

TUBERCULOSIS, ANTI-GLUCOCORTICOIDS AND THE
IMMUNE SYSTEM

BY

SAHAL ABDULAZIZ MOHAMMAD AL-HAJOJ

FROM THE DEPARTMENT OF BACTERIOLOGY

MEDICAL SCHOOL

UNIVERSITY COLLEGE LONDON

THESIS SUBMITTED TO THE UNIVERSITY OF LONDON

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE FACULTY OF SCIENCE

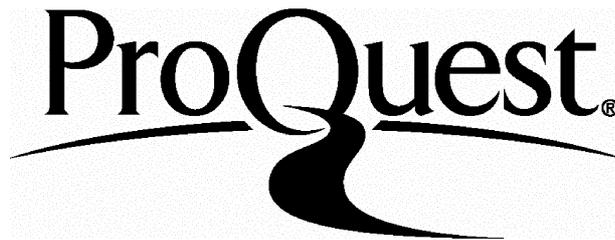
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DEDICATION

I dedicate this work to my son Abdulaziz who was 2-1/2 years old when I started this work. As a consequence I hardly gave him any care or any attention. This work is also equally dedicated to my mother and my wife as without their encouragement, this work would not see the light. Finally I dedicate this work to my brother Falleh who tirelessly kept encouraging me.

ABSTRACT

According to a recent estimate by the World Health Organisation, by the year 2000 tuberculosis (TB) will kill 4 million people a year . This dramatic increase, from 3 million in 1992 to 4 million by year 2000, is an alarming signal for health workers.

Endocrinological abnormalities have been observed in TB that may explain the tendency for patients to develop an inappropriate TH2 response to *M. tuberculosis*. These abnormalities include a fall in the ratio of antiglucocorticoid hormones (dehydroepiandrosterone; DHEA) to glucocorticoid, and a marked decrease in conversion of the major glucocorticoid cortisol to inactive cortisone.

This project was designed to investigate the significance for human tuberculosis of these two endocrine changes. Three approaches were used.

1) The antiglucocorticoid properties of DHEA and some of its metabolites, were tested in order to select the optimum metabolite and schedule for *in vivo* experiments in tuberculosis in mice. 2) The ability of live *M. tuberculosis* to convert DHEA sulphate (present in human serum at up to 4 µg/ml) into active metabolites was examined; 3) The hypothesis that the enzyme that interconverts cortisol and cortisone in the lung is susceptible to local regulation by cytokines was tested.

Antiglucocorticoid effects of DHEA and a series of its metabolites were tested *in vivo* for their ability to protect the thymus from apoptosis induced by corticosterone, and to block the effects of glucocorticoid on non-specific inflammation. The metabolite 3β,17β-

androstenediol (AED) proved most active. The optimum dose level was identified and has since proved strongly protective in a model of murine tuberculosis that is used by collaborators in Mexico.

These metabolites were then tested *in vitro* for their effects on proliferation and cytokine production by murine and human lymphocytes, alone or in the presence of glucocorticoid. DHEA and AED increased the production of IFN γ by murine splenocytes, and antagonised the inhibitory effects of cortisol on IFN γ production by human T cells. The *in vivo* model has subsequently confirmed that AED upregulates TH1 and proinflammatory cytokines, while downregulating TH2 cytokine release.

Mycobacterium tuberculosis cleaved DHEA to a number of metabolites, visualised by thin layer chromatography, including AED and reduced forms of AED that were identified by gas chromatography and mass spectrometry.

11 β -HSD activity was measured in different organs at different intervals after intravenous challenge with live BGC. On day three conversion to cortisone in the lung was reduced in the BCG group compared to normal mice, indicating that the activity of 11 β -HSD was mainly dehydrogenase. However 10 days later the activity shifted towards reductase since conversion to cortisone was markedly increased not only in lung but also in spleen and thymus. The equilibrium point (i.e. the ratio of cortisol to cortisone) did not change in liver or kidney. These results suggest that 11 β -HSD is regulated during the inflammatory response induced by live BCG.

The above data are discussed in relation to the immunological abnormalities that accompany human tuberculosis, and the effects of

manipulating the AED/corticosterone balance in the murine tuberculosis model. They help to explain the changing pattern of immunopathology in human tuberculosis of different ages, and correlation of these disease patterns with age-related changes in DHEAS levels.

SUMMARY OF FINDINGS

The aims of this study were to re-investigate DHEA and some of its derivatives at physiological concentrations, in order to determine their effects on immune system components as follows:

- 1 To determine their effect on cell proliferation and cytokine production, and to determine whether they can protect thymuses against involution caused by glucocorticoids;
- 2 To gain insight into the nature of the active metabolites of DHEA and selected derivatives;
- 3 To investigate whether *M. tuberculosis* can cleave DHEA to active or non-active compounds;
- 4 To determine whether 11 β hydroxysteroid dehydrogenase / reductase activity in lung can be regulated.

Murine spleen cell proliferation induced by ConA was enhanced significantly by 3 β -7 β dihydroxy 5- α - androstan-17-one at 10⁻⁶, 10⁻⁷, or 10⁻⁸M (t- test p < 0.01, 0.05, and 0.01 respectively or by AED at 10⁻⁶ and 10⁻⁷M (p< 0.05, t-test). However, when AED was co-cultured with corticosterone, AED did not antagonise the inhibitory effect of the corticosterone.

AED caused no significant increase or decrease in the production of IL-4 or IL-5 irrespective of the age, sex or strain of mice. However, AED did have an effect on the production of IFN γ , but this seemed to be dependent on the strain and age of the mice and was seen only in old C57Bl mice.

It has been shown that DHEA and AED increased significantly the production of IFN γ by human T-cells when they were co-cultured with cortisol, but did not increase the production of IFN γ alone. However, the current study showed that the effect of such steroids is very much influenced by the status of the blood donors, since when experiments were repeated, with different donors, some of them showed differences although these were not always significant.

The present study showed that the thymuses can be protected from corticosterone induced involution (1.6mg) using very low concentrations of DHEA or AED (0.16 and 0.06mg/kg) respectively.

The effect of *M. tuberculosis* was examined with particular respect to DHEA. *M. tuberculosis*, either in the presence or absence of dipyrindyl, metabolised cholesterol and DHEA to six different products which were identical in terms of colour, mobility and appearance when different indicators were used. An attempt was made to identify these compounds produced as a result of DHEA-metabolism, using gas chromatography and the mass spectrometry. Three compounds were identified as the most abundant compounds from DHEA metabolism by *M. tuberculosis*. These compounds were AED, 7-hydroxy DHEA and a reduced form of DHEA. Also *M. tuberculosis* cleaved DHEA-S to produce DHEA.

Finally this study for the first time shows that the activity of 11 β -hydroxy steroid dehydrogenase/ reductase can be regulated at the site of inflammation caused by infection with BCG. Changing the activity of this enzyme in this model could be due to generation of cytokines caused by such infection.

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ACKNOWLEDGEMENTS

I am deeply grateful to Professor G. Rook for his supervision, guidance, patience and encouragement throughout this study. I am also grateful to Dr. H. Donoghue, and Professor P. Lydyard for their help, encouragement and invaluable discussion during this work.

I would like to express my gratitude to Graham McIntyre for his help. I am indebted to Mr. Abdullah Al-nasser, the educational attaché of Saudi Arabia, for his help, encouragement, care and his tireless effort. My thanks to all my friends and colleagues for their help, support and useful discussions. My special thanks and deep gratitude go to Mr Habeeb Al-jorfy and Mr Abdulmonem Al-jorfi for their invaluable help and support.

My special thanks go to my brother Adnan who always provides support and encouragement. Finally, I am indebted to the Government of Saudi Arabia represented by the Ministry of Health for their support and for paying the costs of this study.

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACTH	Adrenal Corticotrophic Hormone
BCG	Bacillus Calmette Guerin
AED	5 androstene 3β - 7β -diol
AET	5 androstene 3β - 7β - 17β -triol
BSA	Bovine Serum Albumin
CBG	Corticosteroid binding globulin.
CMI	Cell Mediated Immunity
ConA	Concanavalin-A
Cort	Corticosterone
CRH	Corticotrophin - Releasing Hormone
CTL	Cytotoxic Lymphocytes
DCs	Dendritic Cells
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulphate
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme Linked Immunosorbent Assay
ENL	Erythema Nodosum Leprosum

FCS	Foetal Calf Serum
GABA	Gamma Amino Butyric Acid
GCs	Glucocorticoids.
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GH	Growth Hormone
GRE	Glucocorticoid Response Elements
HIV	Human Immuno Deficiency Virus.
HPA	Hypothalamic-Pituitary Adrenal
11β-HSD	11beta-Hydroxy-Steroid Dehydrogenase
hsp-90	Heat Shock Protein-90
ICAM-1	Intracellular Adhesion Molecule-1
IgE	Immunoglobulin-E
IFNγ	Interferon-gamma
IL-1β	Interleukin-1beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL- 6	Interleukin-6
IL-8	Interleukin-8

IL-12	Interleukin-12
IL-1R	Interleukin -1 Receptor Type-I
NFκB	nuclear factor-κB
LTB4	Leukotriene B4
JAK	Janus Kinase
LAM	LipoArabinoMannan
M.tb	<i>Mycobacterium tuberculosis</i>
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
PHA-P	phytohaemagglutinin-P
STAT	Signal Transducer and Activator of Transcription
TCR	T cell receptors
TB	Tuberculosis
TH1	T-helper-1
TH2	T- helper-2
TNFα	Tumour Necrosis Factor Alpha
WBC	White Blood Cells
STAT	Signal Transducer and Activator of Transcription
RNI	Reactive Nitrogen Intermediate
ROI	Reactive Oxygen Intermediate

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CHAPTER ONE

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GENERAL INTRODUCTION

1.1 History of tuberculosis

Tuberculosis caused by *Mycobacterium tuberculosis* (M.tb), killed 3 million people in 1992 and by the year 2000, will kill 4 million people per year world-wide, according to the recent estimate by the World Health Organisation (Zaki & Hibberd 1996). This dramatic increase is an alarming signal for health workers to focus more on this disease. This is mainly a disease of the Third World, but it is rising again in the developed world. Factors such as human immunodeficiency virus (HIV) infection, poverty, socio-economic problems, misusing treatment regimens and the emergence of multi-drug resistance are essential factors in spreading the infection (Fenton & Vermeulen 1996; Zaki & Hibberd 1996).

1.2 Immunopathology of tuberculosis

There are different ways in which pathogens can cause infections and subsequent disease. These include adherence to the host tissue, production of toxins and multiplication inside the host. In order to cause disease all pathogens need to gain access to the host. Also pathogens need to evade the response of the immune system (Mandell *et al.* 1995). The host immune system has different mechanisms to prevent pathogens from producing disease. The most appropriate response of the immune system depends on how the infectious agent causes the disease. There are two types of response, the cellular and the humoral. If the pathogen produces a toxin the humoral response is required to neutralise the toxin (Mandell *et al.*

1995). If the disease is produced by intracellular multiplication of the pathogen or by a pathogen that the immune system cannot kill in any other way, the cellular response is required (Mackaness 1968; Mandell *et al.* 1995; Spencer 1985). Usually both types of response are required to control the pathogen. Some organisms are able to evade the initial response of the immune system by invading host cells and becoming intracellular. Thus the immune system does not recognise the organism unless antigen presenting cells (APC) present the organism as peptides on their major histocompatibility class II (MHCII) (Mandell *et al.* 1995). *Mycobacterium tuberculosis* is such an organism that can survive and grow within host cells. How this organism causes the disease and how the immune system fights against such infection, will be discussed below.

When the pathogen is inhaled, the alveolar cells try to ingest the bacilli and eventually destroy them. This process is controlled by many factors such as the virulence of the strain and the activation state of the alveolar macrophages. If the macrophages are not active, or the bacilli are particularly virulent, the organisms immediately start to multiply (Dannenbergs & Rook 1994). Once the bacilli start to multiply they cannot be controlled until the macrophages are killed and the bacilli are released to be engulfed by other macrophages. This time, the engulferers are non activated macrophages and monocytes (Lurie 1964). Again, the status of the macrophages is important to control the rate of the multiplication. For instance, in human immunodeficiency virus positive (HIV⁺) patients the rate of multiplication of the bacilli is higher compared with normal individuals. When the bacilli are engulfed by peripheral

macrophages, which are considered responsible for the first lesion, the disease enters its second stage (Law *et al.* 1996).

In this second stage, monocytes and complement migrate to the site of the first lesion. These immature macrophages, depending on other factors (sex, race and susceptibility) accumulate in the lesion site. For example, a susceptible rabbit develops disease very quickly and dies in a short time compared to a resistant rabbit exposed to the same dose of the same strain of virulent H37Rv (Lurie *et al.* 1951). It has been shown that black people are twice as susceptible to tuberculosis as white people (Stead *et al.* 1990; Xu *et al.* 1994). However, macrophages keep migrating to the region and keep engulfing new bacilli which can form a symbiotic relationship with the macrophages (Dannenberg 1992; Tsuda *et al.* 1976).

Two to three weeks after infection, the surviving organisms multiply and kill their host macrophages; this is followed by mycobacterial release and subsequent infection of additional host cells (Fenton & Vermeulen 1996). The early exudate contains chemotactic factors such as interleukin-8 (IL-8) that attract circulating monocytes, lymphocytes, and neutrophils, none of which kills the bacteria very efficiently. IL-8 may be partly responsible for the lymphocytic and neutrophilic alveolitis seen in the involved segments of patients co-infected with HIV-1 and *M. tuberculosis* (Law *et al.* 1996). Enhanced production of monocytes, and their early release from bone marrow, can be observed clinically with active tuberculosis and also with active sarcoidosis. Patients with tuberculosis demonstrate a marked increase in proliferation activity of monocytes and premature monocytes which are released from bone marrow (Schmitt *et al.*

1977). Granulomatous focal lesions, composed of macrophage-derived epithelioid giant cells and lymphocytes, begin to form.

Three weeks later, antigen-specific defences develop and contribute greatly to the resolution of infection. With the emergence of a delayed-type hypersensitivity (DTH) response, infected macrophages in the interior of each granuloma are killed as the periphery becomes fibrotic and caseated (Spencer 1985). (The terms DTH and later, CMI, are used in this introduction as descriptive terms without analysis of the immunological mechanisms with which they correlate. These mechanisms are discussed fully in the later section). After 4 to 5 weeks of progressive infection, microscopic granulomas enlarge, as individual foci expand and coalesce (as reviewed by Fenton & Vermeulen, 1996). This results in relatively large areas of necrotic debris, each surrounded by a layer of epithelioid histocytes and multi-nucleated giant cells. These granulomas, or tubercles, are surrounded by a cellular zone of fibroblasts, lymphocytes and blood-derived monocytes. Although *M. tuberculosis* bacilli are unable to multiply within this caseous tissue due to its acidic pH, low availability of oxygen, and the presence of toxic fatty acids, some organisms may remain dormant there for decades. The strength of the host cell-mediated immunity (CMI) responses determines whether an infection progresses or remains arrested (Fenton & Vermeulen 1996). With good CMI, the infection is arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions (Spencer 1985). However, if CMI responses are insufficient, macrophages containing ingested but viable *M. tuberculosis* bacilli may escape from granulomas via the intrapulmonary lymphatic channels. This results

in the rapid spread of the infection to regional hilar lymph nodes. Where CMI is adequate, the host DTH responses stop the multiplication of *M. tuberculosis* bacilli, but at the same time, lung tissue is destroyed, leading to both pulmonary damage and spread of organisms via the lymphatics and the blood. As the disease progresses further, the semi-solid caseous centre of the granuloma begins to soften and liquefy, providing a rich and oxygenated environment for extra cellular mycobacterial replication (Dennenberg 1982). Enlarged lymph nodes can rupture into adjacent airways, releasing liquefied necrotic material and causing tuberculosis bronchopneumonia (reviewed in Fenton & Vermeulen, 1996). At this stage, the organisms can spread in a homogeneous manner via blood causing miliary tuberculosis.

1.3 The role of immune system components

1.3.1 Macrophages and monocytes

Evidence has long existed that murine macrophages have anti-mycobacterial function in tissue culture systems. Macrophages (MPs), activated *in vitro* by cytokines, show various degrees of anti-mycobacterial activity (Fenton & Vermeulen 1996). It has been found that macrophages infected with *Mycobacterium tuberculosis* are activated when interferon gamma (IFN γ) is added to the culture (McDonough *et al.* 1993; Steffen *et al.* 1993). Also, MPs produce cytokines including interleukins 1, 6 and 10 (IL-1, IL-6, IL-10), tumour necrosis factor alpha (TNF α) and transforming growth factor β 1 (TGF β 1) (Denis 1991 b; Fenton & Vermeulen 1996; Toossi 1996). These cytokines are able to exert potent immunoregulatory effects and to mediate many of the clinical manifestations of

tuberculosis. In addition to enzyme and cytokine production, MPs phagocytose mycobacteria, or ingest secreted mycobacterial proteins by pinocytosis, then degrade these proteins into peptides. These peptides are expressed on the cell surfaces in the cleft of the major histocompatibility complex class II (MHCII) molecules. Presentation of specific epitopes by MHCII leads to induction of CD4⁺ lymphocytes (Shima *et al.* 1972; Tsuda *et al.* 1976). Inhibition or destruction of bacilli by murine macrophages have been demonstrated in more than one study. Activation of macrophages with IFN γ and TNF α leads to production of nitric oxide (NO) which is required for the mycobactericidal activity. The mouse macrophages produce nitrogen intermediates, which are able to bind to the Fe-S centre present in some critical enzymes and ribonucleotide (Pabst *et al.* 1988). With human macrophages the situation is not clear. A few people have claimed that they were able to demonstrate killing of virulent TB by human monocytes after stimulation with IFN γ or TNF (Denis 1991 b; Douvas *et al.* 1986). However, it has been demonstrated that the multiplication inside the cells is reduced by adding IFN γ and calcitriol (Denis 1991 a). IFN γ alone induced the growth of the bacilli (Crowle 1990; Rook *et al.* 1986).

Evidence exists for the production by human monocytes of levels of NO high enough to be involved in bacterial killing, though these levels are never as high as those released by murine macrophages. One of the earliest indications of this was provided by reports that human mononuclear cells inhibit platelet aggregation by releasing an NO-like factor (Dugas *et al.* 1995; Moncada & Higgs 1995). Co-stimulation of human monocytes with TNF α , or granulocyte-macrophage colony-stimulating factor (GM-CSF), and

with an avirulent strain of *Mycobacterium avium*, generated nitrite production (Denis 1991 d). Inhibition of nitric oxide synthase (NOS), the enzyme responsible for production of NO, leads to exacerbation of *M.tuberculosis* infection (MacMicking *et al.* 1997).

Human monocytes are able to produce NO upon ligation of CD23, which is a low-affinity IgE receptor. Engagement of CD23 at the surface of normal human monocytes stimulates NO production (Dugas *et al.* 1995; Kolb *et al.* 1995; Ouaz *et al.* 1996) However, this remains controversial because there is one claim that stimulation of monocytes by IFN γ plus IL-4 leads to nitrite accumulation, but not necessarily to the production of NO, and this is enhanced by previous exposure to IL-4 and IFN γ . It was found that L-arginine was not consumed and L-citrulline was not produced upon exposing human macrophages to IFN γ and IL-4. Accumulation of nitrite in this system was thought to be due to nitrate reductase activity in IL-4 and IFN γ (Schneemann *et al.* 1997).

The biochemical mechanisms of NO-induced cytostasis/cytotoxicity are not completely understood. There is an NO-dependent inhibition of key enzymes in the respiratory cycle and the synthesis of DNA in the target cells, in some cases involving a reaction with iron-sulphur centres in these enzymes. More recent studies suggest that NO may inhibit cytochrome c oxidase, the terminal electron transport protein in mitochondria. This blockade could result in the local formation of O⁻² in the respiratory chain and the destruction of iron-sulphur centres by O⁻² itself or via the formation of the potentially damaging species peroxynitrite (ONOO) (Moncada & Higgs 1995). In support of this, it has been shown that

ONOO⁻, but not NO, inhibits the iron-sulphur protein aconitase, previously thought to be a target solely for NO.

Other actions of NO independent of the respiratory chain may also contribute to its cytotoxicity, because after oxidation, NO may cause nitrosative deamination of nucleic acids with consequent double strand DNA breaks and mutagenicity (reviewed in Moncada & Higgs, 1995). The role of reactive nitrogen intermediates (RNI) in infected humans remains a controversial subject since there are no consistent results showing the pathway of RNI production by the L-arginine-dependent pathway. These inconsistencies may simply reflect the differences between human and murine macrophages. One possible difference may be the requirement for the factor tetrahydrobiopterin, which may not be present in sufficient quantities in resting human macrophages (Fenton & Vermeulen 1996). Alternatively, human macrophages may require additional induction signals for RNI production. One study (Nussler & Silvio 1992) showed that a combination of LPS and cytokines IL-1, TNF, and IFN γ was required for human hepatocytes to produce RNI via the L-arginine-dependent pathway (Nussler & Silvio 1992). Another possibility is that human macrophages produce higher amounts of cytokines that suppress RNI production (e.g. IL-4, IL-10, and TGF- β 1) compared with murine cells (Fenton & Vermeulen 1996). The contribution of ROI to defence against TB remains unclear. Several mycobacterial products, including sulphatides and lipoarabinomannan (LAM), can scavenge ROI or inhibit the respiratory burst that generates them (Chan *et al.* 1991; Pabst *et al.* 1988).

1.3.2 T- cells

Enhancement of macrophage microbicidal function can be exerted by T-cells, especially CD4⁺ αβ⁺ T cells that secrete IFN-γ and IL-2 (Fenton & Vermeulen 1996; Orme 1993). IFNγ alone cannot activate macrophages sufficiently to inhibit *M. tuberculosis* multiplication, although combined exposure to IFN-γ and TNF was reported by one author to be sufficient for effective killing (Denis 1991 b), but other authors have been unable to repeat this. Whilst activated alveolar macrophages may kill *M. tuberculosis* effectively, immature monocytes recruited from the periphery are thought to be less effective and to serve as the preferred host cells for *M. tuberculosis* (Fenton & Vermeulen 1996).

In vitro evidence exists that cytotoxic T-cells which recognise mycobacterial antigens do develop in both humans and mice. Transgenic animals with a disrupted β2-microglobulin gene are unable to express MHC class I on the cell membrane, and have a greatly increased susceptibility to tuberculosis. This implies a role for CD8⁺ T cells, possibly cytotoxic cells. Alternatively, it may be the CD4⁻ CD8⁻ CD-1-restricted T-cells that recognise LAM and mycolic acids, since CD-1 is also associated with β2-microglobulin. Interestingly, β2-microglobulin knockout mice were unaffected by infection with BCG or avirulent *M. tuberculosis* H37Ra (Orme *et al.* 1993). One way that an antigen known initially to be taken up into an endosomal compartment could be presented to MHC class I-restricted cytotoxic lymphocytes (CTL) would be by its ability to escape from phagolysosomes into the cytoplasm. There is electron microscopic evidence that virulent *M. tuberculosis* can escape from the phagosome (Xu *et al.* 1994). Following *in vitro* culture of

macrophages with virulent H37Rv, but not with the avirulent *M. tuberculosis* H37Ra or BCG strains, bacteria were observed in the cytoplasmic compartment (Xu *et al.* 1994). It is possible that processing of antigen via the class I pathway is important, allowing the host to kill parasitized cells that are failing to exert bactericidal effects (Orme 1991). This failure could result from an unusual intracellular location of the organisms, or from a failure to present the antigens of such organisms (Orme 1991; Rook & Hernandez-Pando 1996).

1.3.3 $\gamma\delta$ T cells and CD-1 restricted T cells

A large proportion of human peripheral blood $\gamma\delta$ T cells will proliferate in response to mycobacteria. It has been found that the $\gamma\delta$ T cells are able to produce cytokines similar to TH1 cells. A major human $\gamma\delta$ T-cell subset expressing V γ 2 and V δ 2 germline genes, but having diverse junctional sequences, is found in human mycobacterial lesions and responds in vitro to antigens of bacteria and parasites (Tanaka *et al.* 1995; Tanaka *et al.* 1994). In addition, certain haematopoietic tumour cells are specifically recognised and lysed by these T-cells. V δ 2V γ 2-bearing T-cells have been shown to recognise mycobacterial antigens that are protease resistant and phosphatase sensitive as reviewed in Tanaka *et al.* (1995). Those mycobacterial antigens recognized by human V δ 2V γ 2-bearing T cells have been identified as isopentenyl pyrophosphate and related phenyl pyrophosphate derivatives. These are substances involved in the synthesis of complex polyisoprenoid compounds in microbial and mammalian cells. This work provides evidence that $\gamma\delta$ T cells can

recognise naturally occurring small non-peptidic antigens. However, $\gamma\delta$ -T cells are not the only cells that recognise non-peptide epitopes the same is true of CD4⁻CD8⁻ $\alpha\beta$ double negative cells that recognise antigens in association with CD-1 cells.

The CD-1 proteins are expressed on dendritic cells, mantle zone B cells and cytokine-activated monocytes. They show distant homology with MHC molecules, and like MHC class 1 they associate with β 2-microglobulin. However, CD-1 molecules are not encoded within the MHC and, unlike the MHC, they are not polymorphic. Recognition of mycolic acids by CD4⁻ CD8⁻, α/β T cell receptor-bearing lymphocytes has been demonstrated (Beckman *et al.* 1994). Mycolic acids are α -branched, β -hydroxy long chain fatty acids that make up the bulk of the mycobacterial cell wall. Similarly, a CD4⁻ CD8⁻ T cell line grown from the skin lesion of a leprosy patient was found to recognise mycobacterial lipoarabinomannan (LAM). The recognition required mannosides with α (1->2) linkages, and the phosphatidylinositol moiety (Sieling *et al.* 1995). The role of these cells is unknown, but they appear to be cytotoxic, and to secrete the TH1 cytokine pattern, so they may well contribute to immunity (Rook & Hernandez-Pando 1996).

1.3.4 Tumour necrosis factor alpha (TNF α)

Neutralisation of TNF α leads to dissemination of the disease very quickly. However, TNF α may also play a role in granuloma formation, as shown in murine BCG infection (Kindler *et al.* 1989). TNF α can be either beneficial or detrimental to the host. The cells present in the lesion are responsible for determining the role of

TNF α . The role of TNF α depends very much on the kind of cells present, whether the cells are T- helper-1 (TH1) or TH2, and also depends on which cytokines, that have been, produced (Hernandez-Pando & Rook 1994). If the cells present are TH1 associated with IL-2 and IFN γ , the TNF α is potentially beneficial to the host and there is no destruction of tissues. On the other hand, if the cells present are a mix of TH1 + TH2 , or TH0 the TNF α is damaging and tissue destruction is seen.

The kinds of cell present at the lesion site, depend very much on the vaccination protocol. When 10^7 of autoclaved *Mycobacterium vaccae* was used the response was pure TH1, and associated cytokines, IL-2 and IFN γ could be detected. In contrast, when 10^8 or 10^9 cells of the same organism were used, the response was a mixture of TH1 and TH2. If TNF α was injected into the delayed hypersensitivity (DTH) response sites elicited 24 hrs earlier in mice vaccinated with 10^7 , there was no necrosis. In contrast, if mice were vaccinated with 10^8 or 10^9 cells, necrosis developed when TNF α was injected into DTH response sites elicited 24 hrs earlier. These findings suggest that TNF α released into a relatively pure TH1-mediated inflammatory site, acts as a supplementary macrophage-activating molecule, but when released into a mixed TH1 + TH2 or TH0 site, it causes damage (Hernandez-Pando & Rook 1994; Rook & Hernandez-Pando 1994).

It is possible that Thalidomide, which is routinely used for treatment of *erythema nodosum leprosum* (ENL), and of graft-versus-host disease following bone marrow transplantation, works under these conditions by reducing TNF α levels. Thalidomide appears to shorten the half life of TNF α mRNA (Kaplan 1994), thus

it reduces but does not eliminate TNF α . When administered to tuberculosis patients it results in remarkable weight gain and symptomatic relief (Kaplan 1994). This implies that release of TNF α is excessive in relation to the release of TNF α inhibitors. This does not appear to be the case in peripheral blood, where the inhibitors are usually present in considerable excess and free TNF α is rarely detectable (Foley *et al.* 1990). Nevertheless, it is possible that such an imbalance occurs in the lesions (Rook & Stanford 1995).

1.3.5 IFN γ , IL-2 and IL-4

These cytokines are very important in regulating the cellular responses, in particular the responses by T-helper cells. T-helper cells are classified according to the kind of cytokines which they produce. TH1 cells produce mainly IL-2 and IFN γ , and TH2 cells produce mainly IL-4. IL-4 enhances the production of TH-2. However, TH1 is primarily enhanced and stimulated by IFN γ and IL-2. IFN γ is produced mainly by lymphocytes. This cytokine activates mouse macrophages to inhibit *M. tuberculosis*. Human macrophages, when exposed to IFN γ , develop a 1-hydroxylase which converts the circulating inactive form of vitamin D3, 25-hydroxy cholecalciferol (25-OH D3), to its active form (1,25 (OH) $_2$ vitamin D3 or calcitriol). This metabolite of vitamin D3 has a limited ability to increase the resistance of human macrophages to *M. tuberculosis*. However, this effect leads to a high degree of priming for subsequent release of cytokines such as TNF α (Mu & Sewell 1994). Mice, which have had the gene responsible for IFN γ production disrupted, die within three weeks after infection with tuberculosis (Cooper *et al.*

1993). Also, macrophages exposed to IFN γ , are primed for release of super oxide anion, hydrogen peroxide and hydroxyl radicals which are microbicidal to many organisms. Furthermore, IFN γ upregulates nitric oxide synthase and increases the capacity to release NO, which has very important bactericidal activity (Ehlers *et al.* 1994). In the case of human macrophages, IFN γ alone leads to enhancement of the intracellular multiplication of *M. tuberculosis* (Rook *et al.* 1986). However, this is clearly a misleading experiment, and children with defective IFN γ receptors die of BCG infection, and cannot even be saved by treatment with optimal chemotherapy (Jouanguy *et al.* 1996).

