bind to carrier proteins. It is possible therefore that the loss of immunosuppressive activity is due to separation of the active peptides from each other (the oligomer hypothesis) or from carrier proteins during purification. However the oligomer hypothesis seems to be more favorable since the carriers would have to be still very small carrier molecules (Friedrich et al., 2004).

As mentioned above, in this study we also observed immunosuppressive activities in the high molecular weight fraction of TE (HMW-TE) with lower immunosuppressive activity observed compared to the LMW-TE. These HMW immunosuppressive activities are probably similar to those observed by Pöllänen et al., (1988), Sainio-Pöllänen et al., (1990, 1991), Wyatt et al., (1988), and Hedger et al., (1994) in the rat testis. Pöllänen et al., (1988) reported that the molecular weight of immunosuppressive proteins isolated from rat interstitial fluids were 70 kDa and 130 kDa. However, biochemical details of these immunosuppressive proteins have not yet been reported, except for the correction of the molecular weights. Subsequently, this group has also reported several high molecular weight immunosuppressive factors isolated from whole testicular extracts with gel filtration corresponding to Mr 65 and 25 kDa (Pöllänen et al., 1990). In 1993 they then reported that the immunosuppressive factors have a molecular weight of 25 kDa and is a TGF-β-like factor based on its loss of function in the presence of an anti-TGF β antibody (Pöllänen et al., 1993). However, the specific nature of the high molecular weight immunosuppressive factors have still not yet been fully determined.

It is quite difficult to trace the source of these purification problems. However, this is not the first investigation to report this difficulty. Saxena et al., (1988) reported the reduction in immunosuppressive activity in human seminal plasma after purification. They suggested the reduction was due to removal of low molecular weight substances during purification process (Saxena et al., 1988). Similarly, separation of the immunosuppressive factors

in vasectomized boar seminal plasma (Checova et al., 1989) resulted in fragmentation and subsequent reduction of the immunosuppressive activities of some high molecular weight fractions. This is similar to our finding that when the crude TE was dialysed with a 10 kDa cut off dialysing tube, the immunosuppressive activity was reduced.

From these investigations, we would suggest that low molecular weight fractions should be recognised for their contribution to the immunosuppressive activities in the testis. Furthermore, these studies reinforce the notion that there is more than one mechanism of immunosuppression in the testis (Hedger, 1989).

In the present study further investigation on the purification of high molecular weight fractions were not pursued due to time restrictions, and investigations here focused on the low molecular weight compounds because of their potentially unique characteristics. Furthermore, it should be noticed here that the aqueous extraction procedure used limits the recovery of small hydrophobic peptides as does chromatography in PBS. Precipitation by ammonium sulphate has the same limitation as it was developed for and works best with globular proteins or larger molecular size. In other words, it could be a potential risk to miss some of the immunosuppressive activity with aqueous extraction alone. Therefore in the future a complementary approach to extract tissues in an organic solvent and compare the activity with that obtained with the aqueous extraction needs to be carried out.

Further investigation will clarify the mechanisms of immunosuppression in the testis, which appears to be a complex situation involving multiple factors with different immunosuppressive activities (Hedger, 1989). Our data from partial purification of rat TE immunosuppression strongly suggest that immunosuppressive activity seems to be dominated by the low molecular weight peptides. Hayes et al., (1996) reported the presence of low molecular weight (Mr < 10 kDa) immunosuppressive activity related to resident macrophages in the rat testis. Similarly the inhibition of lymphocyte proliferation by fractionated testicular macrophage conditioned medium was demonstrated mainly to exist in the low molecular weight (LMW < 10 kDa) fraction (Kern, 1995). Efforts to purify the testis extract LMW immunosuppressive peptide in the present study were presented in Chapter 4; unfortunately due to time constraints and the complexity of this factor the purification and characterisation of this LMW immunosuppressive peptide could not be completed.

Nevertheless, there are several important concepts that can be addressed from these studies based on the partial purification and characterization of the rat testicular extracts. It is clear that immunosuppressive factors are produced locally in the rat testis. This is possibly a consequence of the immunogenicity of testicular cells and sperm. Local immune regulation is needed to protect these potentially immunogenic components from autoimmune attack. At the same time this local immune regulation creates a general impairment of the immune system at this site, inadvertently favouring allograft survival.

There are several possible mechanisms for down regulating the local immune system in the testis against auto- or foreign antigens at this site. These may involve mechanisms for inducing tolerance, the elimination of auto reactive T cell clones, or the inhibition of immune system effectors in this site. The exact mechanisms responsible for the failure to reject first set allografts in the testis are unknown, however there are strong indicators that immune system effectors are inhibited (Maddocks and Setchell, 1990, Streilein, 1993 and 1995).

The mechanism of the suppression of Con A-activated-T cell proliferation by the rat testicular extracts has also been investigated during these studies. In the present study the mechanism appears to be specific to lymphocytes without the expression of general cytotoxic effects. Interestingly, while the crude and the high molecular weight TE inhibited proliferation of Con A-activated T-cells and resulted in significantly ($P < 0.05$) decreased IL-2 production; the low molecular weight TE impaired proliferation without affecting IL-2 production (Chapter 4). In the context of testicular immunosuppressive activity, this is a novel mechanism since it is not only different from the high molecular weight actions, but also distinct from the mechanism of suppression of activated T cells commonly reported. These results indicate that the immunosuppressive factors in the rat testis act via different mechanisms or at different points of the same pathway (Figure 5.1).

It has been reported elsewhere that the testicular interstitial tissue contain immune cells such as macrophages, NK cells and T cells (Hutson, 1994).