IL-4 is essential for B-cell isotype switching to immunoglobulin-E (IgE) synthesis. In mice lacking functional IL-4 genes, no IgE has been detected. In addition, treatment with monoclonal anti IL-4 at the time of immunisation causes a striking increase in DTH responses, and enhanced INF γ expression, while inhibiting the production of IL-4 and IgE (Mu & Sewell 1994). In contrast, injection of a monoclonal anti-IFN γ antibody is followed by a significant, but not complete, suppression of DTH reactions. IFN γ secretion is also inhibited, whereas IL-4 production and serum IgE levels are increased (Mu & Sewell 1994)

1.3.6 IL-12

IL-12 can be produced by many cells, including mononuclear phagocytes, but not by T-cells. IL-12 produced by these cells, mononuclear phagocytes, has been demonstrated to be important in the development and differentiation of TH1 cells. IL-12 increases

IFN γ production. *In vivo*, IL-12 cures mice of Leishmania infection and also enhances T-cell responses of HIV-infected tuberculosis patients *in vitro* (Wu *et al.* 1993; Zhang *et al.* 1994).

1.3.7 Transforming growth factor beta (TGF- β)

This cytokine is believed to play a role in tissue destruction and fibrosis, since it enhances the production of collagen matrix. In addition TGF β increases the production of macrophage collagenases. Moreover, TGF β is chemotactic for monocytes and polymorphonuclear cells, both of which are rich sources of collagenases and elastases (Toossi 1996). TGF β interferes with the production of reactive nitrogen intermediaries and down-regulates both the production and activity of TNF α and IFN γ (Chantry *et al.* 1989; Ding *et al.* 1990; Shepard & Palladino 1987; Tsunawki *et al.* 1988). Furthermore, TGF β has been found to enhance the intracellular growth of *M. tuberculosis* in human monocytes (Toossi 1996).

1.4 Interactions between the immune system and endocrine system

There is much evidence to show that the cytokines can act on the endocrine system. For instance, IFN γ acts on neural and endocrine cells causing steroidogenesis, melanogenesis and iodine uptake. Another example is that IL-1 and IL-6 cause a release of adrenal corticotrophic hormone (ACTH) and endorphin from a cultured pituitary cell line (Blalock 1994). It has been suggested that IL-1 and

IL-6 may work as hypothalamic-releasing factors. The responsiveness of hypothalamic neurones and pituitary cells to IL-1 suggests that neuroendocrine cells may express IL-1 receptors. Neuroendocrine cells express type I and type II receptors (IL-IR, IL-IIR). In the pituitary gland, Savino and Dardenne (1995) demonstrated that IL-2 and IL-6 alter the proliferation pattern of anterior pituitary cells *in vitro* as well as the secretion of growth hormone (GH) and adrenocorticotrophic hormones. IL-2 stimulates secretion of corticotrophin-releasing factor (CRH) from hypothalamic neurones by increasing NO (Savino & Dardenne 1995). Distinct cytokines including IL-5, IL-7, IL-9 and TGF- β are involved in the regulation of neuronal differentiation (Blalock 1994). The cells of the immune system in both primary and secondary lymphoid organs are able to produce hormones and neuropeptides, whereas classical endocrine glands, neurones and glial cells can produce a variety of cytokines. Moreover, receptors for all of these types of molecules are expressed in the cells of both the immune and neuroendocrine systems (Besedovsky & Rey 1996; Savino & Dardenne 1995). However, it is not clear how IL-1, IL-6 and IFN γ generated by infection and immune responses can signal to the hypothalamus, because very little can cross the blood-brain barrier. At least some signalling is via the vagus nerve. An immune stimulus in the periphery causes a signal via the vagus to the hypothalamus, when IL-1 is released locally (Bluthe *et al.* 1996).

1.4.1 The link between adrenal and pituitary gland

There is a link between the adrenal gland and the pituitary gland via (ACTH). The hypothalamic-pituitary-adrenal (HPA) axis is the main key in stress responses. During psychological stress, the higher centres of the central nervous system cause the release of corticotrophin-releasing hormone (CRH) from the hypothalamus into the portal circulation between the hypothalamus and the pituitary gland. CRH acts on pituitary corticotrophs to elicit ACTH synthesis and its release into the circulation. ACTH acts on the adrenal gland and causes the production of glucocorticoid hormones. These data have drawn attention to the relationship between the immune and endocrine systems in the case of infectious diseases.

1.4.2 Changes in the adrenal gland and consequences for the immune system

In human immune deficiency virus (HIV) patients, the ratio of cortisol / dehydroepiandrosterone (DHEA) increases at the same time as the decline in CD4 count. Also, these changes correlate with the appearance of HIV symptoms in patients (Laudat *et al.* 1995). A similar finding has been observed in TB (Rook *et al.* 1996). It has long been suspected that there is impaired adrenal function in tuberculosis, mainly because of the occurrence of sudden death during treatment resembling acute adrenal insufficiency. It has been found that there are subtle changes in adrenal function in human tuberculosis. Output of both glucocorticoid and androgen (DHEA) derivatives is reduced by approximately 50% in some patients though in others the levels are normal. Nevertheless, cortisol levels are

maintained, with greatly reduced conversion to inactive cortisone, whilst dehydroepiandrosterone sulphate (DHEA-S) levels are not. Some patients have low levels of DHEA-S. In normal humans of either sex, DHEA-S is the major product of the adrenal cortex. Lack of the steroid may have serious consequences because both DHEA and DHEA-S are anti-glucocorticoid. DHEA enhances TH1 activity and inhibits the effects of glucocorticoids, including their tendency to suppress TH1 lymphocytes and enhance TH2. For instance, a single dose of DHEA given before dexamethasone, (the corticosteroid analogue (DEX), which has a longer 1/2 life, and fails to bind to mineralocorticoid receptors), can block the ability of the DEX to cause depletion of thymocytes and temporary unresponsiveness of peripheral T cells to mitogens (Blauer *et al.* 1991). Furthermore, DHEA itself might be not the most active form of the anti-glucocorticoid hormones. For example, DHEA can be converted to 5-androstene-17 β -diol (AED) and 5-androstene-3 β -7 β -17 β -triol (AET) which are claimed by one group to play an important role in up-regulation of the immune system (Loria & Padgett 1992).

A second change in tuberculosis patients, is the abnormal balance of cortisol to cortisone. In normal individuals cortisol is converted to inactive cortisone in the kidney by the enzyme 11 β -hydroxy steroid dehydrogenase 2, (11 β -HSD-2). This cortisone is rapidly converted back to active cortisol in the liver by 11 β -HSD-1, acting as a reductase. Most tissues contains enzymes of this type, three or four different ones are now known.

In 24 hr urine samples from tuberculosis patients, levels of cortisone metabolites are very low and an oral cortisone load is very rapidly converted to cortisol (Baker R., *et al* personal

communication). Therefore, there is either abnormally low dehydrogenase activity, or exaggerated reductase activity. The site of the abnormality is unknown, but it may be the lung, the site of the tuberculosis infection. Therefore, T cells of tuberculosis patients may be chronically exposed to glucocorticoid effects, unopposed by the anti-glucocorticoid influence of DHEA or of its derivatives. This may not only encourage a TH1 to TH2 switch, but even contribute to the fall in CD4 T cell count and in the CD4/CD8 ratio (Rook & Hernandez-Pando 1994; Rook *et al.* 1994; Rook *et al.* 1993). The findings in humans are reinforced by the observation that when mice are infected with tuberculosis by the intra-tracheal route, the adrenals increase in size for two weeks and then decrease to 50% of their normal weight. This is an early stage of the lung infection, and the adrenals themselves are not directly infected. The mechanism leading to the atrophy is uncertain, but it has been found that the changes in cytokines may lead to adrenal abnormality. These changes may be attributable to TNF α .

1.4.3 Factors which may cause adrenal atrophy

TNF α , IL-1 and IL-6, acting via the hypothalamus and pituitary gland to release ACTH, may drive the enlargement of the adrenal in the first three weeks after infection with *M. tuberculosis* (Stankovic *et al.* 1994). In addition, TNF α alone has a direct toxic effect on the adrenal gland even in the absence of infection. *In vitro* TNF α acts directly on adrenal cells to reduce steroid output (Jäättelä *et al.* 1991). Death of tubercle bacilli leads to the release of some of their components such as lipoarabinomannan (LAM) which may have

a role in enhancing the production of TNF α (Moreno *et al.* 1989). A further explanation for the atrophy of the adrenal gland is the presence of TGF β Stankovic *et al.* (1994) showed that TGF β has a direct effect on adrenal cells *in vitro*. Like TNF α , TGF β inhibits DHEA-S more than cortisol. This may be important because TGF β is also abundant in tuberculosis lesions and has been implicated in the suppressive effects of patients' monocytes *in vitro* (Maeda *et al.* 1993).

1.5 Glucocorticoids (GCs)

Glucocorticoids are natural hormones which play an essential immunoregulatory role. Secretion from the adrenal glands is increased during the course of immune responses as a consequence of the interaction between the immune and neuroendocrine systems. The major natural adrenal glucocorticoid in humans is cortisol while in rodents it is corticosterone. The inactive forms of these steroids are cortisone and 11 α -dehydrocorticosterone in humans and rodents respectively. Glucocorticoids (active forms) at high concentration are well known for their suppressive effect on immune system components. Endogenous glucocorticoids may act as a physiological immunoregulators. They control the immune and inflammatory responses. Modulation of cytokine gene expression by corticosteroids has a number of more general implications. It may be anticipated that endogenous production of these hormones in response to environmental stress interferes with the immune response required to control infections that normally elicit a protective TH1 type reaction. Such interaction between the neuroendocrine system and immune

system may help to explain the prevalence of tuberculosis in areas of poverty and malnutrition (Francisco *et al.* 1996; Ramirez *et al.* 1996).

Immuno-suppression in warm blooded animals may be mediated by endogenously or exogenously elevated glucocorticoid (GC) levels. Elevated GC levels can result from a variety of causes including viral infections, bacterial infections, parasitic infections, cancer, some auto-immune syndromes, stress, trauma, post-surgical trauma or burn trauma. In fact it occurs as a secondary consequence of any clinical condition which causes an elevated production of IL-1, IL-6 and TNF α (Besedovsky & Rey 1996).

1.5.1 Immunological effects of glucocorticoids

1.5.1.1 Glucocorticoids inhibit some of the immune system components

Glucocorticoids inhibit production of TH1 cytokines IL-2 and IFN γ (Petrovsky & Harrison 1995). GC can also inhibit the production of pro inflammatory cytokines such as IL-1, IL-6 and TNF α . GC can increase the production of TH2 cytokines including IL-4, IL-10 and IL-13 (Daynes & Araneo 1989; Ramirez *et al.* 1996). These cytokines are important for B-cell development and immunoglobulin class switching. GC also enhances production of pro-TH2 cytokines which direct naive T-cells to differentiate to TH2 rather than TH1. In rodents corticosterone, the analogue of cortisol in humans, has the ability to suppress a wide range of immune system

activities. Glucocorticoids have the ability to enhance the programmed cell death of thymocytes and splenocytes (Wilder 1995). Corticosterone inhibits the proliferation of thymocytes and splenocytes via inhibition of the cytokines that stimulate proliferation e.g. IL-2, and the induction of inhibitory cytokines e.g. TGF β (Ayanlar-Batuman *et al.* 1991).

DEX induces *in vitro* a pattern of lymphokine production characteristic of a TH2 response. An increase was observed in IL-4 production and IL-4, IL-10 and IL-13 mRNA levels and a decrease in IFN- γ and TNF- α synthesis, on reactivation of CD4+ T cells that had been previously cultured for one week in the presence of DEX. IL-4 and DEX appear to act synergistically in that together they induce a high level of production of IL-4 and very low levels of IL-2, TNF α and IFN- γ . A possible explanation is that IL-4 alone promotes the development of a TH2 response without concomitantly inhibiting the development of a TH1 response, while after addition of DEX to the cultures, the development of a TH1 response is inhibited directly or indirectly by DEX. This explanation is consistent with the yield of cells under the following experimental protocol: the number of CD4+ T cells recovered from IL-4 treated cultures after the primary activation and expansion phases was found to be similar to those from the control, but when DEX was added, either alone or together with IL-4, the cell yield was reduced by 78% of control values (Ramirez *et al.* 1996)

GCs not only affects the pattern of cytokine production but they have a wide range of effects on different organs of the immune system. DEX influences the viability of dendritic cells (DCs) which act to present the antigen for T- cells. Also, DEX down-regulates the

expression of co-stimulatory molecules on viable DCs, and strongly reduces their immunostimulatory properties (Moser *et al.* 1995). The mechanism of action includes their lowered production of many lymphokines at both transcriptional and post-transcription levels (Labeur *et al.* 1995).

It has been found that GCs have the ability to disturb the total number of lymphocytes and the subpopulations in human blood. Changes in the concentration of circulating lymphocytes occur within minutes of acute infusion of corticosteroid in humans, are proportional to the dose administered, affect the T-cells more than B-cells and affect CD4⁺ cells more profoundly than CD8⁺ cells (Blalock 1994).

In addition, it has been found that DEX can alter the adhesive properties of endothelial cells. For example, in mice, DEX treatment of the recipient animal perturbs the recruitment and retention of labelled lymphocytes *in vivo*, and alters lymphocyte-endothelial interactions *in vitro*. Lymphocytes from untreated animals adhere poorly to tissue sections of lymph nodes from DEX-treated animals, but lymphocytes from treated animals adhere well to lymph node sections from untreated animals. This suggests that adhesive molecules on endothelial cells, rather than those on the lymphocyte, are altered in the presence of DEX. Indeed it has been shown that the addition of corticosteroid to endothelial cell cultures inhibits the synthesis and expression of endothelial E-selectin and intercellular adhesion molecule 1 (ICAM-1), adhesion molecules that normally occur in response to cytokine stimulation. In intact animals, a direct effect of corticosteroids on the expression of adhesion molecules by endothelium may be complemented by the ability of these hormones

to alter cytokine responses locally, thereby interfering with the response of endothelial cells to local inflammatory signals (Ottaway & Husband 1994).

1.5.1.2 Glucocorticoids activate some immune system components

In addition to the traditional role of glucocorticoids as suppressors of the immune system, at physiological levels glucocorticoids upregulate some aspects of the immune system (Northrop *et al.* 1992). When corticosterone is used at a physiological level, the proliferation of rat splenocytes induced by anti- T-cells receptors (TCR) increases significantly provided that the cells are exposed to corticosterone within the first 60 minutes of initiating the culture (Wiegers *et al.* 1995). Also, splenocytes from adrenalectomized mice pre-exposed to corticosterone for not more than 30 minutes, increase cell proliferation over the next three days compared to the controls. However, if splenocytes are exposed for up to 1 hr there is no effect of corticosterone on the proliferation (Wiegers *et al.* 1994). An earlier study showed that in adrenalectomized mice, replacement with a low concentration of corticosterone implanted subcutaneously restored the responsiveness of ConA-stimulated T-lymphocytes compared with control mice which did not receive corticosterone replacements. In contrast, when adrenalectomized mice were treated subcutaneously with a high concentration of DEX the responsiveness was not restored (Wiegers *et al.* 1993).

1.5.2 Mechanisms of action of glucocorticoids

1.5.2.1 Glucocorticoids downregulate genes responsible for activation of immune system components

Glucocorticoids circulate bound to corticosteroid binding globulin (CBG). Following dissociation from CBG and entry into the cells, the interaction of glucocorticoids with glucocorticoid receptor (GR) in the cytoplasm is the first step towards initiating an effect at the transcriptional level (figure 1-1). Both the concentration of glucocorticoids and the circadian rhythm dictate the receptor (type I or type II), to which the cortisol will bind. Before interaction with GC, glucocorticoid receptors form a complex with heat shock protein 90 (hsp 90). Dissociation of hsp-90 and dimerization of the steroid receptor complex takes place. This dimer is translocated to the nucleus in order to bind glucocorticoid response elements (GRE) in DNA. Interaction of the GC/GR receptor dimer with GRE alters the pattern of gene expression and causes changes in the cell metabolism, such as proliferation, inhibition, apoptosis and production of cytokines (Katzenellenbogen 1980; Wilckens 1995). In addition to binding to the GR, glucocorticoids can also bind to the mineralocorticoid receptor (MR, type I) with even higher affinity. Type II receptors can be, by alternative splicing, spliced into type α and β which might act against each other (Oakley *et al.* 1996). The glucocorticoid-receptor complex can also interact with other transcription regulating factors such as Janus kinase, (JAK), signal transducer and activator of transcription (STAT) and nuclear factor- κ B (NF κ B) (Burg *et al.* 1997; Li Hou & Rothstein 1994; Mezey & Palkovits 1992; Pearec & Yamamoto 1993; Rook *et al.* 1998). It has been demonstrated that genes such as STAT 4, 6 and 1 are very

important in modulating and producing cytokines such as IL-12, IFN γ and IL-4, which play a crucial role in regulating TH1 and TH2 responses (Crabtree 1989; Kaplan *et al.* 1996; Thierfelder *et al.* 1996). In addition, glucocorticoids inhibit expression of the *c-myc* gene which is responsible for production of IL-2 (Northrop *et al.* 1992).

Mechanism of action of glucocorticoids

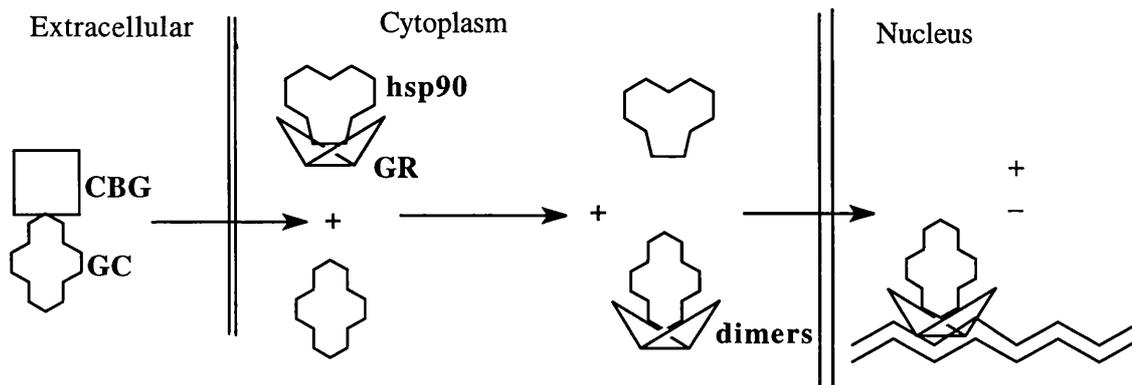
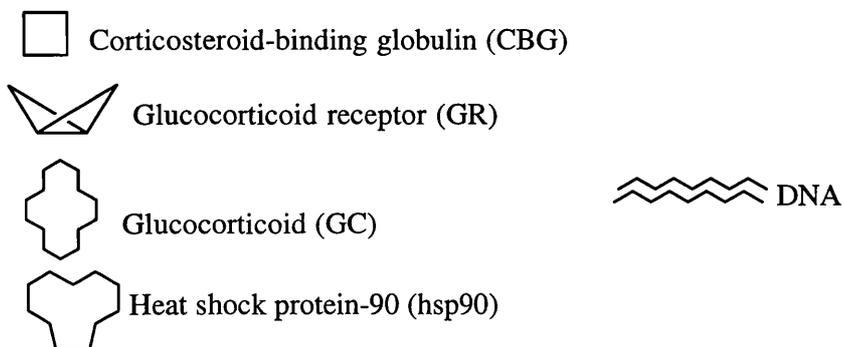


Figure 1-1

The glucocorticoid hormone circulates associated with corticosteroid-binding globulin (CBG). GC enters the cell and interact with heat shock protein-90 (hsp90) - glucocorticoid receptor complex. The GC/GR complex then forms dimers which bind to glucocorticoid response elements (GRE), in the DNA. Upon binding of glucocorticoid to GRE an alteration of transcription occurs. This alteration, depending on which gene encoded, may cause the inhibition (-) or activation (+) of cytokine production.



1.5.2-2 Glucocorticoids activate inhibitory genes in order to exert an inhibitory effect on immune system components.

Glucocorticoids not only inhibit genes to suppress the immune system, but also up-regulate genes to accomplish the job. It has been found that glucocorticoids activate inhibitory molecules such as I κ B α (Burg *et al.* 1997). It has even been suggested that this is not a major mechanism by which GCs down-regulate the production of inflammatory cytokines, and enzymes such as collagenase which are responsible for destruction of tissues. However, recently it has been suggested that binding GCs to GRE is not the ultimate mechanism to inhibit NF- κ B. Upon stimulation, NF- κ B, which is bound to either I κ B α or I κ B β , dissociates from this complex and targets DNA to up-regulate genes, while I κ B α or I κ B β are degraded. For instance, when a cytokine such as TNF binds to its receptors, this leads to destruction of I κ B. NF- κ B then moves into the nucleus, where it activates cytokine production. However, the GC receptor complex binds to NF- κ B, inhibiting any further production of these molecules, and simultaneously the I κ B α concentration increases significantly. GCs increase the rate of transcription of the I κ B α gene into RNA. As a result, the I κ B α concentration within the cell increases, allowing the protein to retain NF- κ B in the inactive form in the cytoplasm even under conditions when it would normally be released to move into the nucleus. This suggests that glucocorticoids activate a gene which, together with the traditional inhibitory method, ultimately stops NF- κ B binding to DNA and therefore halts cytokine production (Auphan *et al.* 1995; Cogswell *et al.* 1993; Marx 1995; Scheinman *et al.* 1995 a; Scheinman *et al.* 1995 b).

1.6 Dehydroepiandrosterone (DHEA)

In contrast to GCs, DHEA is produced in the zona reticulata and can be metabolised to different steroids such as testosterone and oestradiol. Also, DHEA can be metabolised according to the needs of each tissue. It is the intermediate in the biosynthesis of many other hormones. DHEA-S is a sulphated form of DHEA. High levels of DHEA-S are present in the plasma of late-term human foetuses and in new-born infants. Over the first 6 months of (neonatal) life, the DHEA-S level declines dramatically. DHEA-S levels start to rise to intermediate levels at adrenarche, and then rise to a peak at puberty. This peak is sustained until the age of 30 years, then the level starts to decline. By the age of 80 - 90 years, only 5 % of the peak adult plasma DHEA-S levels remain. This decline is concomitant with a decline in the ability of the immune system to counteract all sorts of immunological problems. It has been observed that the deterioration of the immune system correlates well with the DHEA level at two stages in an individual's life-time. The first stage is when plasma levels of DHEA decline sharply at the age of 6 months. The second stage is over the age of 60 years, when the DHEA level declines sharply (Jesse *et al.* 1995; Nestler 1995; Spencer *et al.* 1995). Although DHEA and DHEA-S levels decline with age in men and women, advancing age has no effect on cortisol levels. This also has been linked to the immune system deterioration in advanced age. In view of the fact that cortisol is an immune-regulator at a physiological level and immunosuppressor at pharmacological levels, while DHEA and some of its metabolites may function as anti-glucocorticoids, the imbalance of these steroids may have a serious impact on the ability of the immune system to mount an effective

response. This is supported by data which show that glucocorticoids enhance TH2 cytokines and also enhance the production of TGF β , which has a negative effect on T-cells (Ayanlar-Batuman *et al.* 1991).

1.6.1 Factors which may affect DHEA levels

The reason for the age-related decline in DHEA levels is unknown. However, Nestler (1995) showed that in elderly people insulin levels rise, which increases the metabolism of DHEA-S and DHEA. This suggests that insulin acts to reduce the level of DHEA-S and DHEA in one of two ways: either reducing the production rate by inhibiting one of the key enzymes (17,20 lyase) required to convert the DHEA precursor to DHEA; or by enhancing the metabolic clearance rate. However, Nestler noted that in women there was no alteration in their DHEA-S and DHEA levels. All the variation in DHEA-S / DHEA levels among the elderly group could be accounted for by the existence of different levels of insulin in men. However, this explanation is unlikely and cannot account for the fall in DHEA levels in women. An acute rise in ACTH leads to an increase in DHEA levels in young people while in elderly people DHEA decreases (Vermeulen 1995). Albumin may have some effect, as there is a correlation between albumin levels and DHEA-S. Genetic differences may also affect the levels of both DHEA and DHEA-S. For example, Japanese men have lower levels than their American counterparts (Vermeulen 1995).

1.6.2 Non-immunological effects of DHEA

1.6.2.1 DHEA protects against atherosclerosis

DHEA protects against atherosclerosis, possibly by inhibiting thromboxane B₂ (TxB₂) to prevent platelet aggregation. Barrett-Conner & Goodman-Gruen (1995) noticed an association of coronary vascular disease with a low level of DHEA in men, but not in women. Also, Herrington (1995) noted an inverse relationship between the low level of DHEA in plasma and angiographically-defined coronary diseases. Intriguingly an animal model showed that oral supplementation of the diet with DHEA could prevent the onset of atherosclerosis (Herrington 1995).

1.6.2.2 DHEA controls fat intake in rodents

DHEA at dietary levels has been shown to have some side effects such as reduction in weight, change in liver colour, and increase in liver weight. DHEA increases the production of dopamine 5-hydroxytryptamine (5-HT) (serotonin) and 5-hydroxy indoleacetic (5HIAA) which are responsible for controlling fat intake. Also, DHEA increases the production of serotonin in the lateral hypothalamus (the region involved in food intake), of the obese Zucker rat. Moreover, DHEA alters hypothalamic and regional hypothalamic neurotransmitters which communicate with the central nervous system. This is a mechanism via which DHEA controls the calorie intake (Porter & Svec 1995; Svec & Porter 1995).

1.6.2.3 DHEA activates peroxisomes

DHEA acts as a peroxisomal proliferator, explaining the change in the colour of the liver mentioned in the previous section, and induces key enzymes such as 3-hydroxy- fatty acyl CoA dehydrogenase, which are part of the fatty acid - β oxidation system of liver peroxisomes (Milewich *et al.* 1995). This pathway is considered in greater detail later. Also, DHEA induces hepatic glutathione S-transferase which protects cells against the toxic effects of foreign, harmful compounds. In such cases, DHEA indirectly catalyses the conjugation of these compounds to glutathione to give products harmless to liver cells (Milewich *et al.* 1995).

1.6.2.4 DHEA reduces the production of prolactin (PRL)

DHEA reduces PRL which has shown to have immunoregulatory effects. Both T and B cells contain PRL receptors. Lymphocytes produce and secrete prolactin. PRL increases with advancing age in rodents. Hyper-prolactinaemic animals are characterised by premature albuminuria, elevated IgG and accelerated mortality. Therefore there is an inverse relationship between DHEA and PRL, as the first decreases while the second increases with advancing age (Milewich *et al.* 1995).

1.6.2.5 Possible protective role of DHEA against Alzheimer's disease

The decline in DHEA level may play a role in advancing and developing Alzheimer's disease. This was suggested when it was noticed that a low level of DHEA leads to the development of unwanted amyloid proteins which are common in Alzheimer's patients (Danenberg *et al.* 1995 a).

1.6.3 Immunological roles of DHEA and its derivatives

1.6.3.1 Regulation of systemic resistance to infection by DHEA and its derivatives

There is some evidence that DHEA is able to enhance the ability of the immune system to protect the host from a variety of infectious diseases *in vivo*. It has been found that a single subcutaneous injection of DHEA to inbred male mice resulted in more than 50% protection from *Coxsackie* virus B4-induced mortality (Loria *et al.* 1988). Moreover, a 90% reduction in herpes virus type 2 encephalitis-mediated mortality in female inbred mice was achieved with a subcutaneous injection of DHEA (reviewed in Loria and Padgett 1992). Also, DHEA is able to protect mice from infection with *West Nile* virus, a neurovirulent *Sindbis* virus and *Semliki Forest* virus (Loria *et al.* 1988). In addition to conferring protection against lethal RNA and DNA virus infections, further observations (Padgett & Loria 1994) illustrate that DHEA has a similar protective effect against a lethal *Enterococcus faecalis* infection. DHEA augments the immunisation of old mice to

recombinant hepatitis B vaccine. In addition, DHEA protects challenged animals against live influenza virus (APR/B) (Danenberg *et al.* 1995 b). 5androstene 3 β -17 β diol (AED) and 5-androstene 3 β -7 β -17 β triol (AET) protected the thymuses and the spleen cells against DEX (Padgett & Loria 1994). Also, there is some evidence that AED protects mice against CB4-lethal infection (Loria & Padgett 1992). However, the doses used in the above studies were beyond the acceptable range. Since the normal range in these animals at the highest levels do not exceed 1 ng/ml, all these results should be treated with extreme caution.

1.6.3.2 Effect of DHEA and its derivatives on TH1 cytokines

DHEA and AED protect mice against lethal infections, but the mechanisms of action remain obscure. Although the molecular basis is not fully understood, the immune regulation mediated by DHEA and AED appears to be independent of host haplotype, since the response of animals with different histocompatibility makeups, H-2^d, H-2^b or H-2^q is similar (Loria *et al.* 1988). It has been suggested that these hormones may counteract the immunosuppressive effects of glucocorticoids stimulated by the viral infection itself (Loria & Padgett 1992). Others have also suggested that DHEA blocks the involution of lymphoid organs seen following infection with *West Nile virus* (Loria *et al.* 1988). Such organ involution is similar to the reduction in spleen and thymus size which is due to glucocorticoid increases. Neither DHEA nor its derivative, AED, have any effect directly as anti-bacterial or anti-viral agents (Loria *et al.* 1988; Padgett & Loria 1994). In a similar study (Blauer *et al.* 1991), it was

found that the incubation of lymphocytes with DHEA leads to a significant enhancement of the ability of cells to produce IL-2 and IFN γ . Moreover, in the same study it was shown that incubation with corticosterone produced IL-4 rather than IL-2 and IFN γ . Therefore, it is possible that the effect of these hormones is not by direct inhibition of the viruses or bacteria but is via the regulation of specific immune components i.e. cytokines (Blauer *et al.* 1991; Loria *et al.* 1988; Loria & Padgett 1992; Padgett & Loria 1994).

1.6.4 Possible mechanisms of action of DHEA

The mechanism of action of DHEA and some of its metabolites is not fully understood, nor how they antagonise the suppressive effect of glucocorticoids on the immune system. However, there are some possible hypotheses. For instance, Morfin & Courchay (1994) claimed that DHEA reduces the ability of glucocorticoids to bind to the nuclear receptors, but this has not been confirmed. Spencer *et al.* (1995) described how DHEA-S can up-regulate peroxisome enzyme activities. Peroxisome enzymes, which are responsible for lipid homeostasis, fatty acid degradation, and destruction of leukotriene B₄ (LTB₄), can be activated via peroxisome proliferator-activated receptors (PPARs). PPARs are members of the steroid receptor gene superfamily (Waxman 1996). These receptors can be converted to active transcription factor subunits. Peroxisomal proliferator-activated receptors activate the transcription of genes encoding selected peroxisomal enzymes (Milewich *et al.* 1995). There are three types of PPARs, termed α , β and γ . These receptors can be activated by different molecules, collectively known as peroxisome

proliferators (PPs), which include clofibrate, hypolipidaemic fibrates, some arachidonic acid metabolites and various fatty acids (Soontjens *et al.* 1996). All these activators have a common characteristic structure (Milewich *et al.* 1995). This includes a hydrophobic backbone and the presence of either a carboxylic acid, a functional group that can be metabolized to a carboxylic acid or that otherwise has an acidic function other than carboxylic acid (Milewich *et al.* 1995). This feature exists also in DHEA-S which has been found to act as a peroxisome proliferator (PP). DHEA is considered an endogenous regulator for PPARs, and consequently the fatty acids and their derivatives, in particular, eicosanoids. DHEA and the 17- β -hydroxy derivative (AED) have been found to be active, but the sulphates were more active. This suggests that sulphate is an important mediator of the effects of DHEA-S on the liver enzyme and peroxisome proliferation (Orentreich *et al.* 1984; Waxman 1996). However, 5-androstene-3-17-dione and androstene- 3-17dione, which are metabolites of DHEA, were found to be inactive. It is probable that the effects of DHEA-S as a pp are mediated by PPAR α . PPAR α is expressed in tissues that have high fatty acid catabolism such as the immune system and liver. In the liver, the functions of PPAR α are to induce enzymes which are responsible for the degradation of fatty acids and the detoxification of various xenobiotics. Exposing hepatocytes to DHEA-S results in increases in β -oxidation activity (Devchand *et al.* 1996; Spencer *et al.* 1995). The oxidation is catalyzed by fatty acyl CoA oxidase. This reaction consumes O_2 which is reduced to H_2O_2 . The next step involves the addition of H_2O to the C-2,C-3 double bond in a reaction catalyzed by enoyl CoA hydratase to give the corresponding 3-oxo-fatty acyl CoA, which serves as a substrate for 3-hydroxy-fatty acyl CoA dehydrogenase. The product,

3-oxo-fatty acyl CoA, is degraded by 3-oxo-fatty acyl CoA thiolase (Milewich *et al.* 1995). A decline in β -oxidation activity (as a result in a defect in peroxisome activity) leads to an increase in fatty acid chain length, causing an increase in membrane rigidity which might have an impact on the responsiveness of immune cells to different stimulators. Membrane phospholipid content increases with advancing age in various lymphoid organs. Decreased serum levels of DHEA and its sulphate during ageing, have also been associated with a significant increased risk for the development of several human malignancies including breast, ovarian and bladder cancers as reviewed in Waxman (1996)

It has been suggested that PPAR α controls the anti or pro-inflammatory events by controlling leukotriene B₄ (LTB₄). LTB₄ is a fatty acid derivative which can induce a complex cascade of molecular and cellular events that ultimately recruit cells from the immune system to the site of injury and produce inflammation. However, LTB₄ is inactivated via metabolic degradation by peroxisome β -oxidation. This can be activated by LTB₄ itself via PPAR, or other PPs such as clofibrate or polyunsaturated 3-fatty acid. However, this is not a likely mechanism for the immunological role of DHEA-S, because increased degradation of LTB₄ should have anti-inflammatory effects, while DHEA-S increases inflammation (Sutters *et al.* 1997)

Another possible mechanism is based on a specific complex of protein or macromolecules in the cytosol fraction of peripheral T-cells and murine T-cells hybridomas. This complex has hormone receptor-like characteristics. These receptors exist in both cytosol fractions and intact cells, and appear to be highly specific for DHEA,

although it has been reported that dihydrotestosterone (DHT) can compete for these receptors (Meikle *et al.* 1992). No other group has confirmed the presence of these receptors.

In the central nervous system (CNS), DHEA-S binds to gamma amino butyric acid (GABA_A) membrane-bound receptors which can be regulated by certain neurosteroids (Goodyer *et al.* 1996). Some of these neurosteroids behave as agonists (androsterone and tetrahydroprogesterone) and others behave as antagonists (DHEA-S and pregnenolone sulphate) (Majewska 1992). However, DHEA and DHEA-S at low concentrations function as antagonists for GABA_A receptors (Majewska 1995; Robel & Baulieu 1995). Also, DHEA acts as a potentiator of N-methyl-D-aspartate (NMDA) and sigma receptor function (Baulieu 1996).

There is a possibility that DHEA-S binds to cell-membrane associated receptors in peripheral blood to exert its effect on platelet aggregation. DHEA-S was found to inhibit aggregation induced by arachidonic acid, by inhibiting thromboxane B₂ (Jesse *et al.* 1995).

1.6.5 DHEA metabolism

The major metabolites of DHEA in rodents are AED, 7 α -OH-DHEA, 7 Oxo - DHEA, and 16 α -OH DHEA (Morfin & Courchay 1994). However, in humans androsterone and etiocholanolone are the main metabolites of DHEA-S (Hobe *et al.* 1995). It is possible that DHEA exerts some of its *in vivo* effects via conversion to androgens (testosterone, 5 α dihydrotestosterone), oestrogens (estradiol-17 β , 5

androstene-3 β , 17 β -diol), sulphation (DHEA-S) or 7 α hydroxy-DHEA.

In normal individuals, in addition to etiocholanolone and androsterone, DHEA can be metabolised to produce hormones like AED and AET (Loria & Padgett 1992). However, in TB patients the metabolic balance changes to give increased production of 16 α hydroxylated DHEA (16 α -DHEA) (Rook *et al.* 1996), yet the function of this compound is unknown. For example, 16 α -DHEA occurs in women during normal pregnancy. It has been found that T cells from women with recurrent spontaneous abortion released more TH1 cytokines in response to paternal antigen, than did cells from women who had normal pregnancies which were more biased toward TH2 (Hill *et al.* 1995). Thus, if DHEA promotes a TH1 response, it is theoretically possible that conversion to 16 α (OH) derivatives is a mechanism for the inactivation of DHEA during pregnancy. In this case, 16 α (OH) -derivatives must be immunologically inactive. Also, it has been found that some other steroids contribute to the control of the immune response of the foetus, since it was found that oestrone, β -estradiol and oestriol suppress T-lymphocytes (Medina & Kincade 1994).

1.7 aims of this study

To re-investigate DHEA and some of its derivatives at physiological concentrations, in order to determine their effects on immune system components as follows:

- 1 To determine their effect on cell proliferation and cytokine production, and to determine whether they can protect thymuses against involution caused by glucocorticoids;
- 2 To gain insight into the nature of the active metabolites of DHEA and selected derivatives;
- 3 To investigate whether *M. tuberculosis* can cleave DHEA to active or non-active compounds;
- 4 To determine whether 11 β hydroxysteroid dehydrogenase / reductase activity in lung can be regulated.

CHAPTER-2

The effect of DHEA and some of its derivatives on splenocyte proliferation, on glucocorticoid-suppressed-splenocytes and on cytokine production by murine splenocytes and human T-cells in the presence and absence of glucocorticoids.

2.1 Introduction

2.2 Materials and methods

2.2.1 Stimulation of lymphocytes with mitogens

2.2.2 Determination of inhibition curve of corticosterone *in vitro*

2.2.3 Effect of anti-glucocorticoid steroids on murine spleen cells partially suppressed by corticosterone

2.2.3.1 Part A

2.2.3.1 Part B

2.2.4 Effect of AED on cytokine production from ConA-induced murine spleen cells

2.2.5 Effect of DHEA and AED on production of cytokines by human white blood cells and T-cells

2.2.5.1 Protocol for white blood cells

2.2.5.2 Protocol for T-cells

2.2.6 Statistical analysis of the data

2.3 Results

2.3.1 Stimulation of lymphocytes with mitogens.

2.3.2 Determination of inhibition curve of corticosterone *in vitro*

2.3.3 Effect of anti-glucocorticoid steroids on murine spleen cells partially suppressed by corticosterone

2.3.3.1 Compounds lacking the double bond in ring B

**2.3.3.2 Compounds with a double bond in ring B:
(DHEA and AED)**

2.3.3.3 7-hydroxy derivatives of DHEA and AED

2.3.4 Effect of AED on cytokine production from ConA-induced spleen cells

2.3.4.1 IFN- γ

2.3.4.2 IL-5

2.3.5 Effect of DHEA and AED on production of cytokines by human WBC and T-cells

2.3.5.1 In the absence of cortisol

2.3.5.2 In the presence of cortisol

2.4 Discussion

2.4.1 Variation in the effects of DHEA and its derivatives in an *in vitro* system.

2.4.2 Effect of DHEA and AED on increasing IFN γ production by human T-cells

CHAPTER-TWO

2.1 Introduction

A substantial body of data is accumulating which suggests that DHEA and some of its normal metabolites antagonise the suppressive effect of glucocorticoids on immune system components such as T-cells. However, most of these studies are conflicting. Furthermore it is not known which compound is the most active metabolite.

The following experiments were designed to investigate the *in vitro* effect of DHEA and some of its metabolites on the proliferation of splenocytes, and to determine whether these steroids antagonise the suppressive effect of glucocorticoids on such cells. The effect of these steroids on the production of different cytokines was also investigated.

2.2 Materials and methods

2.2.1 Stimulation of lymphocytes with mitogens.

Concanavalin-A (Sigma)

Concanavalin-A (ConA) was used to stimulate unprimed cells. In cell culture, ConA has the ability to induce mitogenic activity of T-lymphocytes and to increase the synthesis of cellular products.

Nutridoma-SR (Boehringer Mannheim)

Nutridoma-SR is used to replace foetal calf serum in cultures of myeloma, hybridoma and lymphoblastoid cell lines. It is composed of albumin, insulin, transferrin, a cholesterol source and other defined and inorganic growth factors, a serum substitute, a nitrogen source, and may include hormones. The manufacturer does not provide a full list of the components.

Roswell Park Memorial Institute-1640 (RPMI-1640) (Imperial laboratories).

Nutridoma at a concentration of 1%, streptomycin, gentamycin and glutamine were added to RPMI1640 to make the complete medium.

Red blood cell lysis solution

0.83% ammonium chloride (BDH-Analar) and 0.1% potassium hydrogen carbonate (BDH-Analar) were dissolved in 100 ml distilled water then sterilised using a 0.2 μ m filter.

Mice

Female and male C57 BL / 6.J mice aged 3-4 months were kept in the animal house, 6-9 mice per cage with free access to food and water.

Protocol

Two or three mice were killed by cervical dislocation. Spleens were taken under sterile conditions and placed in petri-dishes. Free cells were blown out of the intact spleen using 5-ml complete medium in a syringe, followed by squeezing the spleen to release remaining cells into the medium. Cells were transferred to a universal tube and spun for 5 minutes at 2000 rpm. The supernatant was discarded and the pellet was resuspended in 5 ml of RBC lysing buffer per spleen. The tube was left at room temperature for 7 minutes to lyse the red blood cells. The tube was spun as before, the supernatant was discarded, the cells were washed once in complete medium, and resuspended in 5-ml of complete medium. The cell count and viability were assessed using a haemocytometer and 0.4% (w/v) trypan blue. Cells were adjusted to 2×10^7 / ml. ConA was diluted in complete medium to give 10, 3.3, 1.1, 0.37, 0.12, 0.04, 0.0137 $\mu\text{g/ml}$. Each concentration was dispensed into 24 well plates in 250 μl . The same volume of cell suspension (2×10^7 cells / ml), was added to each concentration of ConA. A control consisting of medium + cell suspension was included. Plates were incubated at $37^\circ \text{C} + 5\% \text{CO}_2$ for 24 hrs for IL-4 assays and for 48 hrs for IFN γ assays. The supernatants were collected and frozen for subsequent enzyme linked immunosorbent assay (ELISA). |Haemocytometer-Improved Neubauer,

ELISA

Coating buffer

This was used to dilute the primary antibody. Sodium hydrogen carbonate (0.84g) was dissolved in 100 ml of sterile (non pyrogenic) water and the pH adjusted to 8.2.

The following monoclonal antibodies were supplied by PharMingen.

Purified rat anti-mouse interleukin-4 (IL-4)

The immunogen was *E. coli* expressed IL-4. It had been purified from tissue culture supernatant by affinity chromatography. This antibody was supplied in phosphate buffered saline (PBS) with 0.1 % (w/v) sodium azide for use as a capture antibody in ELISA.

Biotinylated rat anti-mouse IL-4 monoclonal antibody [ELISA detection]:

This is an IgG1 isotype cloned from BVD - 24 G2. The immunogen was *E coli* - expressed IL-4.

Purified rat anti-mouse interferon gamma (IFN γ)

This is an IgG1 isotype clone of R4-6A2. It is supplied in PBS with 0.1% (w/v) sodium azide and was used as a capture for mouse IFN γ .

Biotinylated rat anti-mouse IFN γ monoclonal antibody [ELISA detection].

This is a rat IgG1 isotype and clone of XMG1.2. This antibody was used as a detector antibody for mouse IFN γ .

Substrate buffer:

Citric acid and di-sodium hydrogen orthophosphate-2 hydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$). Each of these solutions was made separately as follows: 5.25 grams of citric acid was dissolved in 250 ml distilled water. Similarly, 4.447 grams of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ was dissolved in 250 ml distilled water. Citric acid solution was added gradually to $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ until a pH of 4.1 was attained.

ABTS:

0.125mg of 2,2'-Azino-di-3-ethylbenzthiazoline-6 sulphonic acid (Sigma-UK) was dissolved in 10 ml of citrate buffer then 7.8 μl of hydrogen peroxide (BDH-Analar) was added.

Phosphate Buffered Saline -Bovine Serum Albumin (PBS-BSA).

PBS (BDH-Analar)

NaCl 8.0g

KHPO_4 0.2g

NaHPO_4 1.135g

KCl 0.2g

Distilled water 1 litre

pH 7.4

BSA:

3% bovine serum albumin in PBS was used to block the plates after they had been incubated with the primary antibody (capture).

Washing buffer

Polyoxyethylene Sorbitan Monolaurate (Tween-20) (Sigma) 0.5ml was added to one litre of PBS. Tween-20 acts as a detergent to assist washing and blocking of the plates.

Peroxidase-conjugated streptavidin (DAKO)

This was supplied as high activity horseradish peroxidase conjugated to streptavidin from *Streptomyces avidinii*.

Protocol

Cytokines were quantified by a capture ELISA. The primary antibodies were diluted to 3 µg / ml in the coating buffer. A 50 µl volume (3 µg/ml) of an appropriate capture antibody was adsorbed to wells of a 96-well microtest plate (ELISA plates) and incubated overnight at 4°C. The coating buffer was discarded and the wells blocked with 200 µl of PBS-3%BSA for 1hr at 37°C. Plates were well washed 3 times with PBS-Tween. Test supernatants and 2-fold serial dilutions of the appropriate reference cytokines (100 µl/well) were dispensed into the wells and incubated at 37°C for 2 1/2 hrs. After washing as before, 100 µl biotinylated detection antibody (1 µg/ml) was dispensed into each well. Plates were incubated at 37°C for 1-1/2 hrs then washed 3 times. Streptavidin solution (100 µl), diluted 1:1000 in PBS- BSA was dispensed into each well. Plates were incubated 1 hr then washed 3 times. The ELISA plate was developed using hydrogen peroxide (7.8 µl) in 10ml of ABTS solution. After 30 minutes at 37°C, the optical density was determined at 405 nm using a spectrophotometer-Dynatech-500-UK.

The computer programme Assay Zap running on an Apple MAC was used to calculate the concentrations of cytokines in ng/ml.

2.2.2 Determination of inhibition curve of corticosterone *in vitro*

Corticosterone is the major glucocorticoid in rodents and was therefore used in all experiments with cells from mice.

Corticosterone works in a dose- dependent manner to inhibit the proliferation of spleen cells. Choosing the concentration which gives 50% inhibition, enables the assay of enhancers or antagonists of corticosterone-mediated suppression. In the first part of the experiment different concentrations of ConA were tested to determine the optimum concentration which gives the greatest response by murine spleen cells, and then the concentration of corticosterone giving 50% inhibition was identified.

Preparation of corticosterone.

Corticosterone (4-Pregnene-11 β , 21-diol-3, 20 dione) (Sigma) was dissolved in equal volumes (v/v) of ethanol/dimethyl sulphoxide (DMSO, Sigma), to achieve 10^{-2} M as a stock solution. Tenfold dilutions were made in ethanol/DMSO to give concentrations of 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} and 10^{-9} M corticosterone. Each of these concentrations was diluted 1:100 in complete medium to give concentrations of 10^{-5} - 10^{-11} M. The solutions were diluted in culture medium (1:10) and the concentration of ethanol/DMSO was adjusted so the final concentration of ethanol/DMSO in each culture well was 0.1%. This is because high concentrations of ethanol/DMSO are believed to inhibit cell proliferation.

Protocol

Splenocytes were pooled from three normal drug-free C57BL/6J (H-2^d) female mice that had been killed by cervical dislocation. The cells were treated with RBC lysing buffer and washed with complete medium. Viability was assessed and the cell count was adjusted to 2×10^7 cells/ml. Corticosterone was dissolved in ethanol/DMSO (1:1) to achieve concentrations of 10^{-5} - 10^{-12} M. Corticosterone solutions were adjusted so the final concentration of ethanol/DMSO was 0.1% in culture wells. Cultures were set up in triplicate, with each well containing 20 μ l of corticosterone diluted in culture medium, 80 μ l of ConA solution and 100 μ l cells (2×10^7 cells/ml). Control wells (medium with/without 0.1% ethanol/DMSO and medium with /without ConA) were assayed simultaneously with the test wells.

2.2.3 Effect of anti-glucocorticoid steroids on murine spleen cells partially suppressed by corticosterone.

These compounds were prepared in the same way as corticosterone.

Compounds used or discussed in this section were :

DHEA and androstene-diol

1- 5-androstene-3 β -ol-17-one (DHEA) (Sigma)

2- 5-androstene-3 β -17- β -diol (AED) (Sigma)

7-OH derivatives of DHEA and androstene-diol

3- 7-hydroxydehydroepiandrosterone (Celltech)

4- 5-androstene-3 β -7 β -17- β -triol (AET)

5-androstene-3 β -7 α -17- β -triol } (Celltech) a mixture of α and β

Reduced form of DHEA, androstene-diol and their 7-OH-derivatives

5- androsterone

6- androsterone

7- 3 β -7 β dihydroxy 5 α androstan-17-one (Sigma)

8- 3 β -7 β -17 β -trihydroxy 5 α -androstane (Sigma)

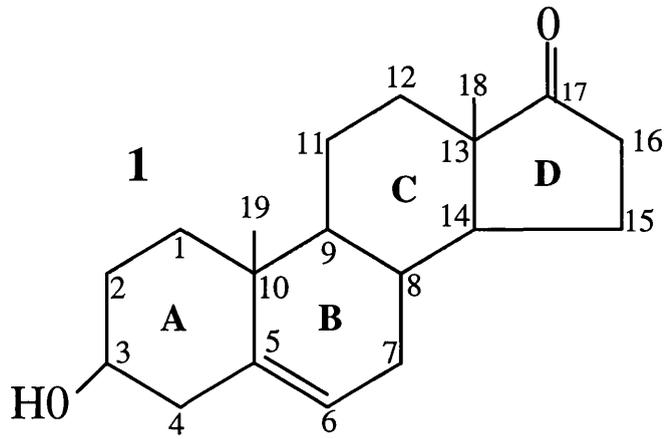
16-OH derivatives of DHEA and androstene-diol

9- 5-androstene-3 β -16 α -diol-17-one (16- α -OH-DHEA) (Sigma)

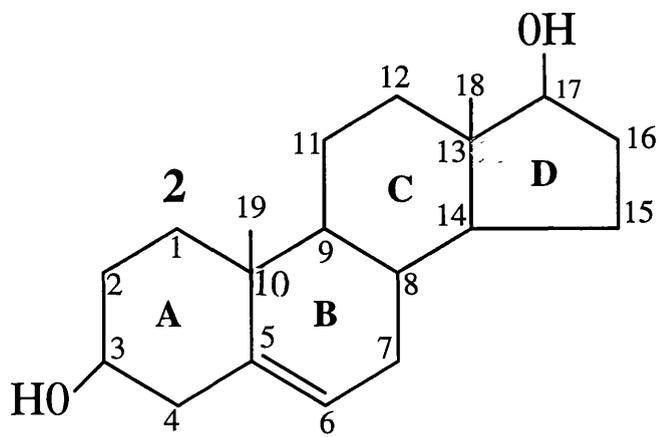
10- 5-androstene-3 β -16 α -17 β -triol

The structures of the above compounds are illustrated overleaf.

DHEA and androstene-diol

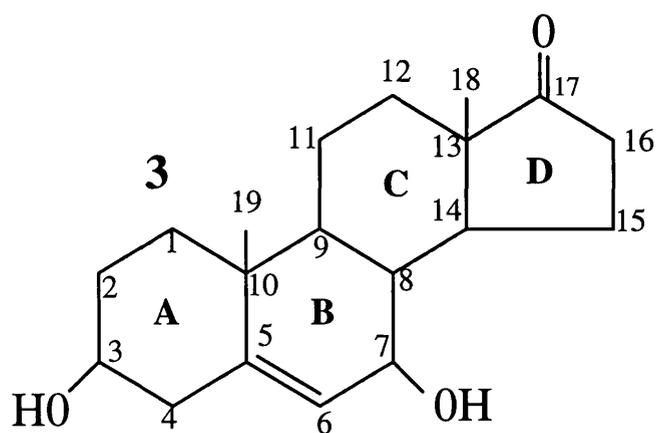


Dehydroepiandrosterone

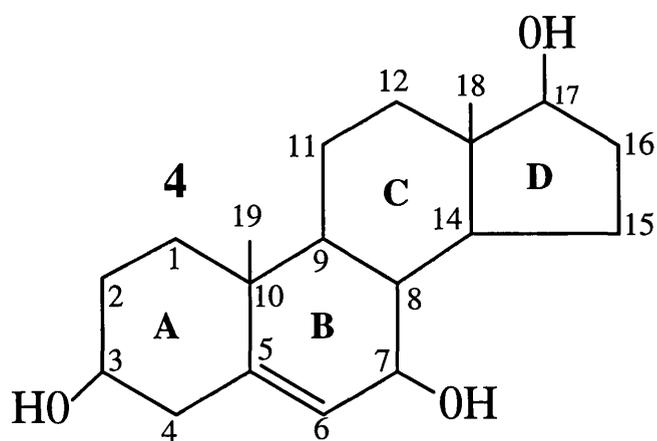


Androstene-3 β -17 β diol (AED)

7-OH-derivatives of DHEA and androstene diol

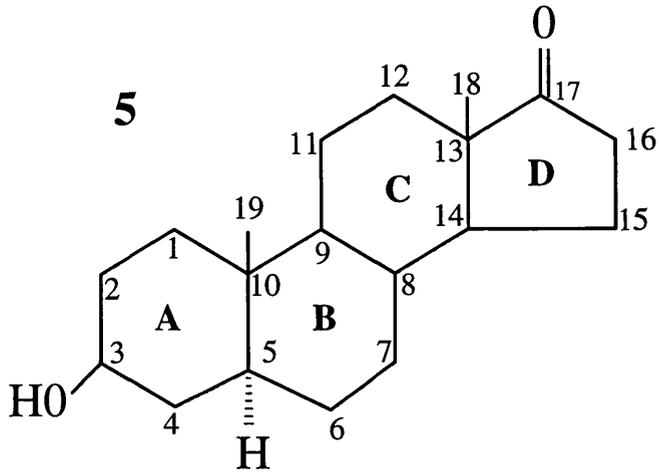


7-hydroxydehydroepiandrosterone

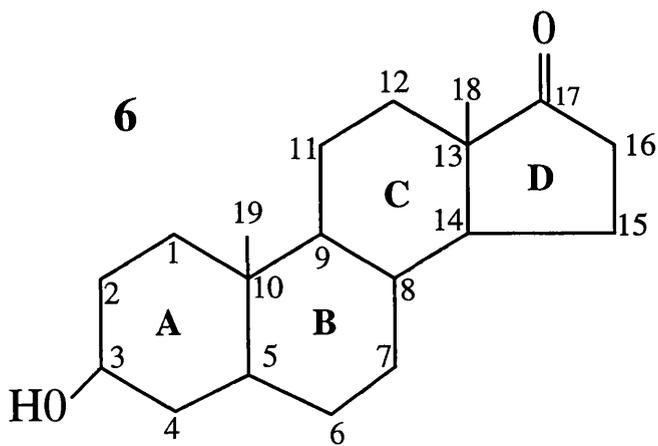


Sandrostene 3β - 7β - 17β -triol (AET)

Reduced form of DHEA, androstene-diol and their 7-OH-derivatives

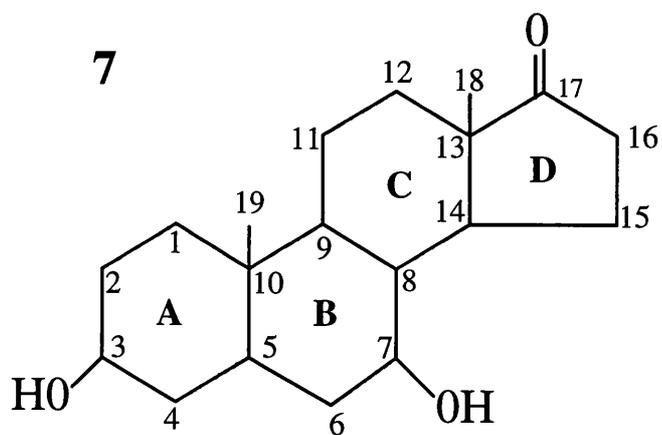


Androsterone

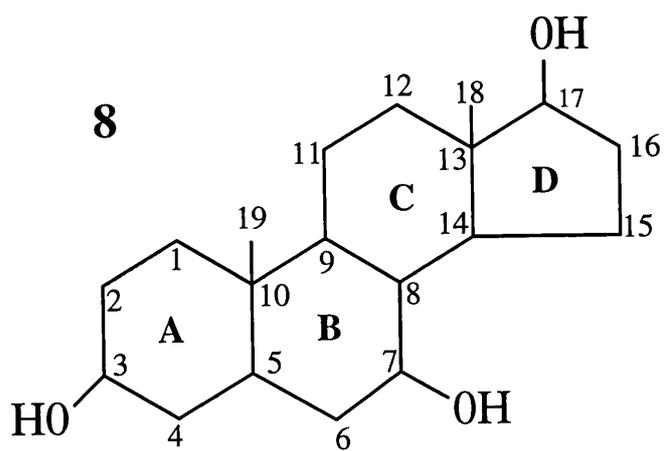


Etiocholanolone

Reduced form of DHEA, androstene-diol and their 7-OH-derivatives

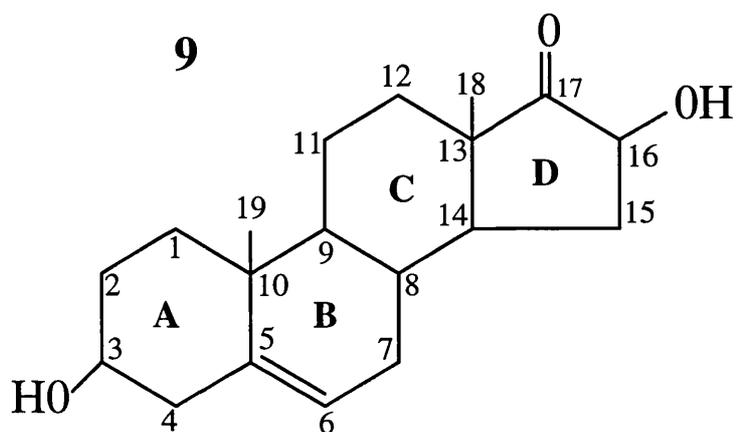


3β-7β dihydroxy 5αandrostan-17-one



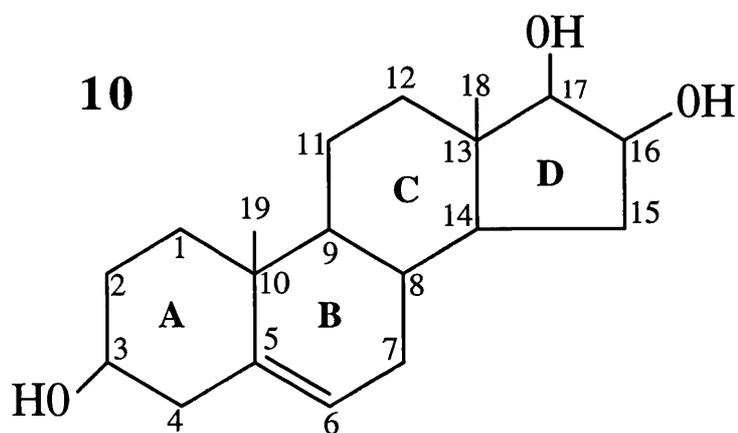
3β-7β-17β-trihydroxy-5α-androstane

16 α -OH-derivatives of DHEA and androstene-diol



Sandrostene-3 β -16 α -diol-17-one

(16- α -OH-DHEA)



Sandrostene-3 β -16 α -17 β -triol

The above diagrams show the structures of steroid compounds which were chosen for investigation. The major derivatives found in urine of normal individual are the reduced compounds whereas in TB patients the 16α compounds are more abundant. AED and AET also exist in normal individuals. Reduced compounds were chosen as a control since they differ from 16α and double bond compounds. 16α and DHEA, AED, AET and 7-hydroxy DHEA all have a double bond in 5 position whereas reduced compounds do not.