Subsequently Tompkins et al., (1998), reported that immune cells in the adult rat testis consist predominantly of $CD8^+$ T cells and NK cells with a relatively minor proportion of $CD4^+$ T cells (see also Hedger and Meinhardt, 2000). Our data show that both $CD4^+$ and $CD8^+$ T cells were suppressed both by HMW-TE an LMW-TE, and that the most potent suppression was of $CD4^+$ T cells. Interestingly at the dilution of 1:8 the HMW-TE could suppress $CD4^+$ T cells but not $CD8^+$ T cells, while at the same dilutions LMW-TE was still able to suppress both $CD4^+$ T and $CD8^+$ T cells significantly. In this context it can be suggested that the presence of HMW-TE and LMW-TE immunosuppressive peptides in the rat testis could contribute in part, directly or indirectly to the different proportions of T cell subsets in the rat testis.

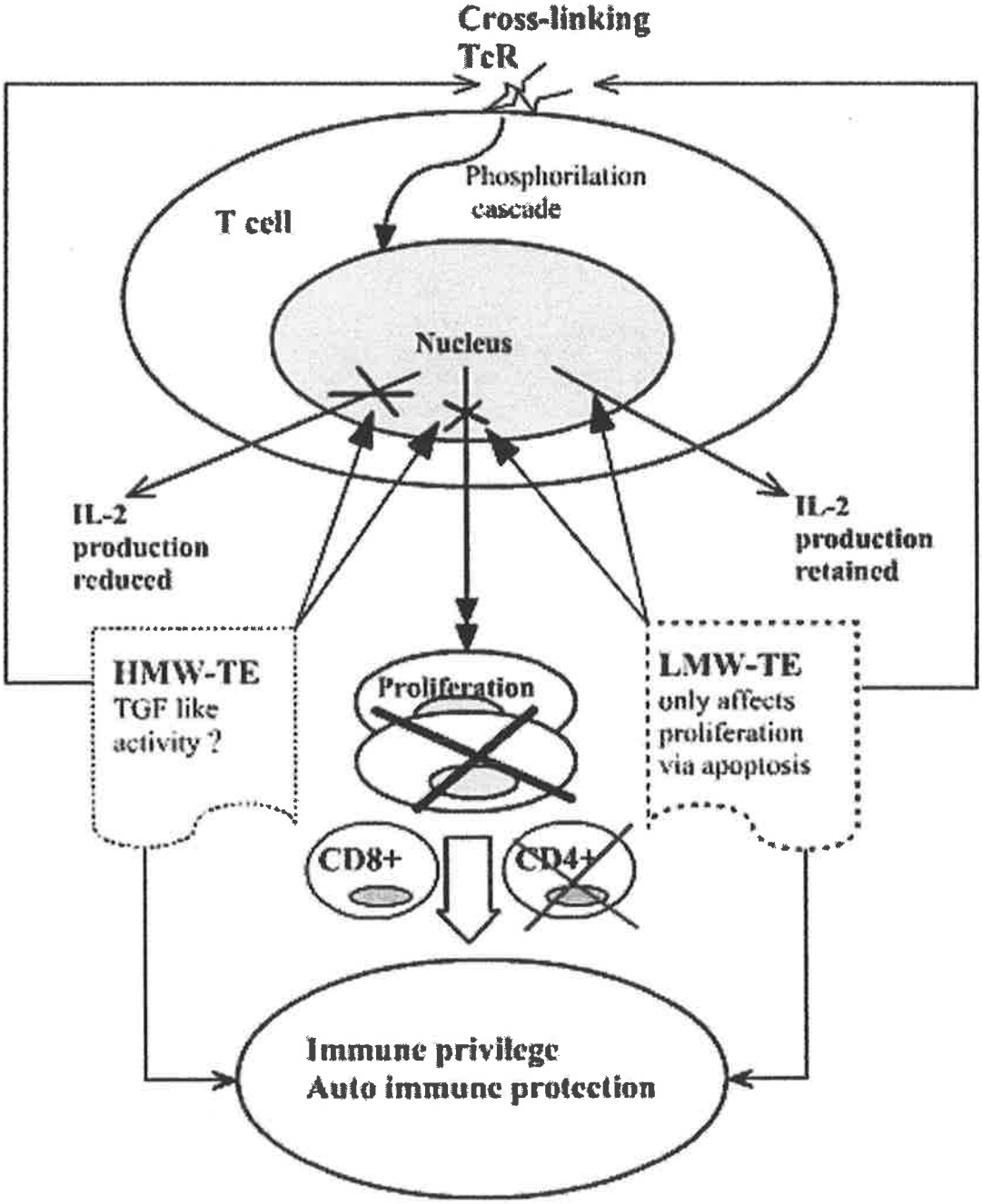


Figure 5.1 Proposed possible roles/mechanisms of action of high- (HMW-TE) and low- (LMW-TE) molecular weight immunosuppressive factors in the rat testis.

In addition to its status as an immune privileged site, the testis can also express normal inflammatory responses (Tung et al., 1987). Some studies have demonstrated that αβ-T cells play a central role in the induction of autoimmune orchitis in a mouse model as shown by the ability of activated αβ-T cells to transfer the disease to naïve mice. Furthermore γδ-T cells regulate the αβ-T cells using their cytokine-producing ability (Mukasa et al., 1998). The presence of HMW-TE and LMW-TE, with their different mechanisms of action in controlling T cell proliferation and cytokine (such as IL-2) expression, could be involved in the regulation of immune privilege of the testis, and inflammation in this site. Details of these events and molecules still need to be elucidated.

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