Protocol

2.2.3.1 Part-A

To investigate the influence of above listed hormones on the proliferation of splenocytes, by using a simple lymphocyte activation assay.

DHEA *in vitro*:

Splenocytes were pooled from two or three normal drug-free control C57 BL/6.J male mice that had been killed by cervical dislocation. DHEA was dissolved in ethanol/DMSO (1:1) to achieve concentrations of 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M. The *in vitro* molar concentrations correspond to levels of 2884 - 0.02884 ng/ml. The DHEA solutions were adjusted so that the final concentration of ethanol/ DMSO was only 0.1% in the culture wells. Cultures were set-up in triplicate, with each well containing 20 μ l of DHEA diluted with culture medium, 80 μ l of ConA solution and 100 μ l of cell suspensions (2×10^7 cells/ml). Control wells were assayed simultaneously as follows:

- 1- cells incubated in presence of ConA
- 2- cells incubated without ConA
- 3- cells incubated with 0.1% ethanol/DMSO and ConA
- 4- cells incubated with 0.1% DMSO/ Ethanol without ConA

Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂. DNA synthesis was measured by adding (³H) thymidine 0.2 µCi/well at 24 hrs. Thymidine incorporation was determined 14 - 16 hrs later by harvesting cells onto Whatman filter paper, followed by scintillation counting. This protocol was followed when other steroids were investigated.

2.2.3.1 Part-B

To investigate the effects of the above listed hormones when co-cultured with corticosterone which known to suppress lymphocyte activation.

DHEA *in vitro* with *in vitro* corticosterone:

The same method was used as in part A but 10 µl of corticosterone (10⁻⁵ - 10⁻⁹ M), and 10 µl of appropriate concentrations of DHEA were incubated in the wells.

2.2.4 Effect of AED on cytokine production from ConA-induced murine spleen cells.

Mice

BALB/c, C57BL/6J and C3H/HeN mice aged between 3 and 7 months were used, either purchased or bred in-house at UCL.

Protocol

The spleen cells were treated as described earlier, and the following dilutions of AED were prepared: 10^{-7} , 5×10^{-8} , 10^{-8} , 5×10^{-9} , 10^{-9} and 5×10^{-10} M. Cultures were set up in triplicate. Each well contained 50 μ l of the relevant concentration of the test agent, 200 μ l of ConA (0.33 μ g/ml) and 250 μ l of cell suspension (2×10^7 cells/ml). Plates were incubated at 37°C in a humidified atmosphere of 5% CO₂. After 24 hrs the supernatants were collected and the presence of cytokines was assessed by ELISA.

2.2.5 Effect of DHEA and AED on production of cytokines by human white blood cells and T-cells

2.2.5.1 Protocol for white blood cells

White blood cells (WBC) were isolated by density gradient separation from whole blood donated by healthy volunteers and cultured with different concentrations of ConA in the presence of DHEA or AED at different concentrations in order to investigate the effect of these steroids on cytokine production.

2.2.5.2 Protocol for T-cells

WBC were isolated as mentioned above, resuspended in Aim-V medium (Imperial laboratories) and introduced into a nylon wool (600mg) column pre-incubated at 37°C in Aim-V medium. After a 30 min incubation at 37°C the unbound cells were collected from the column over a period of 5, 10 and 15 min. Contamination by B cells and macrophages was limited. This was confirmed by staining the surface markers CD14 and CD19, of macrophages and B-cells respectively. T-cells were cultured in serum-free Aim-V medium in the presence of phytohemagglutinin (PHA-P Sigma) (2µg/ml) / phorbol 12-myristate 13-acetate (PMA) Sigma (30ng/ml) with or without cortisol (10^{-7} M). DHEA and AED were added to each appropriate well.

2.2.6 Statistical analysis of the data

Man Whitney U, t- test and Assay Zap were used for data analysis.

2.3 Results

2.3.1 Stimulation of lymphocytes with mitogens.

ConA induced INF γ release from spleen cells, and the maximum release of INF γ was obtained at 1.1 $\mu\text{g} / \text{ml}$ (figure 2-1). IL-4 was not detectable with this treatment.

Different concentrations of ConA were investigated to find the optimum for spleen cell proliferation. Although 1.1 $\mu\text{g} / \text{ml}$ was optimal for cytokine production, it was not optimal for cell proliferation, as a concentration of 1.1 $\mu\text{g} / \text{ml}$ was cytotoxic to the cells. The optimal concentration of ConA for maximum proliferation was 0.156 $\mu\text{g} / \text{ml}$ (figure 2-2).

2.3.2 Determination of inhibition curve of corticosterone *in vitro*

Spleen cells were cultured in the presence of ConA (0.156 $\mu\text{g}/\text{ml}$) and with different concentrations of corticosterone to find the level which gave 50% inhibition. This was found to be 10^{-7} M (figure 2-3).

2.3.3 Effect of anti-glucocorticoid steroids on murine spleen cells partially suppressed by corticosterone

2.3.3.1 Compounds lacking the double bond in ring B

3 β -7 β -17- β -trihydroxy 5 α -androstane at 10^{-6} and 10^{-8} M slightly (although significant it is a trivial effect) enhanced the proliferation of the cells compared to cells incubated with 0.1 ethanol/DMSO

(figure 2-4a) $p < 0.01$ but did not antagonise the immunosuppressive effects of corticosterone (Figure 2-4b).

Murine spleen cell proliferation was enhanced significantly when 3β - 7β dihydroxy 5α - androstan-17-one at 10^{-6} , 10^{-7} , or 10^{-8} M was used (t- test $p < 0.01, 0.05$, and 0.01 respectively; (figure 2-5a).

However, the proliferation suppressed with corticosterone was not reversed by 3β - 7β -dihydroxy 5α -androstan (figure 2-5b), which was therefore not studied further.

2.3.3.2 Compounds with a double bond in ring B: (DHEA and AED)

There was a significant effect of AED on ConA-activated splenocytes at 10^{-6} and 10^{-7} M (figure 2-6a ; $p < 0.05$, t-test).

However, when AED was co-cultured with corticosterone, AED did not antagonise the corticosterone inhibitory effect (figure 2-6b), but when the inhibitory effect of corticosterone was diluted out 10^{-7} M AED enhanced proliferation (figure 2-6b), confirming the enhancement shown in figure 2-6a.

DHEA (5-androstene- 3β -ol-17-one) had no effect on spleen cell proliferation, neither had it any effect on the action of corticosterone (figures 2-7a and 2-7b).

2.3.3.3 7-hydroxy derivatives of DHEA and AED

Figure 2-8a shows that 7-hydroxydehydroepiandrosterone had no effect on the murine spleen cells activated with ConA, neither did it antagonise the suppressive effect of corticosterone (figure 2-8b).

5-androstene-3 β -7 β / α -17 β -triol (AET; mixture of 7 α and 7 β compounds) had no significant effect on lymphocyte proliferation. AET at 10⁻⁶ and 10⁻⁷M did not protect against the *in vitro* immunosuppressive effect of corticosterone (figures 2-9a and 2-9b).

2.3.4 Effect of AED on cytokine production from ConA-induced spleen cells

Since AED was found to be the most active compound, AED and DHEA were chosen to examine their effect on cytokine production.

2.3.4.1 IFN- γ

Production of IFN γ was enhanced significantly ($p < 0.05$, t-test) when C57BL-GrFA-spleen cells, from 7 month-old male and female mice, were incubated with doses of AED ranging from 10⁻⁸ to 10⁻⁹M (figure 2-10)

IFN γ production by BALB/c spleen cells was not influenced by the presence of AED, irrespective of the age or sex of the mice. Similarly, AED had no effect on the production of IFN γ produced from spleen cells of male and female C3H/HeN mice.

2.3.4.2 IL-5 and IL-4

AED had no effect on IL-5 production by spleen cells from any mouse strain, when incubated for 16, 24, 32, and 48 hours (figure 2-11 and table 2-1). IL-4 was not detectable in this system.

In conclusion, AED caused no significant increase or decrease in the production of IL-5 irrespective of the age, sex or strain of mice. However, AED did have an effect on the production of IFN γ , but this seemed to be dependent on the strain and age of the mice.

2.3.5 Effect of DHEA and AED on production of cytokines by human WBC and T-cells

2.3.5.1 In the absence of cortisol

Ficoll/hypaque-separated peripheral blood mononuclear cells were stimulated with ConA in the presence or absence of DHEA or AED. These steroids used in the absence of cortisol did not influence production of IL-4 or IFN- γ .

2.3.5.2 In the presence of cortisol

However, when PHA/PMA-stimulated purified CD4⁺ and CD8⁺ T-cells from the same donors were simultaneously cultured in the presence of DHEA or AED and cortisol, the production of IFN γ was enhanced significantly relative to wells containing control only (figures 2-12 and 2-13). DHEA and AED had no significant effect on the production of IL-4 in this system, and in some experiments IL-4 was undetectable.

The dose response of ConA for induction of IFN γ secretion.

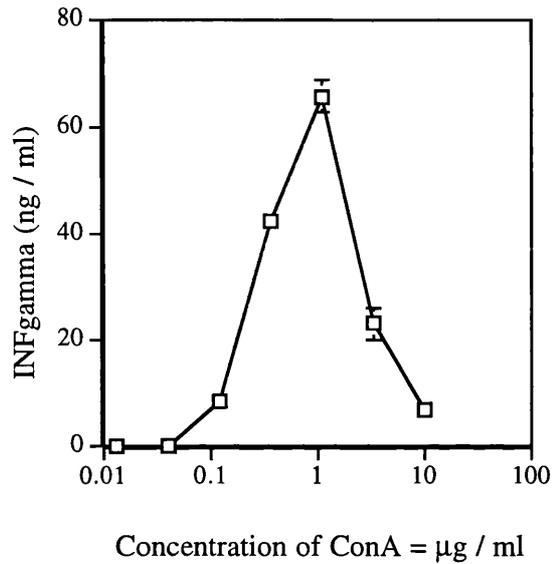


Figure 2-1

Spleen cells were cultured in the presence of different concentrations of ConA. 24 hrs later the supernatant was collected and investigated for the presence of IFN γ . The maximum production of IFN γ was at 1.1 $\mu\text{g/ml}$ of ConA. Other concentrations were either toxic or too low. Each point represents the mean (\pm SD) of triplicate readings. This experiment was repeated twice.

Determination of the proliferation dose response.

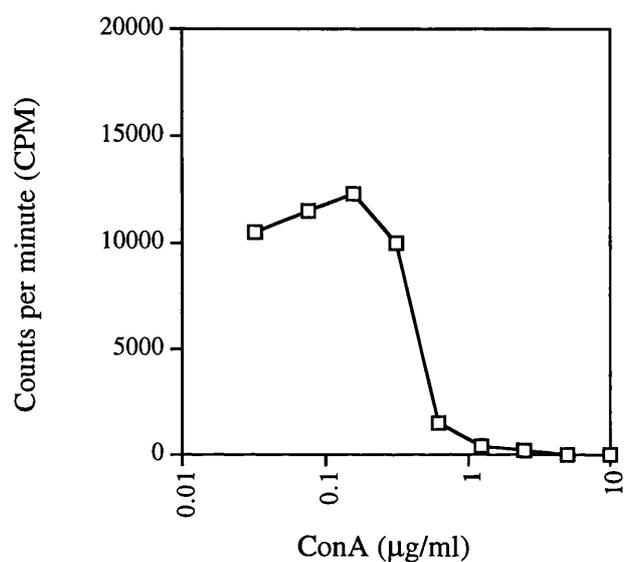


Figure 2-2

Murine spleen cells were cultured in the presence of different concentration of ConA. 24 hrs later the cells were pulsed for 14 hrs, then harvested and thymidine uptake was measured. The optimum dose for maximum proliferation was 0.156 µg/ml. Each point represents the mean (+/- SD) of triplicate readings. This experiment was repeated three times.

The inhibitory effects of corticosterone on proliferation of spleen cells.

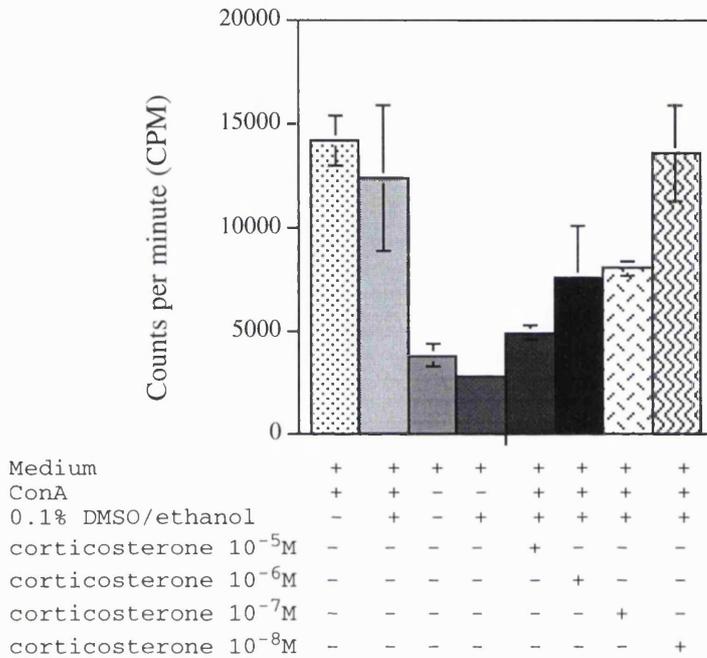


Figure 2-3

Murine spleen cells were cultured for 24hrs in the presence of 0.156ug/ml ConA with different concentrations of corticosterone. Thymidine uptake was measured after 14hrs pulsing. The dose giving 50% inhibition was found to be 10⁻⁷M. Each bar presents the mean (+/- SD) of three readings. This experiment was repeated three times. The standard deviation is shown.

The effect of 3β - 7β - 17β -trihydroxy- 5α -androstane on the proliferation of murine spleen cells.

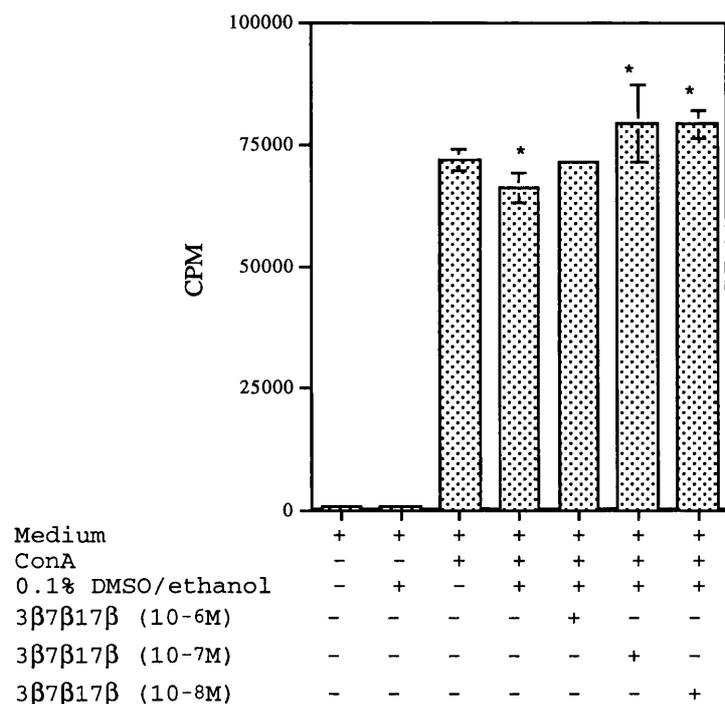


Figure 2-4a

3β - 7β - 17β -trihydroxy- 5α -androstane enhanced the proliferation of murine spleen cells when they were stimulated with ConA. and pulsed with thymidine. * = statistically significant, ($P < 0.05$). The above graph shows the mean (\pm SD) of three readings. The data were reproducible when it was repeated.

The effect of 3β 7β 17β -trihydroxy -5α -androstane on the proliferation of murine spleen cells partially suppressed by corticosterone.

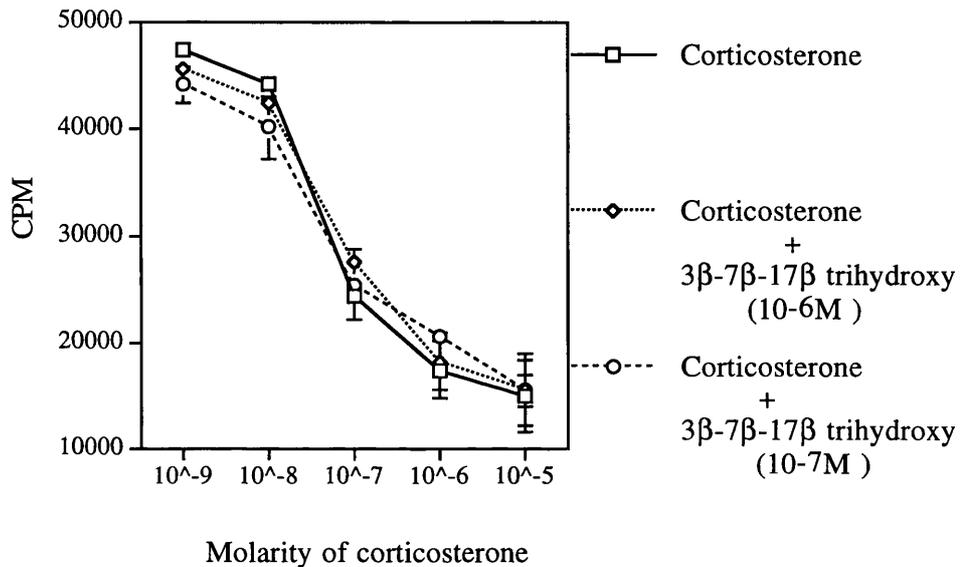


Figure 2-4b

Murine spleen cells were stimulated with ConA ($0.156\mu\text{g/ml}$), in presence of corticosterone with or without 3β - 7β - 17β trihydroxy- 5α androstane. 24hr later cells were pulsed for 14hr and thymidine uptake was measured. The above graph represents the mean (\pm SD) of three readings. The same data was obtained when the experiment was repeated twice. 3β - 7β - 17β trihydroxy- 5α androstane at the doses investigated here did not antagonise corticosterone inhibitory effect.

The effect of 3β-7β-dihydroxy 5α-androstan-17one on the proliferation of murine spleen cells.

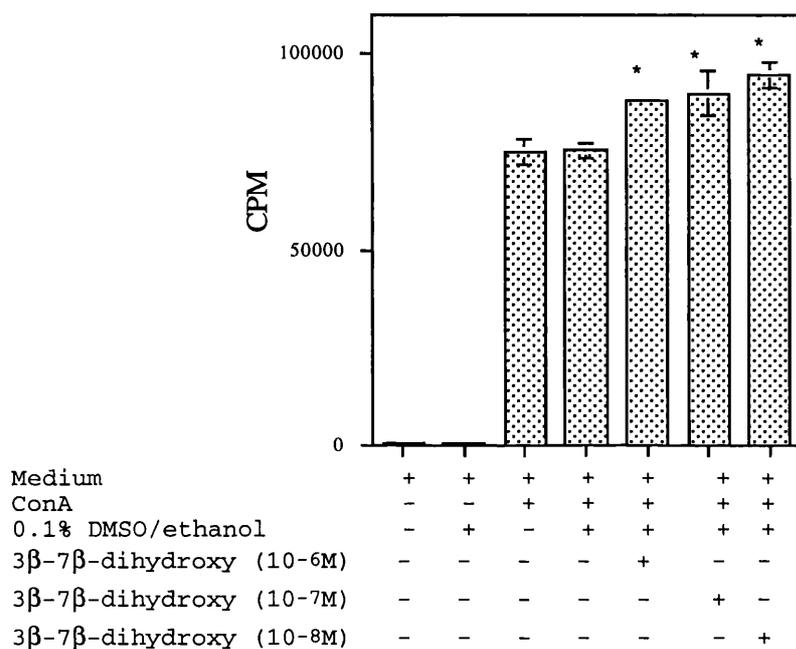


Figure 2-5a

The proliferation of murine spleen cells was enhanced significantly when the cells were stimulated with ConA in presence of 3β-7β-dihydroxy, in comparison to cells stimulated with ConA only. Each column represents the mean (+/- SD), of three readings. * statistically significant (p < 0.05). This data was reproducible.

The effect of 3β - 7β -dihydroxy 5α -androstan-17-one on proliferation of murine spleen cells suppressed partially by corticosterone.

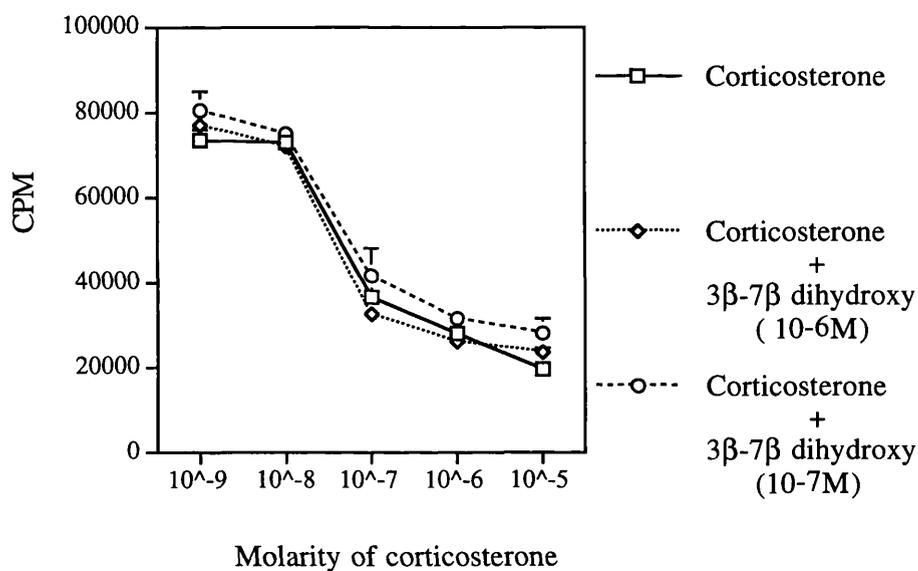


Figure 2-5b

3β - 7β -dihydroxy 5α -androstan-17-one did not antagonise the inhibitory effect of corticosterone, when the cells were cultured in presence of both of them, compared to cells cultured in the presence of corticosterone only.

The effect of 5-androstene 3 β -17 β -diol (AED) on the proliferation of murine spleen cells.

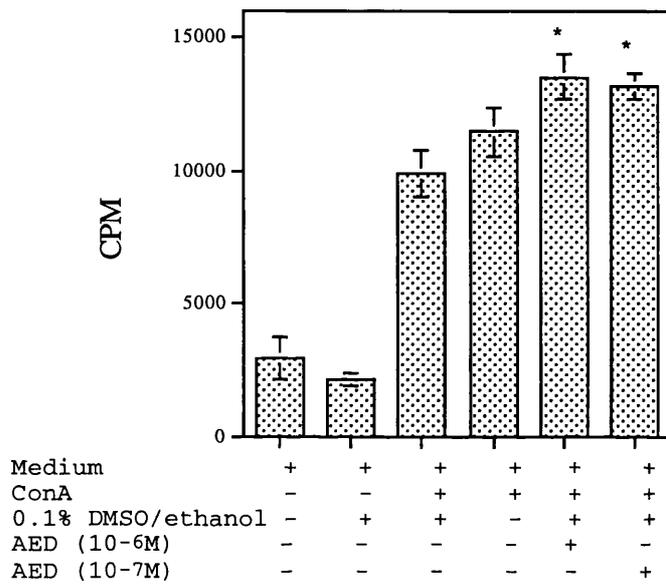


Figure 2-6a

AED enhanced the proliferation of murine spleen cells, when they were stimulated with ConA, in comparison with cells stimulated with ConA only. * statistically significant ($p < 0.03$).

Each column represents the mean (\pm SD), of three readings.

This experiment was repeated three times.

The effect of 5 androstene -3 β , 17 β -diol (AED) on murine spleen cells partially suppressed by corticosterone.

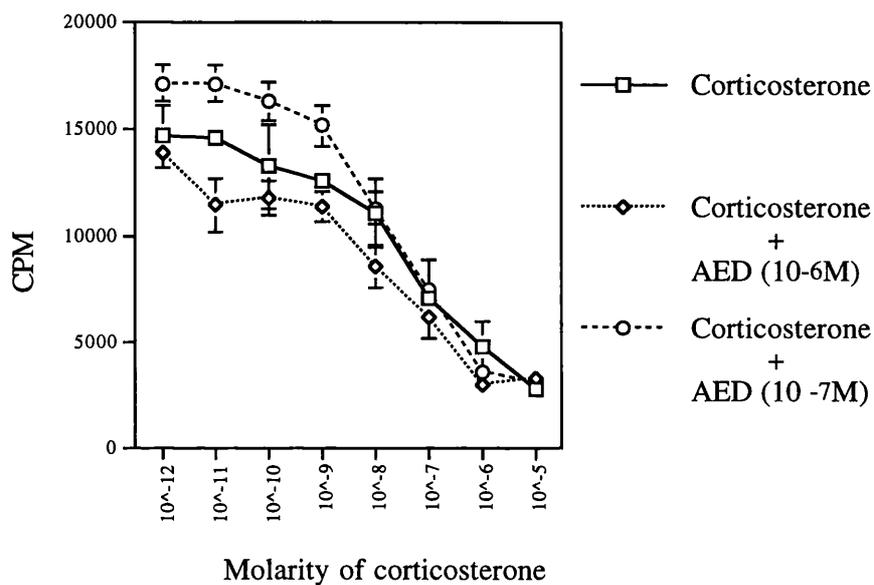


Figure 2-6b

At 10⁻⁷M AED partially antagonised the inhibitory effect of corticosterone compared to cells cultured with corticosterone only. However, AED at 10⁻⁶M inhibited the proliferation further. Each point represents the mean (+/- SD) of three readings. This experiment was repeated three times.

The effect of 5-androstene-3 β -ol-17-one (DHEA) on proliferation of murine spleen cells.

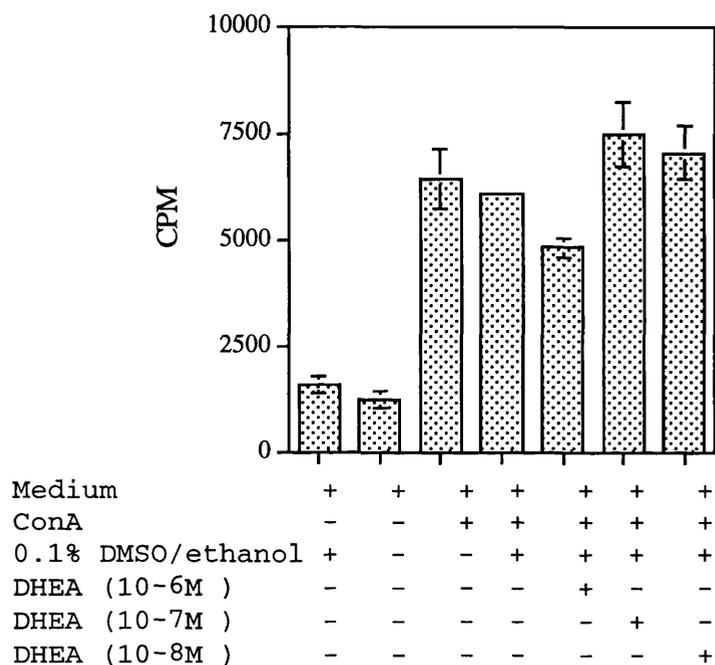


Figure- 2-7a

DHEA at 10⁻⁷ and 10⁻⁸ M did not increase the proliferation of murine spleen cells when they were cultured with ConA. (0.156 μ g/ml). At 10⁻⁶M DHEA tend to suppress the proliferation further, though it is not significant.

**The effect of 5-androstene-3 β -ol 17-one (DHEA)
on murine spleen cells partially
suppressed by corticosterone.**

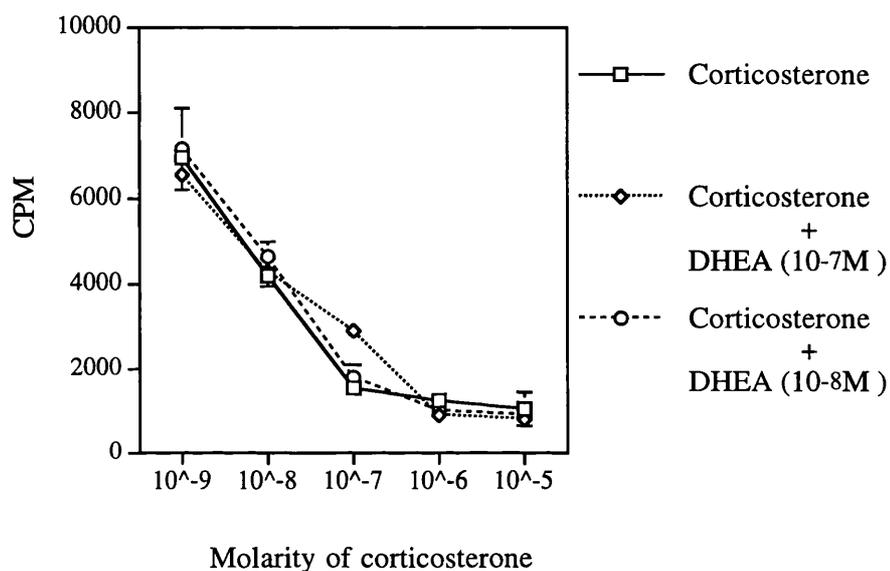


Figure 2-7b

DHEA at both concentrations (10⁻⁷ and 10⁻⁸M) did not antagonise the inhibitory effect of corticosterone when the later was added to spleen cells co-cultured with DHEA and stimulated with ConA.

The effect of 7-hydroxydehydroepiandrosterone on the proliferation of murine spleen cells.

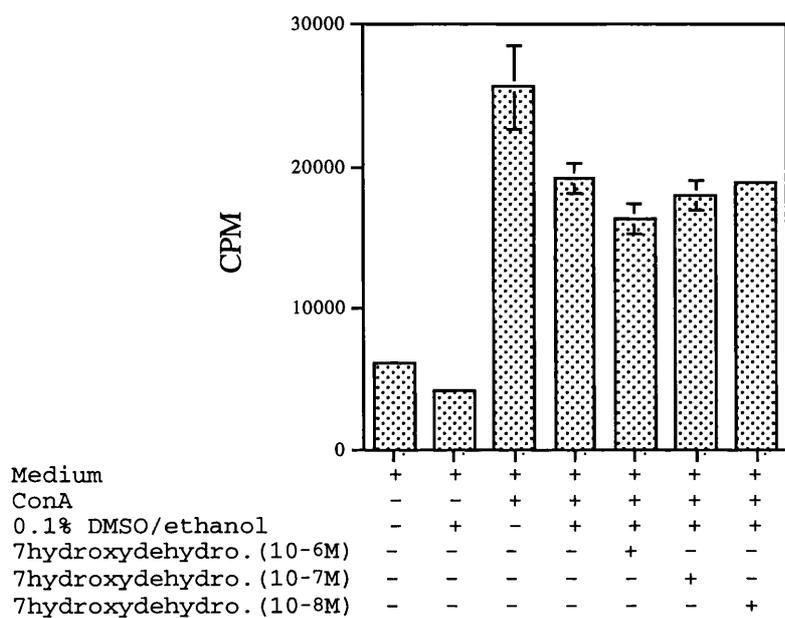


Figure 2-8a

Murine spleen cells were stimulated with ConA the in presence of 7-hydroxydehydroepiandrosterone. Cells were incubated for 24 hrs then thymidine uptake was measured after 14hrs pulsing. 7-hydroxydehydroepiandrosterone did not increase the proliferation of ConA-stimulated murine spleen cell.

The effect of 7-hydroxydehydroepiandrosterone on murine spleen cells partially suppressed by corticosterone.

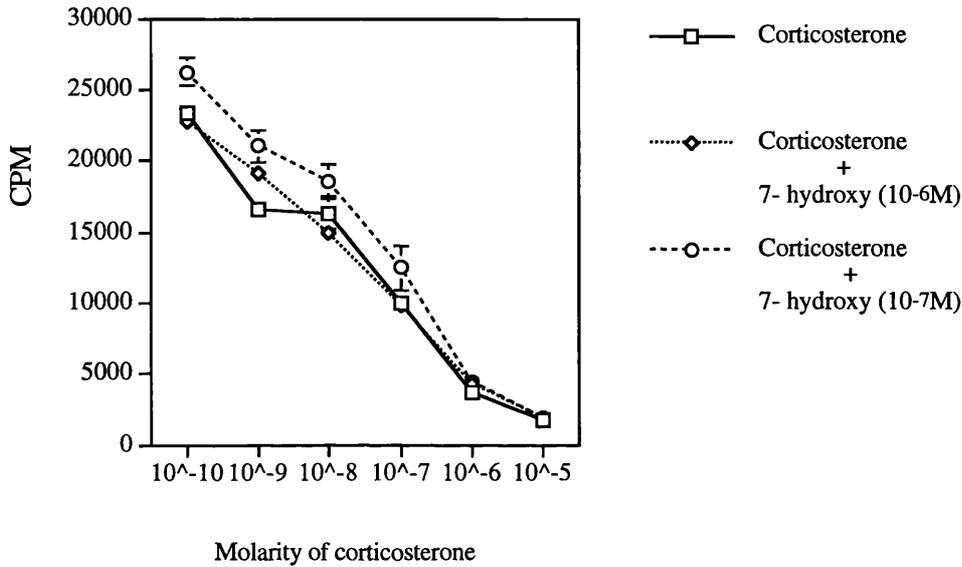
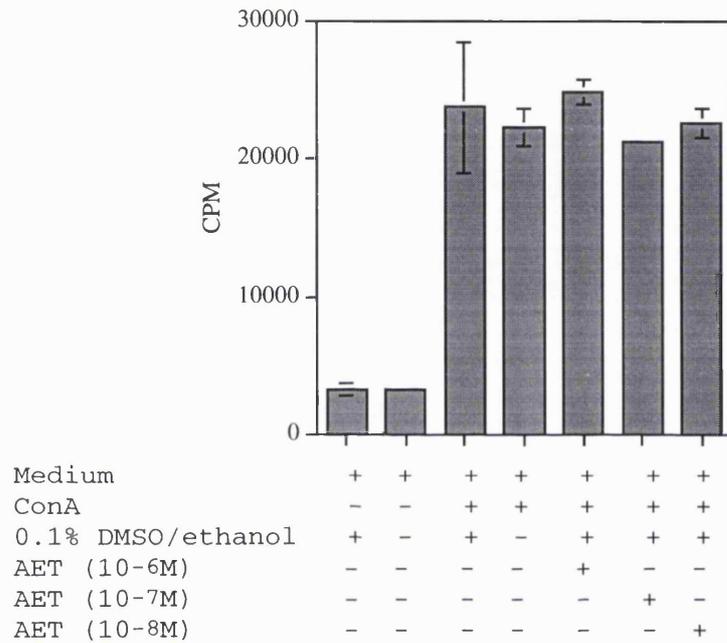


Figure 2-8b

Murine spleen cells were co-cultured with corticosterone in the presence or absence of 7-hydroxydehydroepiandrosterone. This compound did not negate the inhibitory effect of corticosterone.

*
**The effect of 5-androstene-3 β -7 β -17 β -triol (AET)
 on the proliferation of murine spleen cells.**



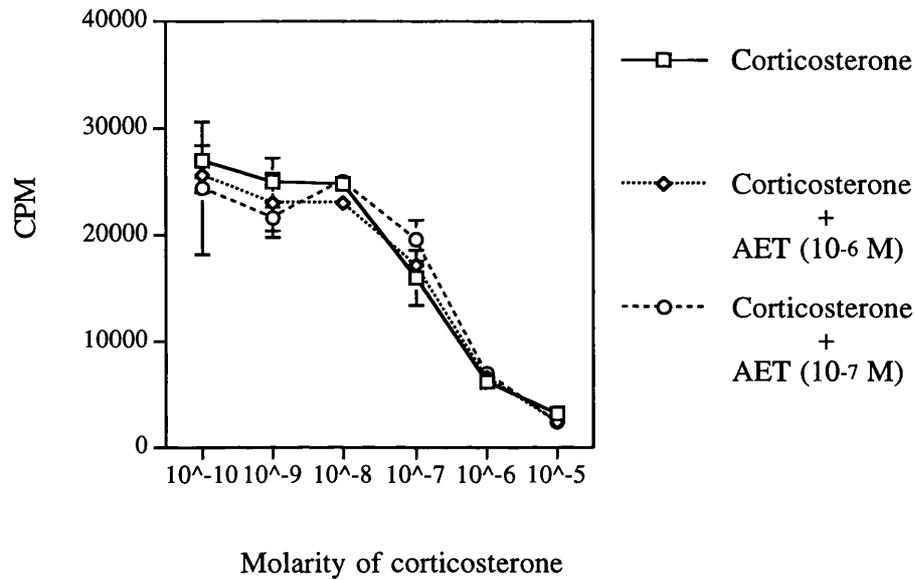
* a mixture of the 7- α and 7- β forms

Figure 2-9a

Murine spleen cells were cultured in presence of ConA with AET at different concentrations. Thymidine uptake was measured which showed that AET did not enhance the proliferation, neither it did inhibit it.

*

**The effect of 5-androstene-3 β -7 β -17 β -triol (AET)
on murine spleen cells partially suppressed by
corticosterone.**



* a mixture of the 7- α and 7- β forms

Figure 2-9b

Murine spleen cells were cultured in the presence of ConA with corticosterone or corticosterone and AET at different concentrations. AET did not antagonise the inhibitory effect of corticosterone.

Effect of AED on production of INF γ by C57Bl/6.GrFA spleen cells.

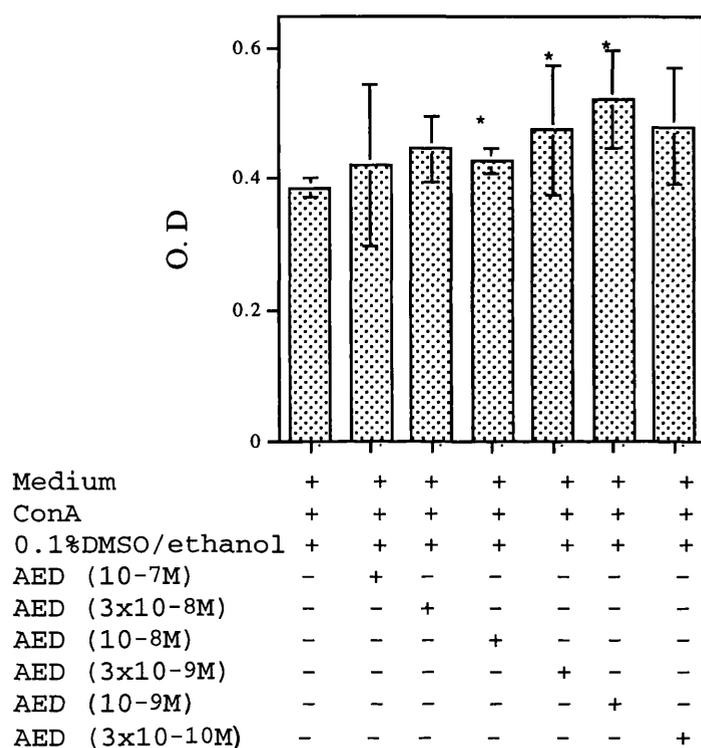


Figure 2-10

ConA-stimulated spleen cells were incubated in the presence (+) or absence (-) of AED. AED at 10⁻⁸-10⁻⁹M enhanced INF γ production significantly (* = p < 0.05), compared to cells cultured without AED. Each bar represents the average of three readings. Standard deviations are shown. The above experiment was repeated three times.

**The effect of AED on IL-5 production
by C57Bl/6.GrFA-spleen cells.**

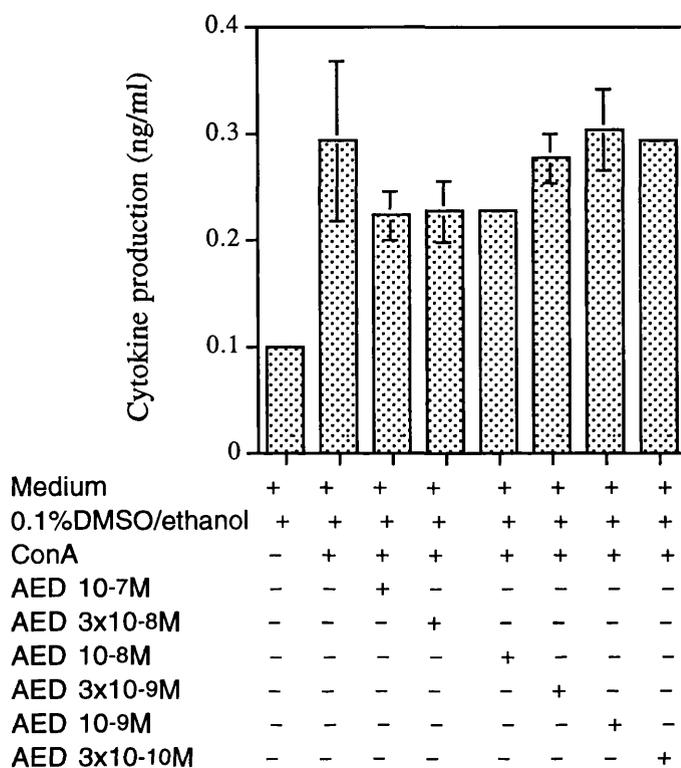
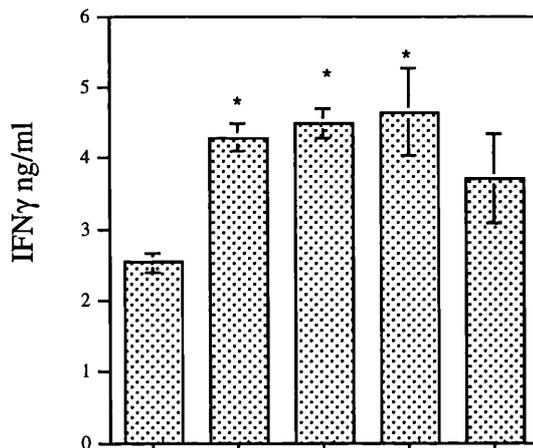


Figure 2-11

Spleen cells were incubated for 24hrs with ConA in the presence (+) or absence of AED. AED had no effect on the production of IL-5. Each bar represents the mean (+/- SD) of three readings. This experiment was repeated three times.

Effect of DHEA on human T-cells partially suppressed by cortisol.



Medium	+	+	+	+	+
0.1%DMSO/ethanol	+	+	+	+	+
PMA/PHA-P	+	+	+	+	+
Cortisol 10 ⁻⁷ M	+	+	+	+	-
DHEA10 ⁻⁷ M	-	+	-	-	-
DHEA10 ⁻⁸ M	-	-	+	-	-
DHEA10 ⁻⁹ M	-	-	-	+	-

Figure 2-12

Human T-cells were cultured the in presence (+) or absence (-) of cortisol and DHEA. ELISA was used to assess the presence of IFN γ in the cell supernatant. IFN γ was suppressed significantly by cortisol compared to IFN γ produced by cells co-cultured with cortisol and DHEA. Therefore DHEA opposed the the suppressive action of cortisol. (* p < 0.05)

Effect of AED on the production of IFN γ by human T-cells partially suppressed by cortisol.

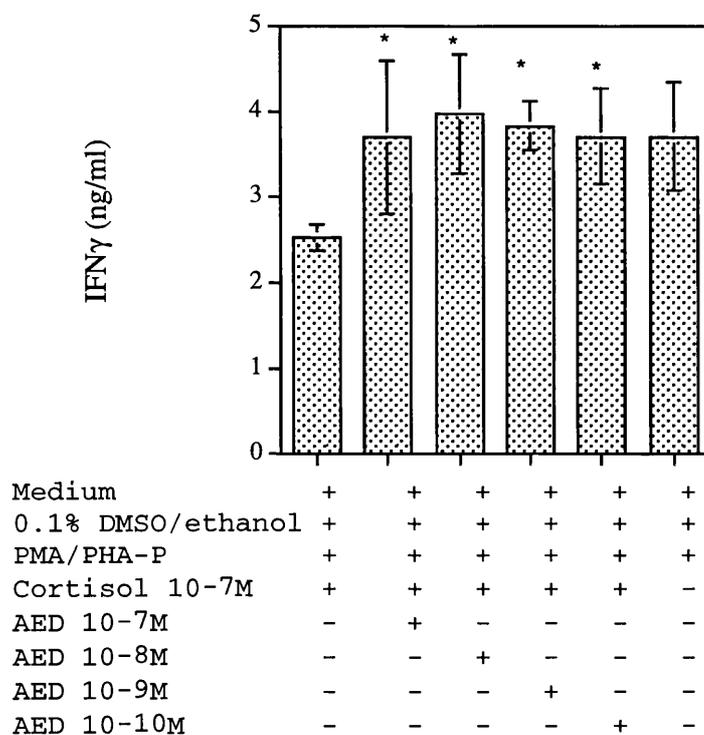


Figure 2-13

Human T-cells were cultured in the presence (+) or absence (-) of cortisol and AED. Cells were stimulated with PHA/PMA followed by 24 hrs incubation. ELISA was used to assess the presence of IFN γ in the cells supernatants. Cortisol suppressed the induction of IFN γ , compared to IFN γ produced by cells co-cultured with cortisol and AED. (* p <0.05)

IL-4 standard curve

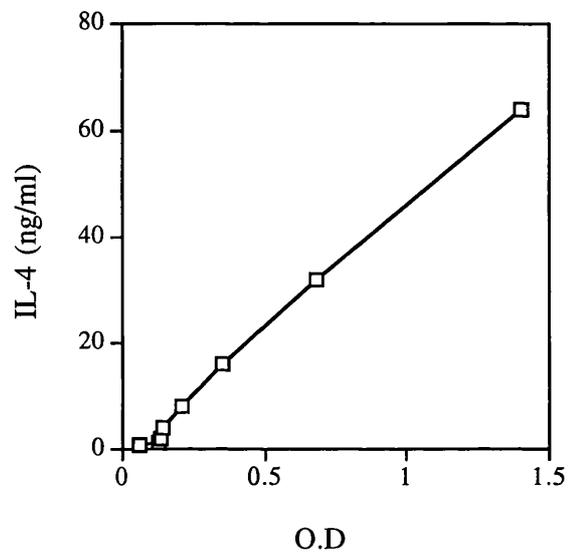


Figure 2-14

Known concentrations of IL-4 plotted against optical density. This a typical standard curve was used to determine unknown concentrations of IL-4 obtained from different experiments.

Production of IFN γ by human T-cells influenced by DHEA.

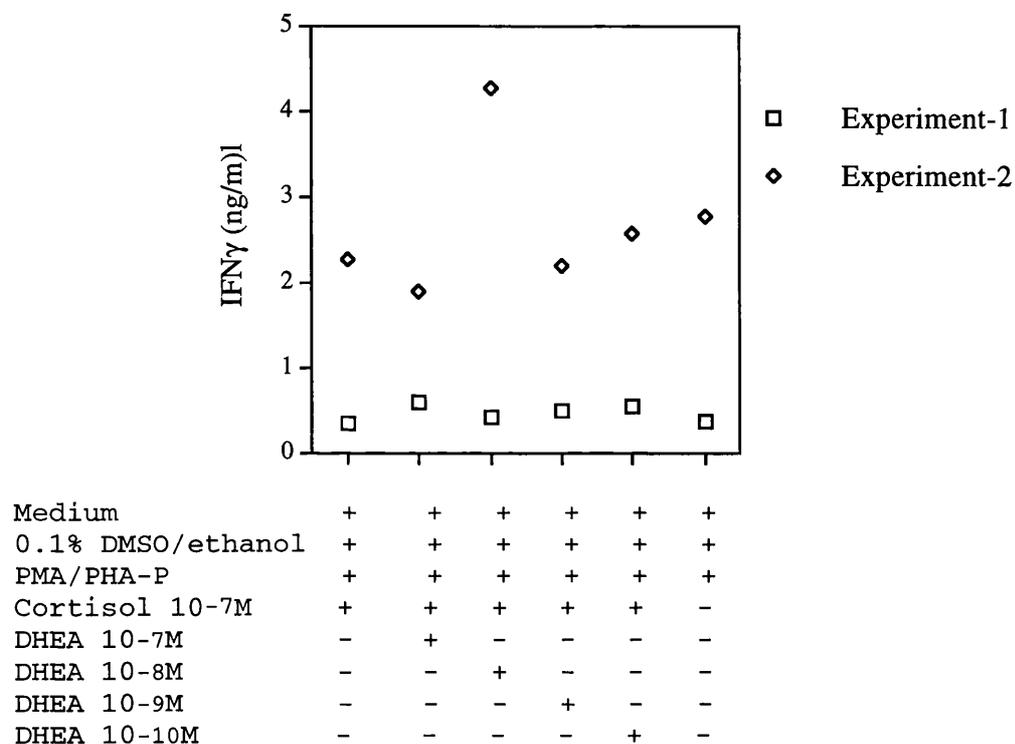


Figure 2-15

Human T-cells were cultured in presence of PMA/PHA-P and DHEA at different concentration. ELISA was used to assess presence of IFN γ in supernatants after 24hrs incubation. DHEA increased IFN γ production, but not always statistically significant.

**The effect of AED (10^{-7} - 3×10^{-10} M) on the
production of IL-5 from murine splenocytes.**

Strain of mouse	Age	Sex	Cytokine production
C57BL-GrFA	Young (12weeks)	Male and female	Normal
	Old (>24 weeks)		Normal
BALB-C	Young	Male and female	Normal
	Old		Normal
C3H/HeN	Young	Male and female	Normal
	Old		Normal

Table 2-1

Splenocytes were stimulated with ConA (0.333 μ g/ml) in the presence or absence of AED and incubated for 16, 24, 32 and 48 hrs (see the procedures for more detail). AED had no effect on IL- 5 production from spleen cells were taken from the strains above in spite of sex and age.

The dose of ConA (0.333 μ g/ml) was different from the optimum concentration determined in figure 2-1. This because it would not have

been possible to see the effect of the steroids in question if the optimum dose had been used

2.4 Discussion

2.4.1 Variation in the effects of DHEA and its derivatives in an *in vitro* system.

One of the aims of this investigation was to determine whether the hormones mentioned earlier are anti-glucocorticoids. To determine this, the dose response must be established. However, the dose response varies depending on the experimental conditions and from one laboratory to another. In this laboratory, where nutridoma was used as a serum substitute, the greatest response of spleen cells to ConA stimulation was at 1.1 $\mu\text{g} / \text{ml}$ in contrast to the findings of others where 5 $\mu\text{g}/\text{ml}$ was reported to be the optimum concentration when serum was used. A possible explanation is that ConA binds to serum glycoproteins. IL-4 could not be detected in the present study. Any effect of these potential anti-glucocorticoids on the proliferation of spleen cells was then investigated, and also, whether or not they antagonised the suppressive effect of corticosterone.

Much interest has centred around DHEA and its capacity to improve the ability of the host to fight infection (Loria *et al.* 1988; Loria & Padgett 1992; O-Garra *et al.* 1987; Panoskaltsis & Sinclair 1989). Most immunological research on steroid hormones has focused on their immunosuppressive and anti-inflammatory effects. However, DHEA provides one of the first avenues by which the immune system can be globally regulated to increase resistance to a variety of lethal infections (Blauer *et al.* 1991; Loria *et al.* 1988).

Since more research has focused on this hormone and its metabolites AED and AET, conflicting results have emerged. For instance *in vitro* DHEA had no significant effect, at 10^{-8}M , on the

proliferation of murine spleen cells (Blauer *et al.* 1991). The trend was toward mild suppression at 10^{-6} and 10^{-7} M concentrations. Padgett & Loria (1994) investigated DHEA, AED ($3\beta,17\beta$) and AET ($3\beta,7\beta,17\beta$) *in vitro*. They found that DHEA, ranging from 5×10^{-6} to 5×10^{-9} M, suppressed proliferation of splenocytes activated with either ConA or LPS, by 20 - 70 % in a dose-dependent manner (Padgett and Loria (1994). However, AED, which is the first metabolite of DHEA, at doses ranging from 5×10^{-6} to 10^{-9} M, had little influence on the response of the cells. In sharp contrast, AET, at doses ranging from 5×10^{-6} to 5×10^{-9} M, potentiated the response with both mitogens to 50 - 70 % above that of the control. When co-culture of these hormones with cortisol was investigated, it was found that DHEA (10^{-7} M) did not counteract the effect of cortisol and AED (10^{-7} M) partially reversed the effect seen with cortisol alone and partially counteracted the suppression attributed to cortisol. In contrast to DHEA and AED, AET (10^{-7} M) negated completely the immunosuppressive effects of cortisol, (Padgett & Loria 1994).

In view of the work of Padgett and Loria (1994) it is not clear why AET had no effect on corticosterone suppression in the experiments reported here. However, this could be due to the fact that the AET used was a mixture of the 7α and 7β -OH compounds provided by Celltech UK. We did not have enough materials to attempt separation of the isomers. Previously, 7β AET but not 7α AET was found to be the active form (Loria *et al.* 1996).

In the present study, DHEA at 10^{-6} M suppressed the proliferation of spleen cell, but at 10^{-7} and 10^{-8} M had no effect. These data are in accordance with the earlier report mentioned above. AED increased the proliferation at 10^{-6} and 10^{-7} M but did not protect

against the corticosterone suppressive effect. 3β - 7β - 17β -trihydroxy- 5α -androstan and 3β - 7β -dihydroxy 5α -androstan-17-one increased the splenocyte proliferation slightly, not significantly, but did not counteract the corticosterone suppressive effect. The apparent effect of these compounds was surprising. The lack of a double bond at carbon 5 resulted in a totally different molecular conformation. It could simply be that the 7β -OH is important, but this cannot explain the effects of AED. It is believed that no one has previously investigated these compounds, either *in vivo* or *in vitro*.

2.4.2 Effect of DHEA and AED on increasing IFN γ production by human T-cells

The influence of DHEA, AED and AET on the production of IL-2 and IL-3 was investigated by Padgett and Loria (1994) who found that DHEA, ranging from 5×10^{-6} to 10^{-9} M, inhibited the production of IL-2 and IL-3. However, AED, ranging from 10^{-6} to 10^{-9} M, had a minimal influence and neither increased nor decreased the production of either cytokine. AET however, ranging from 5×10^{-6} to 5×10^{-9} M, significantly increased the production of both cytokines (Padgett & Loria 1994). In their system AET was more effective in enhancing the production of IL-2 and IL-3 and in augmenting lymphocyte activation and proliferation. The failure of AED and DHEA to do the same was explained by the inability of murine splenocytes to convert them to AET (Padgett & Loria 1994). In addition it has been found that *in vivo* pre-treatment with DHEA or DHEA-S at low doses of 10^{-10} to 10^{-7} M, increased IL-2 production by murine T-cells. Also, glucocorticoid-induced depression of IL-2 synthesis by T-cells or T-cell clones could be overcome by exposure to DHEA (Daynes *et al.* 1990).

In this study, IFN γ production, by spleen cells taken from seven month-old male and female C57Bl/6.GrFA mice, was significantly enhanced in the presence of AED (10^{-8} - 10^{-9} M) compared to cells cultured in the absence of AED. However, AED neither increased nor decreased the production of IFN γ from spleen cells of male or female BALB/c mice, whether young or old. Similarly, IFN γ production, from male C3H/HeN mice, was not affected by the presence of AED irrespective of the age of the animals. The presence of AED had no effect on IL-5 and IL-4 production in any of the mouse strains mentioned above. Despite the variation in the results discussed above, this result together with the findings of others, confirms the fact that DHEA and AED enhance TH1 rather than TH2 cytokines .

It is interesting that C57Bl/6 mice have a particular tendency to give a TH1 response. Daynes and colleagues were able to reverse some age-associated changes in the immune response of mice by giving DHEA. Levels of DHEA decrease in aged humans, but we do not know what happens in mice. Further reasons are discussed in chapter three explaining the variation in the effect of such steroids on the production of cytokines and why the suppressive effect of corticosterone was not negated.

2.4.2 DHEA and AED increased IFN γ production by human T-cells

Suzuki and Tomoko (1991) stimulated purified CD4 $^{+}$ and CD8 $^{+}$ T cells with mitogens or antigens in the presence or absence of DHEA (10^{-8} to 10^{-11} M). IL-2 production by fresh CD4 $^{+}$ cells and by CD4 $^{+}$ clones but not by CD8 $^{+}$ T cells, was increased compared to

cells cultured in the absence of the steroid (Suzuki *et al.* 1991).

Daynes *et al.* (1990b) found that DHEA-treatment alone augmented IL-2 production. These authors also found that lymphocytes exposed to corticosterone and DHEA, followed by their activation *in vitro*, produced normal or enhanced levels of IL-2 and enhanced levels of IL-4, the latter being attributable to the corticosterone, and apparently not opposed by DHEA.

In this chapter it has been shown that DHEA and AED increased significantly the production of IFN γ by human T-cells when they were co-cultured with cortisol, but did not increase the production of IFN γ alone. However, the current study showed that the effect of such steroids is very much influenced by the status of the blood donors, since when experiments were repeated, with different donors, some of them showed differences although these were not always significant. It is known that the circadian rhythm in humans, where the endogenous cortisol peaks in early morning and decreases throughout the day, has a major impact on immune system components (Petrovsky & Harrison 1995). DHEA however, increases at night time and declines over the day, giving a completely opposite picture to the cortisol rhythm. The relationship between cortisol and DHEA can be complicated further by considering the type of receptor to which the cortisol binds and the concentration of circulating cortisol. At low concentrations cortisol may bind to type I receptors and enhance the response. At high concentrations cortisol binds to type I and type II receptors where it exerts its negative effect on immune system components (Scheinman *et al.* 1995 a; Scheinman *et al.* 1995 b).

CHAPTER-3

Effect of DHEA and some of its metabolites on thymuses suppressed by glucocorticoids.

3.1 Introduction

3.2 Materials and methods

3.2.1 In vivo pre-treatment with glucocorticoids and anti-glucocorticoids

3.2.1.1 Determination of effective dose of corticosterone

3.2.1.2 The effect of anti-glucocorticoids

3.2.2 The effect of pre-treatment with anti-glucocorticoids on non-specific inflammation

3.3 Results

3.3.1 In vivo pre-treatment with anti-glucocorticoids

3.3.2 The effect of pre-treatment with anti-glucocorticoids on non-specific inflammation

3.4 Discussion

3.4.1 Determining the most protective doses of DHEA and AED

3.4.2 AED is more protective than DHEA in vivo

3.4.3 Factors which may determine the effectiveness of DHEA and AED

3.1 Introduction

There were three objectives. First, having established that AED is active in the *in vitro* system, it was important to establish its activity *in vivo*, and to determine the optimum dose for *in vivo* work. DHEA, AED and AET were studied *in vivo* by other investigators (mentioned earlier) but the doses used were far too high to be used in animals which do not possess more than 1 ng/ml of DHEA at the most. Therefore, these compounds were re-investigated with respect to the doses. The dose identified has subsequently been used by collaborators in an *in vivo* model of murine tuberculosis.

Second, DHEA metabolism in TB patients is different from that in normal individuals. This prompts the question whether the changes seen in tuberculosis divert the metabolism of DHEA towards active or non-active compounds. DHEA is metabolised by normal individuals to give mostly androsterone and aetiocholanolone, whilst in TB patients and particularly in pregnancy, there is increased production of 16 α -OH-DHEA and 3, 16, 17 androstene-triol (Diamond *et al.* 1995; Medina & Kincade 1994). This is quite important as some normal metabolites of DHEA are antagonists of cortisol. The abnormalities found in the metabolic pathway of DHEA in TB patients could be one way in which *Mycobacterium tuberculosis* inactivates compounds that potentially block the effect of cortisol.

Cortisol is well-known for its inhibitory effect on T-cells. TB patients have more cortisol metabolites in their urine than of cortisone, the inactive form of cortisol (Rook *et al.* 1996). Also, TB patients convert cortisone to cortisol at a much higher rate than in

normal individuals following consumption of a cortisone tablet (Baker *et al.* 1997). Concomitantly, TB patients produce little DHEA and the metabolism of this DHEA is not normal. The experiments described here were designed to answer the question whether the metabolites of DHEA which are found in TB patients are active or not compared to the metabolites found in normal individuals.

The third question was whether DHEA can also block effects of glucocorticoid on non-specific inflammation not involving T-cells.

3.2 Materials and methods

Chemicals

In addition to AED, DHEA and 5-androstene-3 β -16 α diol 17-one, the following compounds were used: aetiocholan-3 α -ol-17-one, 5 α -androstan-3 α -ol-17-one and 5-androstene-3 β -16 α -17 β -triol. These compounds were purchased from Sigma.

Statistical tests

Mann-Whitney U and T unpaired tests were used to analyse the data.

3.2.1 In vivo pre-treatment with glucocorticoids and anti-glucocorticoids

Corticosterone is susceptible to enzymes such as 11- β hydroxysteroid dehydrogenase, which convert biologically active glucocorticoids to their inactive 11-keto forms, corticosterone to 11-dehydrocorticosterone or cortisol to cortisone. For this reason dexamethasone (DEX) has been used by other authors. However, for the present study, corticosterone was investigated *in vivo* and it was found that this could also be used in place of DEX. Corticosterone was preferable because it is the physiological glucocorticoid in all rodents. It was necessary, therefore, to determine the concentration which leads to a 50% reduction in thymus weight in mice.

3.2.1.1 Determination of effective dose of corticosterone

Protocol for corticosterone dose response

Groups of (five), male C3H/HeN mice received different doses of corticosterone (6.4, 1.6, 0.8, 0.4 mg/dose) in 40µl DMSO/ethanol. The control group of 5 mice, received the vehicle only, twenty four hours later the mice were killed by cervical dislocation and the thymuses were weighed.

3.2.1.2 The effects of anti-glucocorticoids

Protocol

Groups of 10 male C3H/HeN mice received three daily subcutaneous injections of either different concentrations of DHEA (48, 24, 5, 1.6, 0.552, 0.16, 0.06, 0.02 mg/kilo/day) or ethanol/DMSO (40 µl) in a dorsal skin fold. On the third day, half of the mice in each group received either corticosterone (1.6 mg in 40µ ethanol/DMSO) or an equal volume of ethanol/DMSO (40 µl). On day 4, the mice were killed and thymuses were weighed and the SD determined. The same protocol was followed when other steroids were investigated.

A similar protocol was followed to determine the effect of anti-glucocorticoid alone.

3.2.2 The effect of pre-treatment with anti-glucocorticoids on non-specific inflammation

Carrageenan Lambda Type IV (Sigma) is a polysaccharide extracted from seaweed, and was used to induce inflammation in the mouse foot pad. It was dissolved in PBS and filter-sterilised before use.

Protocol

Groups of 10 male C3H/HeN mice received three daily subcutaneous injections of either DHEA (1.2 mg/dose = 48 mg/kilo/day) or ethanol/DMSO (40 μ l) in a dorsal skin fold. On the third day, half of the mice in each group received either corticosterone (1.6 mg) or an equal volume of ethanol/DMSO (40 μ l). After 2-1/2 hrs the thickness of the mouse foot pads was measured. Next, carrageenan lambda (300 μ g in 30 μ l PBS) was injected into each foot pad. The thickness of all foot pads was measured 4 1/2 hrs later. The same protocol was followed when different doses of DHEA and AED were applied.

Statistical test

Unpaired t - tests were used for statistical analysis.

3.3 Results

3.3.1 In vivo pre-treatment with anti-glucocorticoids.

The concentration of corticosterone that gave a 50% reduction of thymus weight was found to be 1.6 mg (figure 3-1).

With the exception of AED and DHEA, none of the steroids tested antagonised the suppressive effect of corticosterone.

AED at 48 mg/kilo/day (1.2mg/dose) did not protect the spleen cells against a single dose of corticosterone (1.6 mg/dose). Also, AED did not protect the thymuses against atrophy. At the above concentration the thymuses treated with AED / Cort were of lower weight compared with thymuses treated with Cort alone. This suggests that at this concentration (48mg/kilo/day), AED is harmful rather than protective. Therefore, lower concentrations were used, and it was found that thymuses were protected against corticosterone action at very low concentrations (0.16 and 0.06 mg/kilo/day = 0.004 and 0.0015 mg/dose) of DHEA and AED respectively (Figures 3-2, 3-3 and Table 3-1).

3.3.2 The effect of pre-treatment with anti-glucocorticoids on non-specific inflammation

DHEA (48mg/kilo) appeared to slightly increase the inflammation, but this was not significant (figure 3-4). DHEA did not antagonise the inhibitory effect of corticosterone. AED neither increased nor decreased the suppressive effect of corticosterone on the inflammation which was triggered by carrageenan (Figure 3-4). Both DHEA and AED were investigated using lower concentrations (0.125 mg/dose = 5mg/kilo/day), but neither of them showed any effect.

Corticosterone dose response in vivo

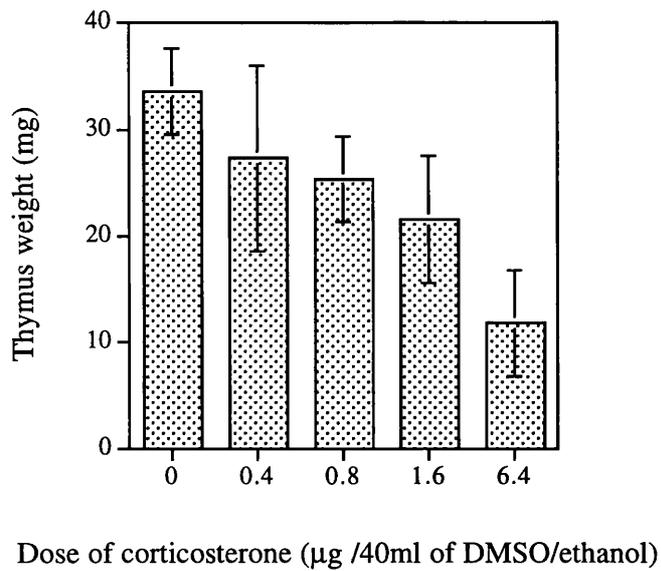


Figure 3-1

Different concentrations of corticosterone were injected intraperitoneally to different groups of mice. 24hrs later the mice were killed and their thymuses weighed. 1.6mg of corticosterone was chosen as the most appropriate dose to give approximately 50% reduction in thymus weight.

Pre-treatment with DHEA and AED to correct the immunosuppressive effect of corticosterone.

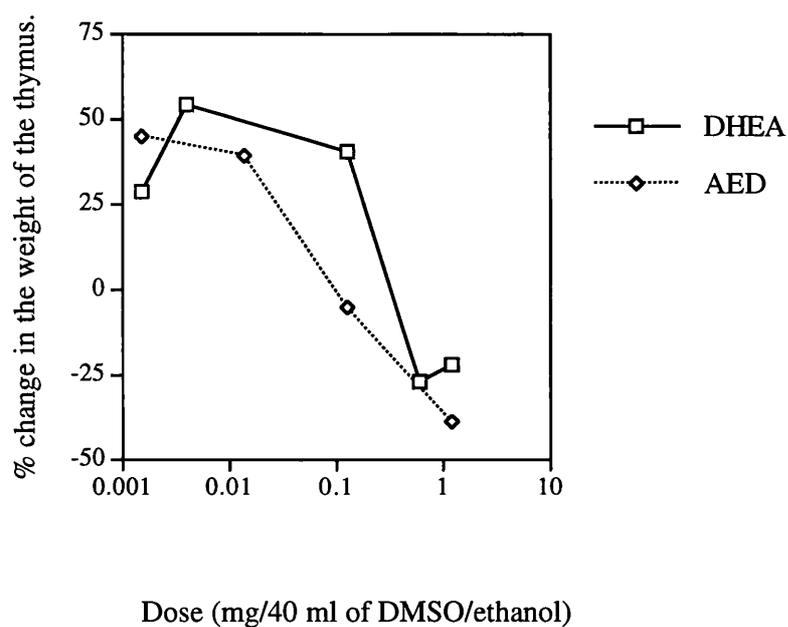


Figure 3-2

The above figure represents the correction in the thymus weight after involution caused by corticosterone. Each dose was given to 5 mice. The experiment was repeated twice and was reproducible.

The effect of AED on thymus weight in the absence of corticosterone.

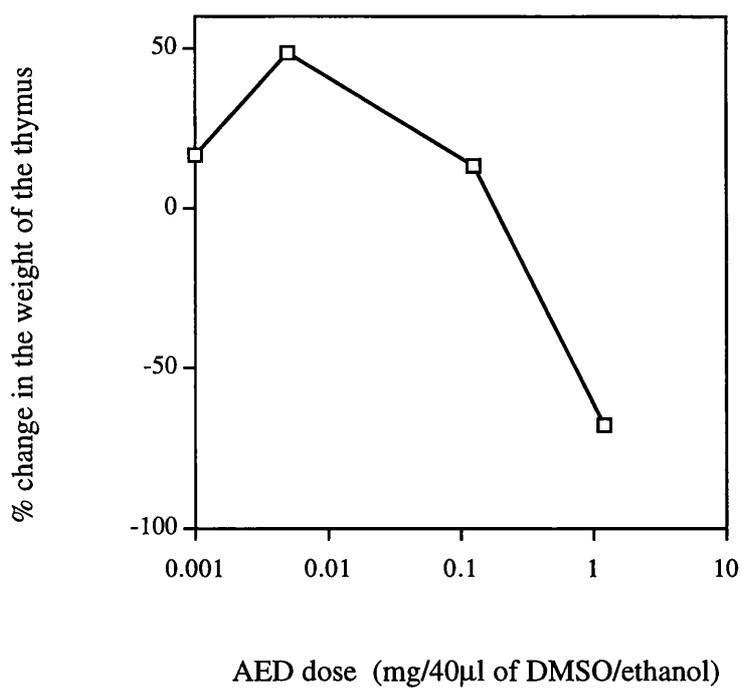


Figure 3-3

The above graph shows the increase in the thymus weight after treatment with AED. 5 mice were used in each group. The experiment was reproducible when it was repeated.

% correction of corticosterone-induced thymic atrophy

Doses (mg)	mg/kilo	aetiocholanolone	5-androstene-3 β -16 α -diol 17-one	5- α androstan-3 α -ol-17-one	5-androstene-3 β -16 α -17 β -triol	AED	DHEA
1.2	48	4.6	22.16	19.28	19.28	-39	-22.22
0.6	24	16.7	8.9	-3	-3		-27.47
0.125	5	-3.6	5.02	-8.95	-8.95	-5	40.08
0.04	1.6	0.0829	19.92	16.1	16.1		29.54
0.0138	0.552	0.33	25.8	-4.85	-4.85	38.9	22-22
0.004	0.16	-1.3	20.8	2.3	2.3		54.29
0.0015	0.06	5.8	5.2	-25.31	-25.31	44.49	28.53
0.005	.005	9.6	-7.36	-7.416	-7.41	12.60	13

Table 3-1

The effect of DHEA (1.2mg/dose) on mouse foot pad inflammation triggered by carrageenan.

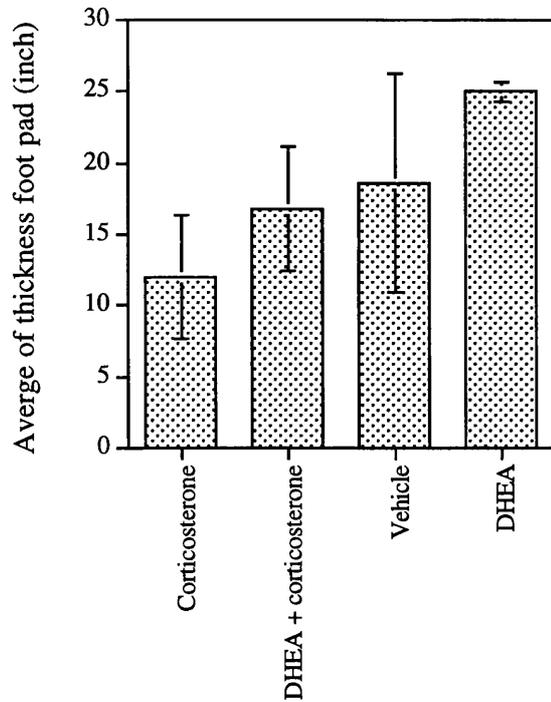


Figure 3-4

A group of mice were treated with DEA for three days, then animals were divided into groups (as shown above) to receive either corticosterone or vehicle. Foot pads were measured before and after carrageenan was given. DHEA with corticosterone increased non specific inflammation of the footpads, but not significantly, in comparison with corticosterone alone which did the opposite.

3.4 Discussion

3.4.1 Determining the most protective doses of DHEA and AED

Different compounds, some of them increased in the urine of TB patients and all of them found in urine of normal individuals, were investigated for any anti-corticosterone or pro-inflammatory effect. The compounds were AED, DHEA, 5-androstene-3 β -16 α diol 17 aetiocholanolone, androsterone, 5-androstene-3 β -16 α -17 β -triol, and 3 β 16 α -diol 17-one (16- α -OH-DHEA). Only DHEA and AED showed activity against the thymocyte killing effect of corticosterone. In TB patients, it has been reported that DHEA metabolism is altered to produce increased levels of 16 α - hydroxylated compounds (Rook *et al.* 1996). However, these compounds did not antagonise the suppressive effect of corticosterone. Therefore, it is suggested that the alteration of the DHEA metabolic pathway in TB patients could be one of many mechanisms whereby TB converts DHEA metabolism to give rise to non-active products. The effect is likely to be due to activation of the liver enzyme CYP-3A7 by cortisol, while in treated patients, the effect is exaggerated by rifampicin which is also a potent activator of this 16 α -hydroxylase (McAllister *et al.* 1983)

In normal individuals, aetiocholanolone and androsterone are common products of DHEA metabolism, but in the present model aetiocholanolone showed no protection against corticosterone. This finding is in accordance with Loria *et al.* (1988). who found that aetiocholanolone was not protective against systemic Coxsackie virus B4 and herpes simplex type 2, either as a subcutaneous injection, or when it was fed long-term to the animals. AED suppresses the thymus further when given at a high dose (48mg/kilo). This may be because

DHEA and AED can be metabolised to give androgen hormones which have a negative effect on the thymus. When DHEA or AED were given at a low concentrations (0.16 0.06mg/kg), respectively they did protect the thymus against the immunosuppressive effect of corticosterone. The optimum dose identified in this study is 1000th (10^{-3}) of that used by previous workers. Since the compounds are easily converted to testosterone, the effects seen in the earlier studies may not have been due to DHEA or AED themselves. Therefore data generated with such doses must be treated with caution.

3.4.2 AED is more protective than DHEA in vivo

DHEA (60mg/kg/day) prevented the thymus and spleen atrophy caused by DEX when it was given subcutaneously. AED protected the animals against a lethal infection of Coxsackie virus (CB4) (Blauer *et al.* 1991; Loria & Padgett 1992; Rook *et al.* 1994). The present work shows that DHEA (48mg/kilo/day) did not protect the thymus. At this dose (48 mg/kilo/day) DHEA increased thymus atrophy as did AED. This may be an effect of conversion to androgens at least in mice. A significant protective effect of DHEA has been previously demonstrated in studies of two lethal viral infections in a mouse model: systemic Coxsackie virus B4 (CVB4) and herpes simplex type 2 encephalitis (HSV2). The most protective dose of DHEA was found to be 1g /kg (Loria *et al.* 1988). DHEA at 0.4 % of the food pellets, protected mice from a lethal dose of CVB4 and HSV2. Other doses of DHEA (2g, 500 mg and 250 mg/kg) were investigated, none of these doses were protective. Loria and Padgett (1992) using this model, investigated AED and found that 320 mg/kg protected the mice

against the above two viruses (Loria and Padgett 1992). Therefore, they claimed that AED is 3 times more active than DHEA. In another study, (Loria & Padgett 1992), they found that 10 mg/kilo of AED effectively protected SWR/J mice from a lethal viral infection . They reported that AED at a dose of 10 mg/kg was 100 times more potent than DHEA, which was protective at 1.0g/kg. In the C57BL/6J in-bred mouse model, a higher dose of 70 mg/kg of AED was required to achieve 50% protection. Compared to the C57BL/6J in-bred strain, animals of the SWR/J strain were more resistant to the enterovirus infection and were more responsive to lower doses of AED. This study showed AED is 3x more active than DHEA and both protected the thymus at very low concentrations. The oral administration of either DHEA or AED was associated with considerably lower levels of protection than when the hormones were delivered by subcutaneous injection. In contrast, when AED was injected as a single dose of 320 mg/kg intraperitoneally, protection from CB4 mortality was not observed. Both DHEA (1g/kg) and AED (320mg/kg) showed a similar protective effect against *Entameba faecalis*. However, they had no direct effect (*in vitro*) on the bacterial growth rate. DHEA (10mg/kg) enhanced influenza immunisation in aged mice (Danenberg *et al.* 1995 b). DHEA (120mg/kg/day) partially restored the ability of DEX-suppressed spleen cells, to respond to activation by ConA and endotoxin. In addition, DHEA reduced the colonisation of the gut by *Cryptosporidium parvum* (Rasmussen *et al.* 1993). DHEA (60mg/kg) negated the suppressive effect of DEX on murine spleens (Blauer *et al.* 1991). DHEA and AED protected mice against lethal infection with CB4, but AED was 100x more effective than DHEA (Loria *et al.* 1988; Loria & Padgett 1992). Blauer *et al.* (1991) investigated the effect of DHEA *in vivo*

on thymuses and spleens suppressed with DEX and found that DHEA (60 mg/kilo day) inhibited DEX-induced involution in both thymus and spleen weights when the DHEA/DEX treated group was compared with the DEX-treated group. DHEA alone caused a moderate increase in spleen weight compared to the control groups.

The present study showed that immune components such as thymuses can be protected with using a very low concentrations of DHEA and AED (0.16 and 0.06mg/kg) against a high dose (1.6mg) of corticosterone. This is in accordance with the finding that 10 μ g of DHEA-S injected subcutaneously 3hrs before immunisation with pneumococcal polysaccharide increased the numbers of plaque-forming cells and the antibody titre (Garg & Bondada 1993). The present data prove that DHEA and AED can give protection against the suppressive action of corticosterone.

The amount of DHEA circulating in the blood of a human is far greater than that laboratory animals such as the rat, rabbit or mouse (Charles *et al.* 1995). Treating such species (which circulate < 1ng/ml of DHEA) with high doses of steroids [1.0g/kg of DHEA, 320mg/kg of AED (Loria *et al.* 1988; Loria & Padgett 1992), 60 mg/kg (Blauer *et al.* 1991) and 1mg/kg (Morfin and Courchay 1994a)], is far beyond the acceptable range not only for animals but also for humans. Therefore, any use of these animals to investigate the effect of this steroid at high doses is inappropriate.

3.4.3 Factors which may determine the effectiveness of DHEA and AED

It seems that results with AED and DHEA depend very much on when and how they, or other steroids, are used. For instance, Loria *et al* fed the animals for several weeks with AED in addition to the doses given at the challenge time with the infectious agent. AED was active in such a model. On the other hand, Hernandez-Pando and Rook (personal communication) gave the AED three weeks after the animals were challenged with TB. Their results showed that under such conditions the AED did not work, nor did DHEA. Instead the animals died very quickly due to inflammation and the migration of many inflammatory cells to the lung. However, when AED was used at the challenge time with TB, it partially protected the mice from developing granulomas compared with control mice. The concentrations of these hormones appears to be very important, because at very high concentrations they suppress rather than protect the thymus.

The route and the animal strain used also play a role. Loria *et al.* (1988) found that AED was most active when it was injected subcutaneously. Also, they found that AED was protective at a lower concentration (10µg) with SWR/J mice, compared with C57BL/6J mice, when a higher dose was required (Loria & Padgett 1992).

It has been found that thymus cells express steroidogenic enzymes which are capable of synthesising steroids such as glucocorticoids. Inhibition of these enzymes results in thymic atrophy implying a role for glucocorticoids in maturation and protection of thymic cells. The low level endogenous glucocorticoid protects the

thymic cells from programmed cell death triggered by recognition of self peptides during the development of the T cells repertoire (King *et al.* 1995). It has been found that endogenous glucocorticoid can protect when stimulation occurs simultaneously via T-cell receptors (TCR) (Besedovsky & Rey 1996). However under circumstances where the animal receives a high dose of exogenous glucocorticoid or is exposed to stressful conditions, the resulting high levels of glucocorticoid increase thymic involution.

Genetic factors play an important role. For example, it is known that the C57BL in bred mouse strain exhibits resistance to particular infectious agents indicative of a tendency to produce a TH1 pattern of immune response. However, it is insufficient to state that this mouse strain is simply more "TH1-like" than other mouse strains. The reason why the C57BL mouse strain mounts more "TH1 like" immune responses may lie in differences in the enzyme 11 β HSD, which converts the active form of glucocorticoids to an inactive form (Daynes *et al.* 1990). This enzyme is more active in the lymph nodes of strains of mice which are known to be resistant to infectious agents. On the other hand, it has been found that mice such as BALB/c, which are susceptible to infections, have a very low lymph node activity of 11 β -HSD compared with mouse strain C3H/HeN. That is why BALB/c mice are more susceptible than strains such as C3H/HeN (Daynes *et al.* 1990; Hennebold *et al.* 1996). Also, this may correlate with the theory that IFN γ pushes the response towards TH1 rather than TH2. This could explain and support other findings that the anti-glucocorticoids act via the enhancement of TH1-cytokines (Daynes *et al.* 1990; Loria & Padgett 1992). Furthermore, by showing that AED does not enhance the TH2 cytokines (IL-5 and IL-

4), this supports the suggestion that these hormones shift the balance of cytokines towards TH1.

The role of DHEA as a precursor hormone may play a role in determining the immunological activities of this steroid. DHEA can be converted not only to aetiocholanolone, androsterone, AED and AET but also to androgens which can be metabolised further to give sex hormones such as testosterone and oestradiol (Bellino *et al.* 1995). The weak effect of DHEA as an anti-glucocorticoid may result from the conversion of other steroid hormones.

Circadian rhythm is one of the most important factors which might dictate the kind of response. In humans cortisol increases during the day time and decreases at night, whereas other hormones such as melatonin does the opposite. These variations coincide with an increase in IFN γ production by activated whole blood cells (*in vitro*) at night. On the other hand, IL-10, produced by TH2, increases during the day. A rising level IFN γ at night when cortisol is at its lowest level and rising IL-10 when cortisol is at its highest level could indicate the balance between TH1 and TH2 (Petrovsky & Harrison 1995). This could dictate the out-come of experiments such as those in the present study which were carried out during the day. The circadian rhythm can be abolished under circumstances such as lack of sleep, stress and deficiency in energy. It has been shown that cortisol loses its circadian rhythm in military trainees due to the above reasons. In such situations, cortisol peaks to maximum levels (Opstad 1994).

CHAPTER-4

Investigation of the metabolism of DHEA-S and DHEA by *Mycobacterium tuberculosis*

4.1 Introduction

4.2 Materials and methods

4.2.1 Medium preparation

4.2.2 Preparation of cholesterol and 2,2' dipyridyl

4.2.3 Extraction methods

4.2.4 Developers used

4.2.5 Effect of incubation in medium alone on cholesterol breakdown

4.2.6 Effect of *Mycobacterium vaccae* on cholesterol breakdown

4.2.7 Effect of *Mycobacterium vaccae* on DHEA breakdown

4.2.8 Effect of *Mycobacterium tuberculosis* on cholesterol

4.2.9 Effect of *Mycobacterium tuberculosis* on DHEA and DHEA-S

4.2.10 Identification of products using gas chromatography and mass spectrometry

4.2.10.1 Principle of gas chromatography and mass spectrometry

4.2.10.2 Protocol-1

4.2.10.3 Protocol-2

4.3 Results

4.3.1 *Mycobacterium vaccae* and cholesterol

4.3.2 *Mycobacterium tuberculosis*, DHEA and DHEA-S

4.4 Discussion

4.1 Introduction

Mycobacterial species are used commercially to generate steroid precursors from cholesterol. Among these species are *Mycobacterium aurum*, *M.vaccaea*, *M. phlei*, *M. smegmatis*, *M. fortuitum*, *M. thermophilus* and *M. butyricum*. These species have been used to manufacture many different steroids originating from a single precursor substance. For instance, using *M. aurum*, progesterone and 1- dehydroprogesterone can be synthesised from sterols (Prome *et al.* 1987), or from cholesterol (Horhold & Bohme 1990). Another example is the use of *M.vaccaea* to cleave steroids to various hormones (Mahato & Subhadra 1997).

Steroids, such as DHEA and related hormones, play important physiological and immunological roles. From an immunological point of view, DHEA and its derivatives act as immuno-stimulants and as anti-glucocorticoids, which are known for their role as suppressors of TH1 cytokines. In order to possess immunological activity the DHEA precursor DHEA-sulphate (DHEA-S), needs to be converted to DHEA locally, since DHEA-S is not the active compound. This is supported by data which shows that inhibition of such conversion results in fewer inflammatory cells infiltrating into the sensitised area compared with animals which receive no inhibitor (Suitters *et al.* 1997). Also, DHEA is not the most active compound since it has been shown that AED or AET are more active than DHEA itself (Padgett & Loria 1994). Therefore, conversion of DHEA or its precursor is an essential step towards exerting immunological activity at its highest level.

In normal individuals DHEA-S is present at around 4 µg/ml (1.38 x10⁻⁵ M) and can be converted to DHEA. Metabolic conversion of DHEA-S in tuberculous lesions could be very significant, since the sulphatase is present in macrophages, infected macrophages, and tissues containing dead macrophages. These macrophages must contain large quantities of DHEA or its metabolites (Hennebold & Daynes 1994). However, it is not known whether *Mycobacterium tuberculosis* converts DHEA-S or DHEA to further active or inactive compounds. This question was the aim of the work reported below

4.2 Materials and methods

Middlebrook medium (Difco) was used for bacterial culture. Analar grade D-glucose, dichloromethane, ethyl acetate, tungstophosphoric acid and chloroform were all purchased from BDH. Dimethyl formamide, 2,2' dipyridyl, (a chelating agent which stops the microbe from digesting the nucleus of cholesterol) and cholesterol were purchased from Sigma.

Mycobacterium vaccae. The strains used were NCTC 10916 and NCTC 11659. Both strains were from National Collection of Type Cultures and were subcultured in the Bacteriology Department, UCL Medical School.

Mycobacterium tuberculosis. The strain used was H37Rv. This strain is not drug resistant and was obtained from the Department of Bacteriology.

4.2.1 Medium preparation:

Middlebrook-casitone-glucose broth. Glucose (40mg) was dissolved in 100 ml of distilled water and autoclaved. 4.7 grams of Middlebrook medium-7H-9 and casitone (5g) were dissolved in 975 ml of distilled water. This preparation was aliquoted into 100 ml and autoclaved. Glucose solution (2.5ml) was dispensed aseptically into each flask containing Middlebrook and casitone broth.

4.2.2 Preparation of cholesterol and 2,2' dipyridyl:

Cholesterol (50mg) and 20 mg of dipyridyl were both dissolved in 2 ml ethanol. Another 50 mg of cholesterol only was dissolved in 2 ml of ethanol. To each flask of culture medium with growth of *M. vaccae* cholesterol plus dipyridyl or cholesterol alone were added. The flasks were placed in an orbital incubator for three days at 37°C. The contents of each flask were then acidified by adding few drops of hydrochloric acid diluted in distilled water.

4.2.3 Extraction methods

The contents of each flask were poured into a 250 ml separation funnel. Dichloromethane (5ml) was added to each funnel and the contents shaken well and left to separate. When the separation was complete the dichloromethane fraction containing the cholesterol was collected into clean vials. The contents of the vials were washed twice with distilled water and the pH brought to neutrality. The vials were centrifuged and the water discarded. The contents of the vials were kept at 4°C for subsequent investigation.

Silica gel 60-A (Whatman) plates were used for thin-layer chromatography. 10 µl of each sample was loaded onto the plates. The plates were left to dry and placed in a tank containing 100 ml of solvent consisting of 85 ml of chloroform and 15 ml of ethyl acetate. A mixture of petroleum spirit, 40 ml + ethyl acetate 60 ml, was also tested

Silica gel 60-F (Merck) plates were used for UV- light chromatography.

4.2.4 The following developers were used:

1- Phosphotungstic acid (BDH-Analar)

A 10% (w/v) solution of phosphotungstic acid was prepared in 100 ml of ethanol. The plates were immersed in the solution for 1-2 minutes, then left to dry. The plates were heated in the oven for 2-5 minutes at a temperature of 183°C.

2- Iodine crystals (BDH-Analar)

Vaporised iodine was used by placing the plate in a closed atmosphere together with iodine crystals at room temperature.

3- Sulphuric Acid (BDH-Analar)

A 2% solution of sulphuric acid in ethanol was used for both UV-light plates and ordinary plates.

4- Liebermann- Burchard Reagent

The plates were immersed in the reagent which consists of 1 ml sulphuric acid, 20 ml of acetic acid anhydride, and 50 ml of chloroform. This was used with the two plates, UV and ordinary plates.

4.2.5 Effect of incubation in medium alone on cholesterol breakdown

Protocol

This was a control to examine the possibility that cholesterol might be broken down by medium only.

Three flasks of culture were used:

- 1- Medium only
- 2- Medium + cholesterol
- 3- Medium + cholesterol + dipyrindyl

All were kept in the orbital incubator for 3 days at 37°C and a duplicate for 10 days.

4.2.6 Effect of *Mycobacterium vaccae* on cholesterol break down

Protocol

Six cultures of *M. vaccae* were inoculated as follows:

- 1- *M. vaccae* NCTC10916 + cholesterol + dipyrindyl.
- 2 - *M. vaccae* NCTC10916 + cholesterol only.
- 3- *M. vaccae* NCTC11659 + cholesterol + dipyrindyl
- 4 - *M. vaccae* NCTC11659 + cholesterol only.
- 5 - Medium + cholesterol + dipyrindyl.

6 - Medium + cholesterol only.

The flasks were kept at 37 °C in the orbital incubator until substantial growth was observed. Cholesterol with or without dipyrindyl was added to each flask. After three days, all the flasks were harvested and the supernatants were extracted and kept in the cold room ready to be run on the TLC plates.

4.2.7 Effect of *Mycobacterium vaccae* on DHEA breakdown.

The following cultures were set up:

1 - *M. vaccae* NCTC10916 + DHEA + dipyrindyl.

2 - *M. vaccae* NCTC10916 + DHEA - dipyrindyl.

3 - *M. vaccae* NCTC11659 + DHEA + dipyrindyl.

4 - *M. vaccae* NCTC11659 + DHEA - dipyrindyl.

DHEA with or without dipyrindyl, was added after a substantial growth of *M. vaccae* was seen. Three days later the contents of the flasks were harvested as described earlier.

4.2.8 Effect of *Mycobacterium tuberculosis* on cholesterol

Control cultures were set up as follows:

- 1- *M. tuberculosis* + cholesterol + dipyrityl.
- 2- *M. tuberculosis* + cholesterol - dipyrityl.
- 3- *M. tuberculosis* + dipyrityl .
- 4- *M. tuberculosis* - dipyrityl.
- 5- cholesterol + dipyrityl
- 6- cholesterol - dipyrityl.

Three days later, the contents of flasks were harvested and the cholesterol was extracted and kept for subsequent investigation.

4.2.9 The effect of *Mycobacterium tuberculosis* on DHEA and DHEA-S

Cultures were set up as follows:

- 1- *M. tuberculosis* + DHEA + dipyrityl.
- 2- *M. tuberculosis* + DHEA - dipyrityl.
- 3- *M. tuberculosis* + DHEA-S + dipyrityl

A slight modification was introduced into the extraction method by adding 3x more dichloromethane than the volume of medium. The amount of DHEA and DHEA-S were reduced to 5 µg /ml following pilot experiment.

4.2.10 Identification of the products using gas chromatography and mass spectrometry

4.2.10.1 Principle

Gas chromatography and mass spectrometry are two powerful techniques used in conjunction to recognise a mixture of compounds such as steroids or pheromones. Gas chromatography can separate a mixture of compounds which are detected as peaks. The separation is dependent on the partition of the component between the stationary liquid phase and a mobile gaseous phase. Mass spectrometry can subsequently identify these separated compounds according to the mass of substances after ionization. There are many ways of ionizing molecules including electron impact (EI). In the EI method, energetic electrons are fired through a small volume that contains the gaseous sample. If sample molecules are denoted by M , where M is a compound in question, electrons have a close encounter with molecules and the energy transferred to M is sufficient to cause an electron to be ejected from the molecule, forming a molecular ion. Also, energy transfer causes fragmentation of molecular ions. Measuring the mass is not enough to identify a molecule since there are so many molecules have same molecular mass (M_r). However, during the ionisation considerable amounts of energy are imported to the initially formed-ions called molecular ions. The excess of energy causes some of the molecular ions to fragment. The mass spectrometer also measures the masses of all of the charged products of this fragmentation. Therefore a mass spectrum is the abundance of molecular and fragment ions plotted against the mass.

4.2.10.2 Protocol-1

1-Extracts from 24 and 48 hrs cultures were dried then reconstituted with 200µl of 2% methoxyamine hydrochloride (Mo-HCl, Sigma) and incubated at 37 °C for 1 hr in order to attach MoHCl to the carbonyl group of steroid molecules.

2- N-trimethyl silylimidazole (TMSI, Pierce), (100 µl) was added to the extract followed by an overnight incubation at 100°C in order to add TMSI to the hydroxy group of steroid molecules.

3- The samples were cleaned of lipids by using lipophilic hydrophobic gel (Packard). The samples were dried and reconstituted with 100 µl cyclohexane (BDH-Analar).

4- The samples were injected into the gas chromatograph and mass spectrometer.

5- The data were interpreted and investigated using a computer programme (U.K) set for this purpose. The above procedures were performed mainly by Dr. John Honour and his assistant in the Endocrinology Laboratory, UCL.

4.2.10.3 Protocol-2

1-Extracts of 72 and 96 hr cultures were dried and reconstituted with 0.5 ml of dichloromethane.

2- 5 ml of 0.5 M sodium acetate buffer, pH 4.6. and 5 mg of sulphatase were added then incubated at 37°C for 24 hr. This step was to remove the sulphate group.

3- The sample was eluted using Sep -Pak cartridges (Water Corporation), then the sample was dried. The remaining procedures were the same as in protocol-1.

No differences between the two protocols could be observed when the analysis was carried out.

4.3 Results

4.3.1 *Mycobacterium vaccae* and cholesterol

Pilot experiments were performed with non-pathogenic *M. vaccae*.

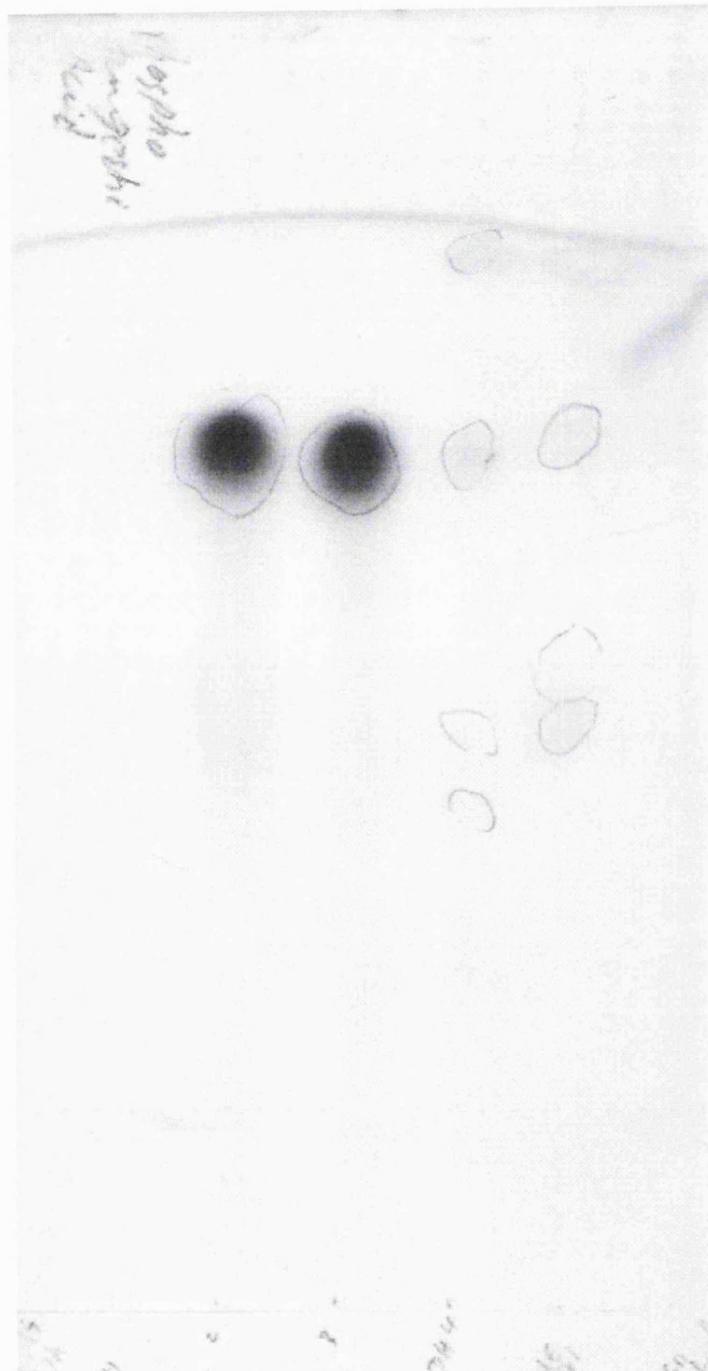
It was found that *M. vaccae* cleaved cholesterol in at least three sites. The activity of strain NCTC 11165 was unaffected by dipyriddy. However, when cholesterol (without dipyriddy) was incubated with strain NCTC 10916, the cholesterol disappeared completely as a result of digestion of cholesterol nucleus. This demonstrates that these two strains have different enzymes. Some of these enzymes are responsible for nuclear degradation and can be inhibited by using the dipyriddy as a chelating agent. Strain NCTC 10916 apparently has such enzymes, since it was influenced by the chelating agent. Strain NCTC 11165 does not have the enzymes responsible for nuclear degradation, since dipyriddy made no difference. These data could be valuable taxonomically. More interestingly, it was found that the enzymes responsible for cleaving cholesterol were secreted into the medium. When cholesterol was incubated with filtered supernatants from both strains, the cholesterol was cleaved and spots were seen on the chromatography plate. This result could mean that the steroids do not need to enter the organisms in order to be cleaved. This could be the case *in vivo* in TB infections.

4.3.2 *Mycobacterium tuberculosis*, DHEA and DHEA-S

The effect of *M. tuberculosis* was examined with particular respect to DHEA. *M. tuberculosis*, either in the presence or absence of dipyrindyl, metabolised cholesterol and DHEA to six different products which were identical in terms of colour, mobility and also in appearance when different indicators were used. Of these, four spots were seen when phosphotungstic acid was used, and the same four spots were seen when sulphuric acid and fluorescent plates were used. However, when sulphuric acid plates were heated and examined under the UV- lamp, two more spots in each track, either with or without dipyrindyl, were seen. All spots were seen when the Liebermann reaction was used. An attempt was made to identify these compounds produced as a result of DHEA-metabolism, using gas chromatography and the mass spectrophotometer.

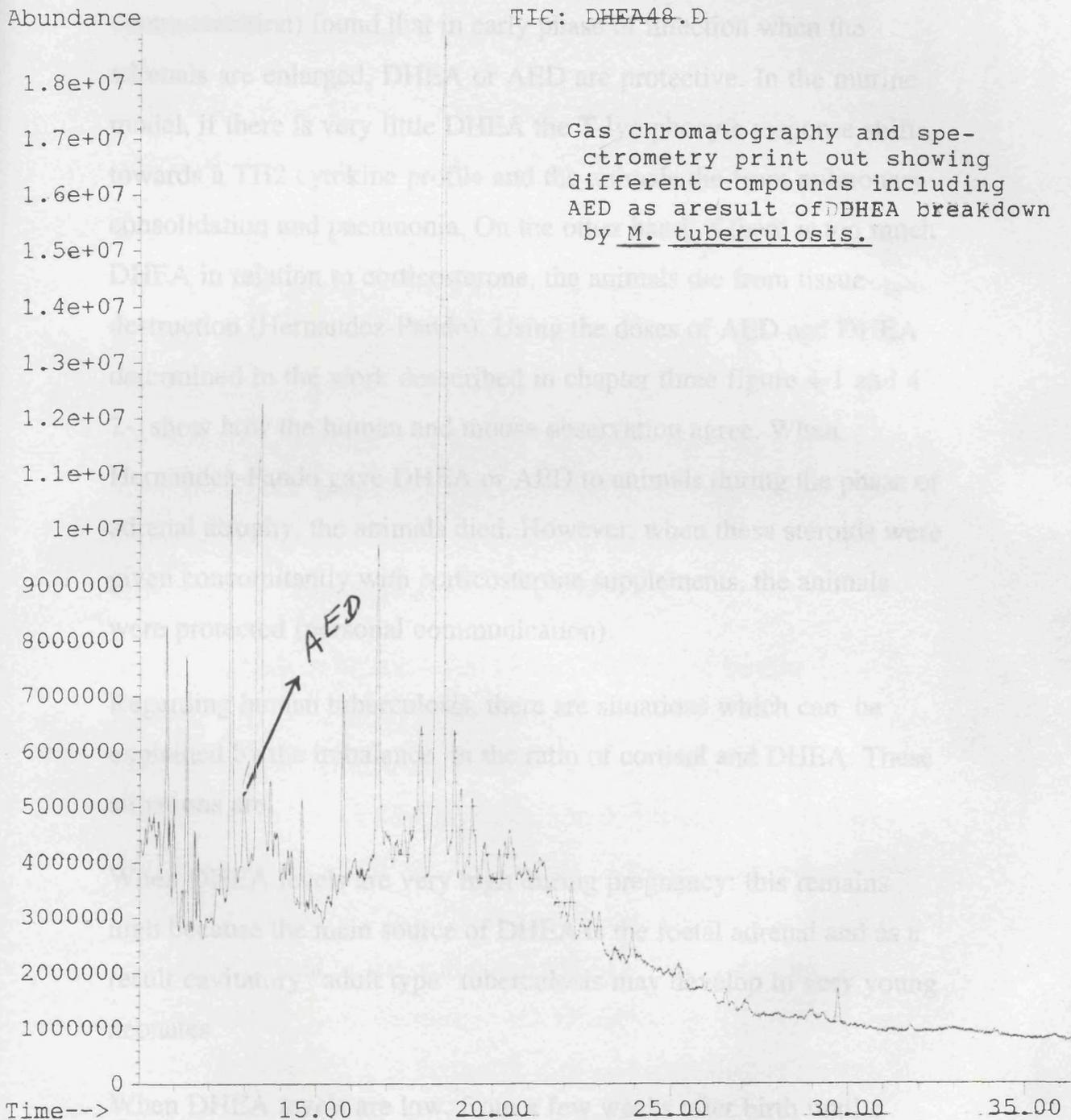
Three compounds were identified as the most abundant compounds from DHEA metabolism by *M. tuberculosis*. These compounds were AED, 7-hydroxy DHEA and a reduced form of DHEA.

Also *M. tuberculosis* cleaved DHEA-S to produce DHEA.



A model of a thin layer chromatography (TLC), plate showing breakdown DHEA. DHEA was incubated with *M. tuberculosis* broth culture for varying intervals. DHEA products were extracted, then samples were run on TLC-plates. Colour developers were used to reveal the areas of steroid on the plates.

File : C:\HPCHEM\1\DATA\DHEA48.D
Operator :
Acquired : 29 May 97 3:03 am using AcqMethod USP
Instrument : HP5970
Sample Name:
Misc Info :
Vial Number: 1



4.4 Discussion

The ratio of glucocorticoids to DHEA is very important. In murine tuberculosis, the adrenals first increase in weight, and then atrophy to 50% of their normal size. Hernandez-Pando (personal communication) found that in early phase of infection when the adrenals are enlarged, DHEA or AED are protective. In the murine model, if there is very little DHEA the T-lymphocyte response shifts towards a TH2 cytokine profile and the animals die from pulmonary consolidation and pneumonia. On the other hand, if there is too much DHEA in relation to corticosterone, the animals die from tissue-destruction (Hernandez-Pando). Using the doses of AED and DHEA determined in the work described in chapter three figure 4-1 and 4 2- show how the human and mouse observation agree. When Hernandez-Pando gave DHEA or AED to animals during the phase of adrenal atrophy, the animals died. However, when these steroids were given concomitantly with corticosterone supplements, the animals were protected (personal communication).

Regarding human tuberculosis, there are situations which can be explained by the imbalance in the ratio of cortisol and DHEA. These situations are:

When DHEA levels are very high during pregnancy: this remains high because the main source of DHEA is the foetal adrenal and as a result cavitary "adult type" tuberculosis may develop in very young neonates.

When DHEA levels are low, from a few weeks after birth until 5 years of age. At this time, consolidation and pneumonia are the dominant features.

When DHEA is about 50% of maximum at the age of 5-10 years, TB is rare. It is hypothesised that this is because the ratio of glucocorticoids and DHEA is in balance.

When DHEA is high again in adolescence: at this time, cavitation and necrosis are the features of this type of adult TB.

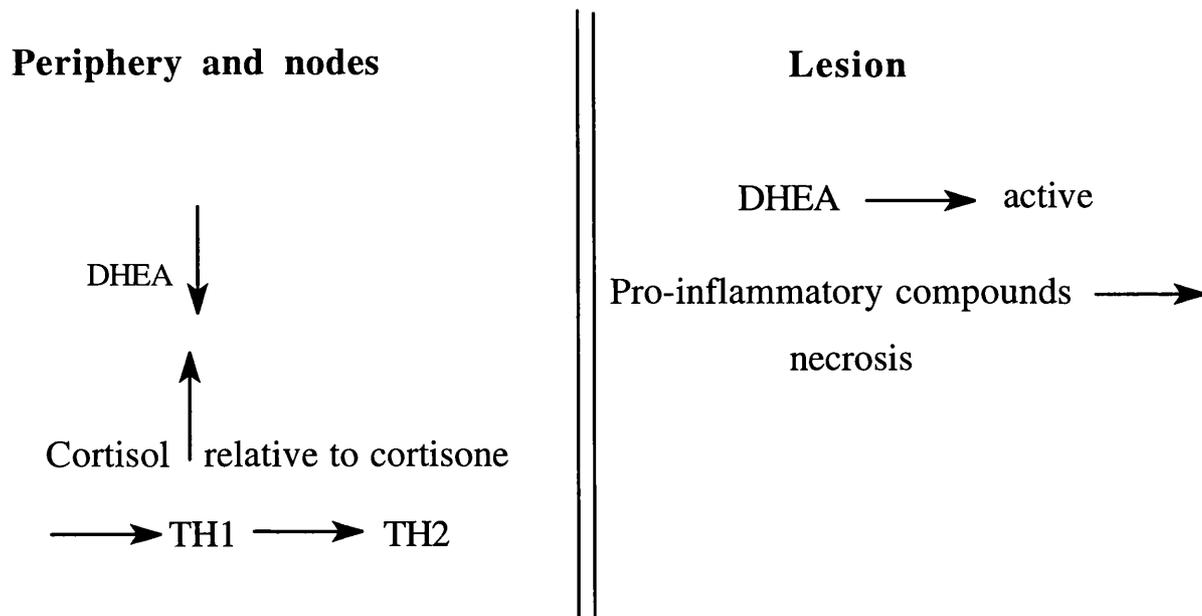
In the light of the above findings and information, the results of the present study can be interpreted as follows:

When *M. tuberculosis* converts DHEA-S to DHEA this could shift the balance towards more DHEA in relation to cortisol. Similarly, when *M. tuberculosis* cleaves DHEA to AED and 7-hydroxy DHEA, this also could shift the balance as above. This means more active anti-glucocorticoid activity is present in the lesion site. The presence of more active anti-glucocorticoids may lead to the type of tuberculosis whereby the patients die from excessive migration of all sorts of inflammatory cells, cavitation and necrosis.

DHEA-S needs to be converted either to DHEA or further down to AED or AET in order to possess maximum activity. However, the ratio of these steroids or the ratio of the most active one of these steroids has to be in a delicate balance with glucocorticoids as was explained above. Therefore, the activity of the enzymes responsible for the conversion is a two-edged sword. It is known also that cortisol can be inactivated by the action of 11 β HSD. This apparently is another control point, because it was found that in TB patients the activity of 11 β HSD is in favour of cortisol rather than cortisone. It has been suggested that this activity takes place in the lung (Baker *et al.* 1997). Therefore, the balance of these steroids in the lesion site can be disturbed either by producing too high a quantity of active

compounds as suggested by the data above, or by producing too much cortisol. Alternatively, both cortisol and DHEA and its derivatives could be produced in excess.

The paradox can be explained as follows :



In the lymph nodes and periphery DHEA level is low while cortisol level is high relative to cortisone. This disturbed balance leads to promotion of the TH2 response. However, in the lesion site the presence of inflammatory cytokines such as TNF α leads to necrosis since DHEA promotes production of more TH1 cytokines.

Survival of mice with pulmonary TB: Treatment from d60 with androstenediol (AED) alone, or with AED & corticosterone
Data from R. Hernandez-Pando et al.

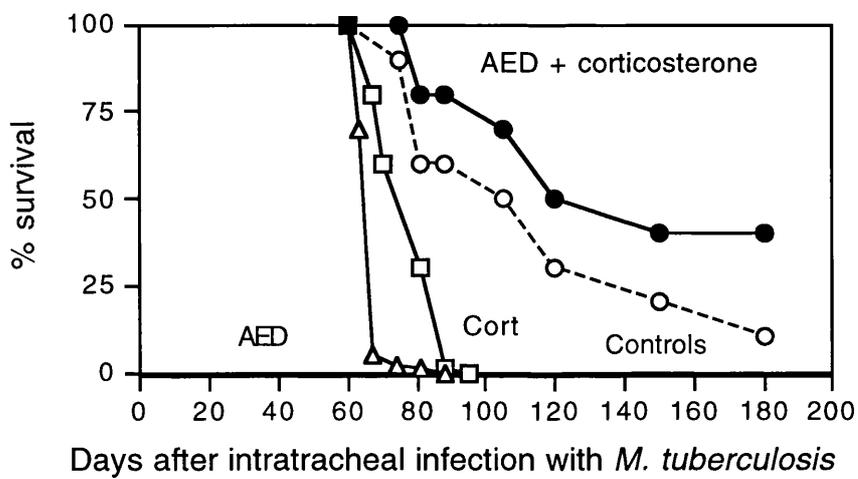
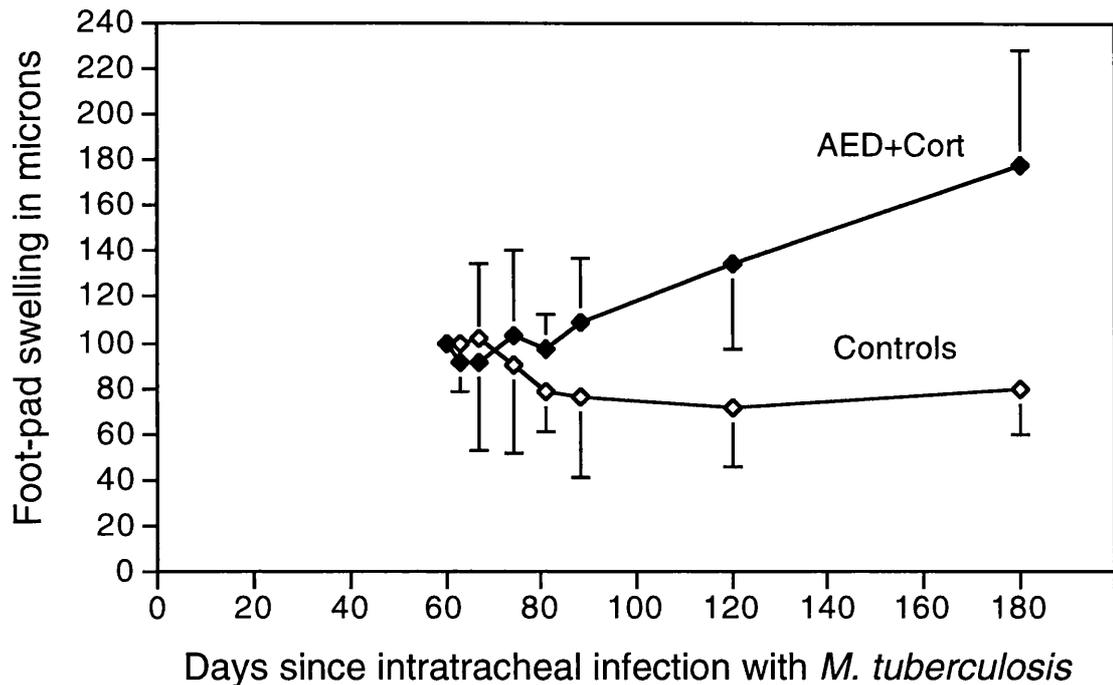


Figure 4-1



Foot-pad swelling (delayed type hypersensitivity; DTH) was measured 24 hours after injecting 20 μ g of soluble antigen of *M. tuberculosis* into the foot-pad.

Figure 4-2

Comment

AED 25 μ g was given by subcutaneous injection dissolved in olive oil, 3 times/week (Mon, Wed, Fri). Corticosterone was added to drinking water at 3 μ g/ml. Either steroid used by itself was rapidly fatal. The combination of both steroids increased survival (upper Fig) and caused a return of skin-test positivity

(R. Hernandez-Pando, G. Rook, et al., manuscript in preparation)

CHAPTER-5
and murine
The regulation of human 11beta-hydroxysteroid
dehydrogenase (11 β -HSD).

5.1 Introduction

5.1.1 Factors which may affect 11 β -HSD

5.1.2. End organ metabolism and shifting from TH1 to TH2

5.1.3. Cytokines and regulation of 11 β -HSD

5.2 Materials and methods

5.2.1 Assay of 11 β -HSD activity in intact cells

5.2.1.1 Protocol-1

5.2.1.2 Protocol-2

5.2.2 Harvesting and homogenising CCD 19 Lu

5.2.2.1 Protein assay

5.2.2.2 Measuring 11 β -HSD activity in CCD 19 Lu
lysate

5.2.3 Measuring the activity of 11 β -HSD in lysate of
different organs.

5.2.4 Measuring the activity of 11 β -HSD *in vivo*

5.2.5 Effect of BCG on the activity of 11 β -HSD

5.3 Results

5.3.1 CCD 19-Lu and cortisol conversion

5.3.2 Activity of 11 β -HSD in vivo

5.3.3 Effect of BCG on the activity of 11 β -HSD

5.3.3.1 Three-day groups

5.3.3.2 7-day group

5.3.3.3 10-day group

5.4 Discussion

5.1 Introduction

11 β -hydroxysteroid dehydrogenase (11 β -HSD) is a tissue enzyme capable of efficiently converting biologically active glucocorticoids to their inactive 11-keto forms (cortisol to cortisone, or corticosterone to 11-dehydrocorticosterone). Human 11 β -hydroxysteroid dehydrogenase (11 β -HSD) is an intracellular enzyme found in many tissues. At least two isoforms of 11 β -HSD exist, one of which plays an important physiological role in the kidney (Mercer & Krozowski 1992; Nath *et al.* 1993; Page *et al.* 1994^a; Page *et al.* 1994). In the distal nephron, 11 β -HSD rapidly metabolises physiological glucocorticoids to their 11-keto products which do not bind to or activate intracellular receptors. This type of 11 β -HSD is named 11 β -HSD-2, which is found in placenta and kidney. 11 β -HSD-2 is NAD-dependent and is localised in the distal nephron (figure 5-1). In addition, this enzyme is expressed not only in classical aldosterone target tissues (kidney, salivary gland and colon) but also in adrenal gland and placenta, suggesting the possibility of other roles for 11 β -HSD-2, in addition to that of excluding physiological glucocorticoids from mineralocorticoid receptors (MR) in kidney, colon and other aldosterone target tissues. In addition to the expected localisation in classical aldosterone target tissues, 11 β -HSD-2 is widely expressed in the female reproductive tract and in the rat adrenal cortex (Roland & Funder 1996).

Another isoform of 11 β -HSD termed 11 β -HSD-1, which is NADP-dependent and shown to be a reversible enzyme, has dehydrogenase and reductase activities (Low *et al.* 1994). 11 β -HSD-1 exists in many tissues where it most often acts as a reductase in intact

cells e.g. liver. Also, 11 β -HSD-1 is found in testis, lung and renal proximal tubule (Funder 1996).

5.1.1 Factors which may affect 11 β -HSD-1

There are many factors which direct the action of 11 β -HSD-1 including the internal pH but not the external (Jamieson *et al.* 1995). The context of the cells is a major factor in determining the action of 11 β -HSD. When some cells /cell lines are intact, 11 β -HSD-1 mainly acts as a reductase, but when cells are lysed both activities, dehydrogenase and reductase, can be demonstrated. This suggests that when this enzyme is removed from its normal cellular context it is capable of acting as a dehydrogenase. Also, when 11 β -HSD-1 was transfected into CHO or toad bladder cell lines, it showed both dehydrogenase and reductase activities (Duperrex *et al.* 1993). The NADP/NAD ratio has an effect on the activity of 11 β -HSD-1 in the lysate (Page *et al.* 1994). Page *et al.* found that addition of NADP increases the reductase activity and the conversion of cortisone to cortisol is almost twofold higher in the presence of NADPH. 11 β -HSD is a glycoprotein that utilises NADP⁺ as an electron acceptor and does not require any other protein for activity (Agarwal *et al.* 1989). When NADP is disrupted by treating the hepatocytes with KCN or sodium azide, the activity of the enzyme is reduced dramatically (Jamieson *et al.* 1995). However, the reaction direction was not affected by altering the ratio of NADP/ NADPH. 11 β -reductase activity was increased by dexamethasone and decreased by insulin (Jamieson *et al.* 1995).

5.1.2 End organ metabolism and shifting from TH1 to TH2

The presence of an 11 β -HSD isoform, possibly a novel type, in both primary and secondary lymphoid organs suggests that the variations in 11 β -HSD could be acting as a lymphoid tissue regulator of bioactive glucocorticoid concentrations in specific tissues. Significant 11 β -HSD activity has been observed in the spleen, peripheral lymph nodes, and thymus. However, most of the enzyme activity resides in the stromal cell fraction of these organs. The activity of this enzyme is significantly higher in the stroma of C3H mice than of BALB/c mice. This results in a 3-4-fold higher rate of conversion of corticosterone to 11-dehydrocorticosterone. Similar findings were observed regarding the activity of this enzyme in the lungs of both type of mice (Daynes *et al.* 1991). These data support the concept that end-organ metabolism influences the immune outcome. A lower rate of glucocorticoid catabolism may, therefore, create a microenvironment that selectively promotes a type II cytokine pathway, whereas higher rates of glucocorticoid catabolism would lower the active glucocorticoids within lymphoid tissues and favour a type I cytokine pathway (Daynes *et al.* 1991). Also, it is believed that DHEA-S is locally converted to its active form DHEA, which is responsible for promoting TH1 type cytokines. This process of site-specific end-organ metabolism provides a means to restrict anatomically the presence, and therefore the activity, of bioactive steroid hormones that circulate normally in non-active forms (Daynes *et al.* 1991). These two ways of conversion of DHEA-S to the active form and converting cortisol to the inactive form can dictate the immune outcome.

5.1.3 Cytokines and regulation of 11 β -HSD

It has been found that dehydroepiandrosterone - sulphatase (DHEA-S), which converts the precursor of DHEA to DHEA, can be inhibited by exposing murine macrophages to inflammatory cytokines such as INF α , β , or TNF α (Hennebold & Daynes 1994). Also, it has been found that the conversion of cortisol to cortisone can be reduced by inhibiting or blocking the enzyme 11 β -HSD. In addition inhibition of 11 β -HSD *in vivo* was shown to reduce TH1 cytokines and enhance type 2 cytokines (Hennebold *et al.* 1996). Moreover, cortisone administered to TB patients has been found to be converted to cortisol at faster rate than in normal individuals or in cured TB patients. Furthermore, it has been noticed that more metabolites of cortisol relative to metabolites of cortisone, are present in the urine of TB patients compared to normal individuals (Baker *et al.* 1997; Rook *et al.* 1996).

In lymph nodes 11 β -HSD-1 plays an important role in regulating the T- lymphocytes to produce type 1 or type 2 cytokines. Spleen and peripheral lymph nodes possess high 11 β -HSD activity and harbour T-cells with the greatest capacity to produce a dominant type 1 cytokine response. A significant rate of catabolism of corticosterone in the peripheral lymphoid organs has been observed (Hennebold *et al.* 1996).

Some pro-inflammatory cytokines (IL-1 and TNF α) have the ability to influence the activity of 11 β -HSD1 (Escher *et al.* 1997). These cytokines increase the 11 β -HSD reductase activity in glomerular mesangial cells. In another study, 11 β -HSD was also shown to be responsible for a high level of injected prednisolone

compared to prednisone in liver (Escher *et al.* 1994). *In vitro* dexamethasone, was found to increase the production from T cells of TGF β , which has a negative effect on T-cells, yet dexamethasone increases the reductase activity of 11 β -HSD (Ayanlar-Batuman *et al.* 1991; Jamieson *et al.* 1995; Parker *et al.* 1995).

Since TB patients have more cortisol metabolites than cortisone metabolites in their urine, and also produce a huge amount of inflammatory and anti-inflammatory cytokines, 11 β -HSD could be a target for cytokine regulation, particularly in the case of infectious diseases such as TB, where all sorts of inflammatory cytokines are produced. The following experiments were designed to investigate whether 11 β -HSD can be regulated by cytokines.

5.2 Materials and methods

Steroids

Cold cortisol and cortisone were purchased from Sigma. Tritiated cortisol [1,2,6,7-³H(N)] was purchased from Du Pont NEN. It was packed as 1mCi/ml (37MBq/ml) in ethanol solution. This solution was kept at -20°C at all times.

CCD 19 Lu Human Caucasian fibroblast cell line, derived from normal lung tissue of a 20 year old Caucasian female, was purchased from the European Collection of Animal Cell Cultures (ECACC). The culture was despatched in a 25 cm² tissue culture flask filled with culture medium. On the arrival, cells were incubated at 37°C in 5% CO₂. The cells were maintained in minimum essential medium MEM (Imperial), 10% foetal calf serum (Imperial) and 1% non-essential amino acids.

Mice

They were BALB/c females 5-6 weeks old, purchased and kept in UCL-animal house with free access to food and water.

Bacille Calmette Guerin (BCG)

A freeze-dried preparation of a living attenuated strain of bacteria derived from *Mycobacterium bovis*. The vaccine was purchased from Evans Medical Limited and kept refrigerated until use.

Hypotonic lysis buffer

Ethylenediaminetetra acetic acid, MW 372.2 (Sigma 0.5583g, 1.5mM), Magnesium chloride 6-hydrate (BDH-Analar 0.30495g, 1.5mM) and Trizma hydrochloride MW 157.6 (Sigma, 0.788g 5mM), were dissolved in 1 litre of pure water and pH was adjusted to 7.4. This preparation was used to homogenise cells.

Potassium chloride KCL MW 74.55 (BDH-Analar) 11.1825 g (1.5M) dissolved in 100 ml of pure water. This solution was used to restore the isotonicity of the homogenate.

Medium preparation

Mycobacterium 7H-11 agar-Difco (21 grams) was added to 900 ml distilled water containing 5 ml glycerol (Sigma) and boiled until the agar dissolved completely. The medium was sterilised at 121°C for 15 minutes. Aseptically 100 ml of Bacto Middlebrook OADC enrichment (Difco) was added when the temperature was between 50 - 60°C. The medium was mixed well and dispensed into petridishes in 25 ml aliquots. The plates were left to solidify then stored at 4°C.

*The supernatants were transferred into universal tubes then ethyl acetate (3x the supernatant volume) was added, mixed thoroughly then centrifuged for 1/2hrs at 2500 rpm. The aqueous layers were transferred into new tubes. These steps were repeated three times to insure maximum extraction of the steroids.

5.2.1 Assay of 11β -HSD activity in intact cells

5.2.1.1 Protocol-1

To investigate the possibility of cortisol conversion using CCD 19 Lu.

The cells were harvested using trypsinised EDTA, the viability was determined using methylene blue exclusion, and the cell number was adjusted to 1×10^6 cells/ml. 1ml of cell suspension was dispensed into each 25cm² flask which contained 3 ml of culture media dispensed earlier. The cells were incubated overnight to allow the cells to regain their health and receptors. This was followed by dispensing 10^{-9} M cortisol in 500 μ l of culture medium which contained 1 μ Ci of labelled cortisol. Another 500 μ l of medium was dispensed. The total volume of medium was 5 ml, with a final concentration of 10^{-9} M of cortisol, in each flask. Control flasks were treated similarly but contained no cells. All flasks were incubated for different intervals, 24, 48, 72, 96, and 120 hrs. The supernatants were transferred into universal tubes then centrifuged for 1-2hrs at 2500 rpm. The aqueous layers were transferred into new tubes. Ethyl acetate was evaporated and the steroid was re-dissolved in 100 μ l of ethanol containing 3mg/ml of cold cortisol and cortisone. 10 μ l of each tube was run onto fluorescent-coated Merck silica gel plates. Cold cortisol and cortisone, as controls, were run separately, parallel to the test spots. The spots of labelled cortisol and cortisone were located under 254nm UV-light, cut, transferred to scintillation vials and the reading of radioactivity of each spot was performed using a β -counter.

Calculation of conversion of steroid:

$$\% \text{ conversion} = \frac{\text{CPM in spot of cortisone}}{\text{CPM in spot of cortisol} + \text{CPM in spot of cortisone}} \times 100$$

The above equation was used throughout this chapter to calculate % conversion of cortisol to cortisone.

5.2.1.2 protocol-2

To investigate the effect of supernatants of ConA and PHA-P-activated WBC cells on 11 β -HSD activity.

White blood cells (WBC) were isolated by density gradient separation from whole blood donated by healthy volunteers, adjusted to 2x10⁶ cells/ml and cultured with 5 μ g/ml of ConA or 2 μ g/ml of PHA-P then incubated for 24, 48 and 96 hrs. The supernatants were collected and stored at -20 °C until further use.

CCD 19 Lu cells were seeded into a 24-well plate in 1ml volumes of minimum essential medium supplemented with 10% FCS and 0.1 % non-essential amino acids. Into a further two wells medium was dispensed without cells to determine non-specific cortisol oxidation. The plates were incubated for up to 4 days at 37°C in an atmosphere of 5% CO₂. The cells then were washed with 1 ml of pre-warmed serum free-medium. To each well, 1ml of serum free-medium containing different concentrations, 200, 150 100 and 50 μ l, of ConA-activated WBC

supernatant, and 1µl of labelled cortisol were added. Each concentration was run in triplicate. To the control wells the same amount of cortisol was added. Incubation was carried out for 6, 24 and 32 hrs. The contents of each well were transferred into a new tube and steroid was extracted with 3x of ethyl acetate. The rest of the procedure was as in protocol-1. Unpaired t-tests were used for statistical analysis.

5.2.2 Harvesting and homogenising CCD 19 Lu

Two different types of homogenate were made as follows:

Homogenate A

CCD 19 Lu cells were collected by scraping with a rubber policeman, washed twice with PBS and resuspended at 10^6 cells in 100 µl of Dulbecco's modified Eagles' medium (DMEM)-HEPES-[15 (mM) HEPES, pH 7.4, 0.1 (mM), PMSF -Sigma, and 2 (mM), EDTA (mM)]. The cells were homogenised in the same buffer using a dounce homogenizer.

Homogenate B

The cells were homogenised in 0.5 ml of hypotonic lysis buffer, described earlier. The isotonicity of this buffer was restored by adding 50 µl of potassium chloride (1.5mM).

Both homogenates were transferred into centrifuge tubes and centrifuged at 2500 g for 10 minutes at 4°C. The supernatants were

collected and processed to adjust protein concentration using the protocol detailed below.

Homogenate B was made after homogenate A was found to be unsuccessful in converting steroid.

5.2.2.1 Protein assay

To measure the protein concentration in the CCD Lu19 cell line.

The principle:

It is a calorimetric assay based on the reaction of protein with alkaline copper tartrate solution and Folin reagent. In the first step the copper reacts with the protein in an alkaline medium. Subsequently Folin reagent is reduced by the copper-treated protein.

Preparation of reagents:

The reagents for measuring the protein were purchased from BioRad.

20 μ l of reagent S was added to 980 μ l of reagent A, an alkaline copper tartrate solution.

Protocol:

Protein standard was prepared to achieve 0.2, 1.5 and 2 mg/ml protein. This was followed by dispensing 100 μ l of standards or samples into clean test tubes. 500 μ l of reagent A (prepared in the first step), was added to each test tube, then followed by 4 ml of reagent B, dilute Folin reagent, and all test tubes were vortexed. After 15 minutes absorbances were read at 750 nm. The concentration of protein in the samples was

calculated. The protein concentration was adjusted to 5 mg/ml and frozen at -20 °C for subsequent investigation.

5.2.2.2 Measuring 11 β -HSD activity in CCD 19 Lu lysate

Using homogenate A

10 μ l of 5 mM β - nicotinamide adenine dinucleotide phosphate (β -NADP- Sigma) was added to 85 μ l of protein supernatant. This was followed by adding 5 μ l of labelled cortisol. Triplicate tubes were incubated for various time at 37°C in a shaking water bath.

Using homogenate B

350 μ l of the homogenate was incubated with 75 μ l of β -NADP 5 mM and 25 μ l of labelled steroid. Mouse liver homogenate was treated, once as homogenate A and another as homogenate B, as a control. Another control was included which was a preparation without lysates. At the end of the incubation the steroid was extracted and treated as mentioned above.

5.2.3 Measuring the activity of 11 β -HSD in lysate of different organs

Liver, lung, spleen and kidney of BALB/c mice were harvested and weighed. 50 mg of each organ were homogenised using hypotonic lysis buffer. The isotonicity of the homogenate was restored by adding 100

μl of 1.5M KCl to each 900 μl of homogenate. Homogenates were centrifuged at 6000 rpm at 4 °C, then the supernatants were collected. Of each supernatant 100 μl was transferred to a glass screw cap culture tube. This was followed by adding assay buffer (PBS). The tubes were incubated for 30 mins at 37°C in a shaking water bath. During this incubation 4mM of NADP⁺ was prepared. Of this preparation 100 μl was added to each tube, followed by 10 μl of labelled cortisol. Another 90 μl of PBS was added so the total volume in each tube was 1000 μl . The tubes were re-capped and replaced in the shaking water bath for 2, 4 and 24 hr. At the end of the incubation period the steroid was extracted and the samples were treated as described earlier in this chapter.

5.2.3 Measuring activity of 11 β -HSD in vivo

Groups of animals (4 mice each) were injected intraperitoneally with 2 μl (2 μCi) of labelled cortisol in 500 μl of PBS. The animals were dissected 30 mins later, their organs, liver, kidney, spleen and lung were removed and snap frozen in liquid nitrogen. The organs were ground to very fine powder using a pestle and mortar. This process was carried out in dry ice and acetone, to prevent the organs melting and therefore enzyme activity was halted. Stopping the enzyme activity was important since after lysis of cells the enzyme catalyses the reaction in both directions and might reach equilibrium. The powder of each organ was transferred into a falcon tube with ethyl acetate. Each tube was vortexed for 2 minutes and centrifuged for 30 mins at 2500 rpm. This step was repeated three times to ensure maximum extraction of the steroid. The organic layer, ethyl acetate, was transferred into a new

tube, dried and reconstituted with 100 μ l of ethanol containing 2-3 mg/ml of cold cortisol and cortisone. Each sample was loaded into fluorescent (Merck) thin layer chromatography plates. Control steroids were run parallel to the samples then the plates were run in 100 ml solvent which consisted of dichloromethane 95 ml and 5 ml of methanol. The spots were viewed under the UV lamp (254 wavelength). The spots of cortisol and cortisone were cut, transferred into β -counter vials, 5 -10 ml of scintillation fluid were added and the radioactivity was measured by the β -counter machine. The percentage of conversion was calculated as described earlier.

5.2.5 Effect of BCG on the activity of 11 β -HSD

Groups of animals (4-10 mice each) were injected intravenously with 0.1 ml of PBS that contained 5×10^6 cells. Control groups were injected with PBS only. On days 3, 7 and 10, labelled cortisol was given as described above, except that the volume was increased to 6 μ l (6 μ Ci). The remaining procedures were as above. Another group of animals (3-4 mice each), received BCG only without steroid, for single cell suspension, counting and culturing the organism. The lungs were homogenised individually in 500 μ l of PBS and diluted 1/100. Each homogenate was diluted and cultured in two plates, which were incubated at 35 $^{\circ}$ C for up to 3 weeks.

5.3 Results

5.3.1 CCD 19 Lu and cortisol conversion

None of the experiments performed with above cell line worked. The reason for this was that this cell line has a very low activity of 11 β -HSD which is contrary to what was reported by Page *et al.* (1994). This was confirmed when a homogenate was used. Labelled cortisol was incubated with lysate of CCD 19 Lu lysate in the presence of NADPH as a co-factor. Compared to mouse liver homogenate which was treated the same as CCD19 Lu, CCD19 Lu did not convert the cortisol to cortisone (figures 5-2 and 5-3).

For the above reason, such experiments were brought to a halt. Therefore, an attempt was made to investigate the activity of 11 β -HSD in an animal model.

5.3.2 Activity of 11 β -HSD *in vivo*

Homogenates were prepared from different mouse organs in order to measure the activity of 11 β -HSD *in vitro*. Figure 5-4 shows the presence of 11 β -HSD activity in all lysates of organs investigated. All lysates converted cortisol to cortisone. However, activity of this enzyme was higher in the liver than the other organs. NADPH was used as a cofactor which has the ability to increase 11 β -HSD activity in a homogenate rather than in intact cells.

Figure 5-5 shows the activity and the direction of 11 β -HSD *in vivo*. As can be seen, in the liver < 5% of the cortisol was converted to

cortisone, whereas in the kidney 23% of the cortisol was converted to cortisone. This result is very much in accordance with the fact that 11 β -HSD activity in the liver is exclusively reductase (11 β -HSD-1), although it may act as dehydrogenase, whilst in the kidney the activity is dehydrogenase attributable to 11 β -HSD-2. This keeps the aldosterone receptors free and unoccupied by cortisol. Also, the figure shows that the activity of 11 β -HSD in the lung is at a level between that in kidney and the liver. This may be due to the presence of the two isoforms of 11 β -HSD. Spleen and thymus demonstrated conversion of cortisol to cortisone, but the activity in both of them was not high compared to kidney or even lung. Thymus and spleen are also similar to the lung, with a conversion rate between kidney and liver.

5.3.3 Effect of BCG on the activity of 11- β -HSD

Bacterial colony forming units were counted from growth on plates which were prepared from lung homogenate of mice which received only BCG intravenously. Growth of BCG was observed from all lung homogenates from mice were killed on days 3, 7, and 10 after vaccination. 1:100 dilution was most suitable for counting since a direct culture from lung homogenate was uncountable. The observation of the growth was to confirm the success of the vaccination, since the purpose of intravenous injection was to get the BCG into the lung.

5.3.3.1 Three day group

Liver and kidney

There was no difference in the ratio of cortisol / cortisone conversion between the control group and BCG group (figures 5-6A, 6-5B).

Lung

The conversion rate of cortisol was reduced in the BCG group compared to the control group (P value is 0.046) (figure 5-6C).

Thymus and spleen.

The equilibrium point did not change in both BCG and control groups.

5.3.3.2 7 day group

In no organs was there any change in cortisol/cortisone conversion rate.

5.3.3.3 10-day group

Liver and kidney

Again there was no difference in the equilibrium point between BCG and control groups.

Lung, spleen and thymus

In comparison to control groups, BCG-groups showed a significant difference in conversion rate of cortisol to cortisone. P values were

0.012, 0.01, and 0.083 respectively. For the above results please see figures 5-6C - 5-6E .

Conversion of cortisol to cortisone and vice versa by 11 β -HSD.



11- β -hydroxy steroid dehydrogenase (11 β -HSD-2)

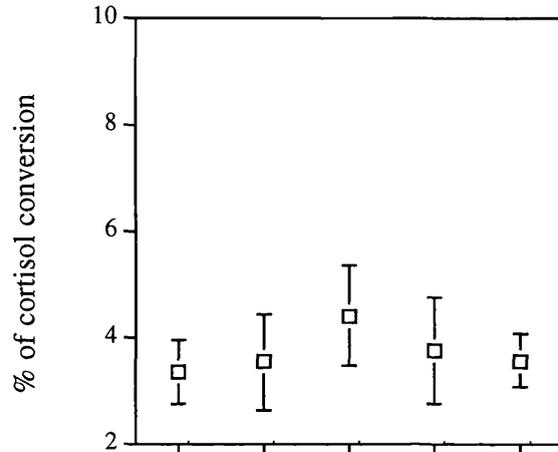
Located largely in distal and collecting tubules to inactivate the cortisol and therefore keeps mineralocorticoid receptors unoccupied by cortisol and ensures aldosterone selectivity in the face of 100 to 1000-fold excess of circulating cortisol.

11- β -hydroxy steroid dehydrogenase (11 β -HSD-1)

The above enzyme is believed to exist in many tissues, including liver and lung. 11 β -HSD-1 catalyses both oxidation and reduction of glucocorticoids. In addition, 11 β -HSD-1 action and its direction are tissue specific. 11 β -HSD is found even in proximal tubules of the cortex and in the interstitial cells of the medulla.

Figure 5-1

% conversion of cortisol to cortisone by supernatant of ConA-stimulated WBC.



Medium	+	+	+	+	+
CCD19-Lu	-	+	+	+	+
Supernatant (200 ul)	-	+	-	-	-
Supernatant (150 ul)	-	-	+	-	-
Supernatant (100 ul)	-	-	-	+	-
Supernatant (50 ul)	-	-	-	-	+
Cortisol (2ul labelled)	+	+	+	+	+
Cortisol (10^{-9} M)	+	+	+	+	+

Figure 5-2

The above figure showed that intact cells of CCD 19 Lu cannot convert cortisol to cortisone.

Each point represents the mean (+/- SD) of 3 readings.

This experiment was repeated three times.

This graph is a summary of a variety of negative results obtained from incubating cortisol with the intact cells of the above cell line.

Conversion of cortisol to cortisone using different lysates.

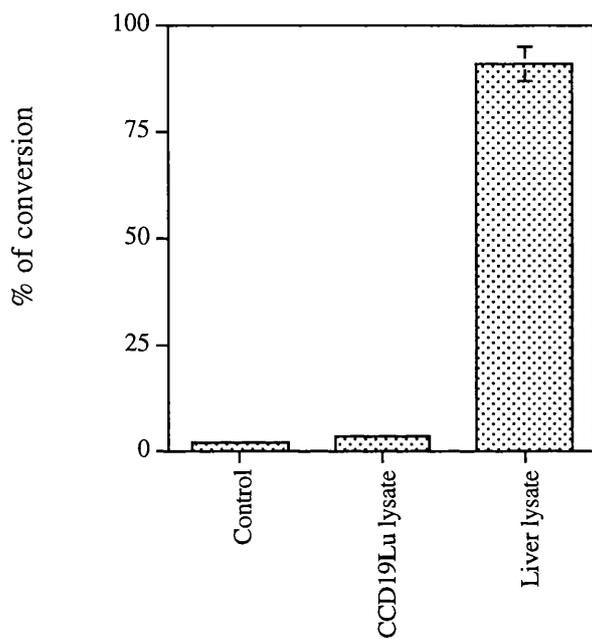


Figure 5-3

As can be seen there is no activity of the enzyme in the lysate prepared from the CDD 19 Lu cell line in comparison with liver lysate. Each point represents the mean (+/- SD) of four readings. The above experiment was repeated twice.

**Conversion of cortisol to cortisone
by lysates of different organs.**

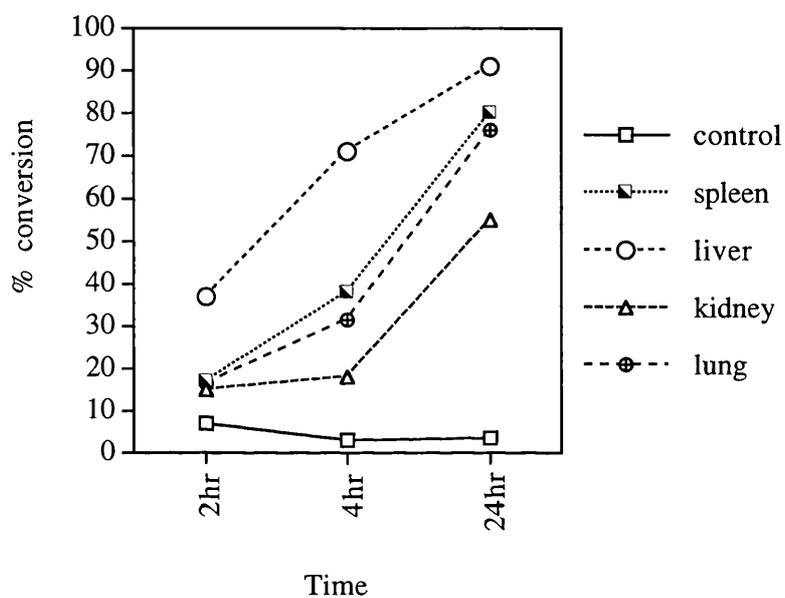


Figure 5-4

The homogenates of all organs investigated converted cortisol to cortisone. Each point represents the mean (+/- SD) of four readings. This result showed the existence of 11beta HSD activity in the all organs tested above.

IN VIVO EXPERIMENTS.

Conversion of cortisol to cortisone by 11β -HSD in different organs.

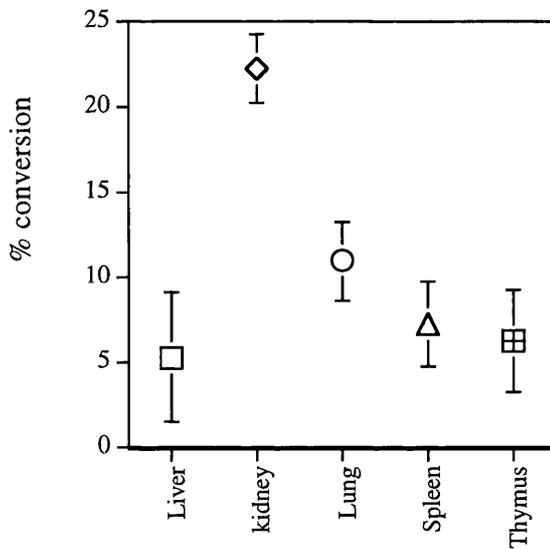


Figure 5-5

The above figure showed the interconversion of exogenous cortisol to cortisone *in vivo*. Kidney has the highest conversion rate, whereas the liver has the lowest. Lung, spleen and thymus have conversion rate which is intermediate. Each point represents the mean (+/- SD), of four readings. This result was reproducible.

Effect of BCG on the activity of 11 beta hydroxysteroid-dehydrogenase (11 β -HSD), in liver and kidney.

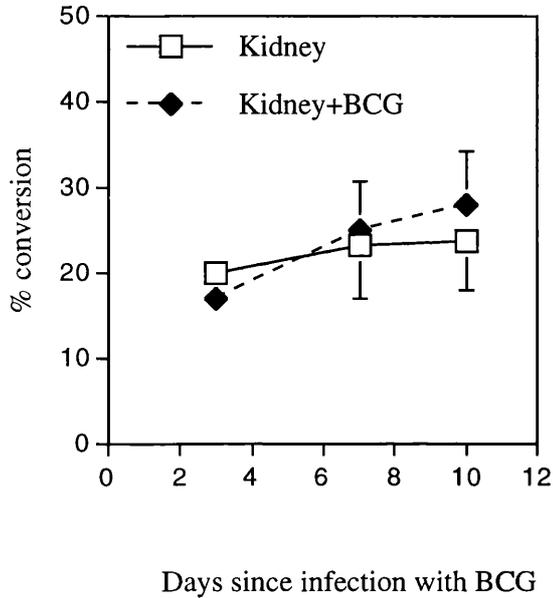


Figure 5-6A

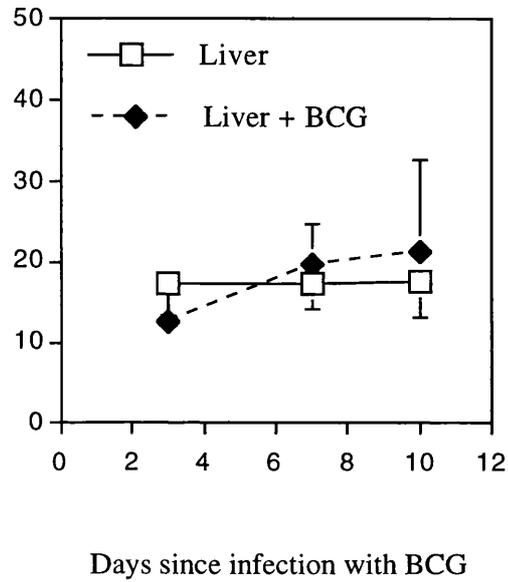


Figure 5-6B

BALB/C mice were used to investigate the effect of BCG on the activity of 11 β -HSD. The above figures showed that the conversion rate of cortisol to cortisone was not statistically different in all organs at all intervals. The results represents the mean (+/- SD) of up to 10 mice.

Effect of BCG vaccination on 11β -HSD activity in the lung.

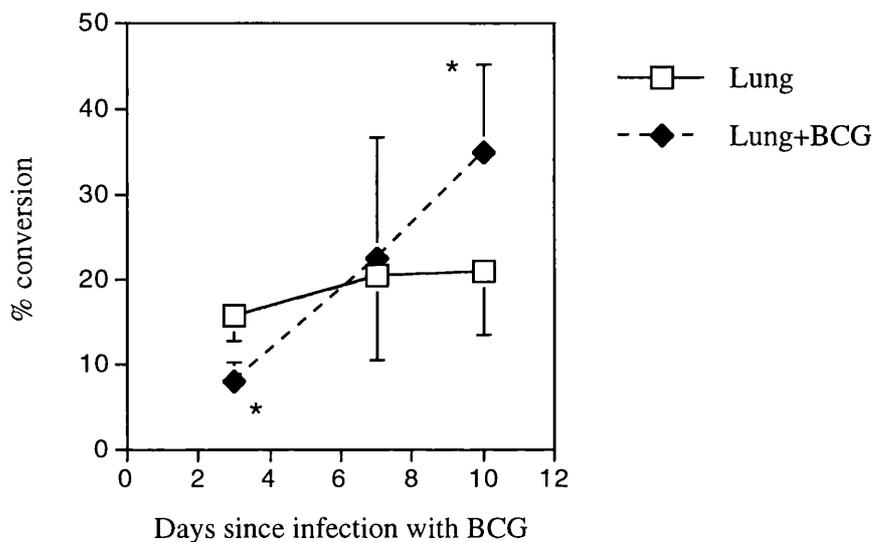


Figure 5-6C

This shows that, the activity of 11β -HSD was affected by BCG vaccination. On day 3 the cortisol conversion rate was reduced in the BCG group ($P= 0.046$). There was no change in the day-7 group. The day-10 group showed that the cortisol conversion rate was significantly increased, since more cortisone was observed ($P = 0.012$). Each point of day three and 7 represents the means (\pm -SD) of four organs while day 10 group represent the mean of 10 organs.

* = statistically significant.

The effect of BCG on the activity of 11beta-HSD in the spleen.

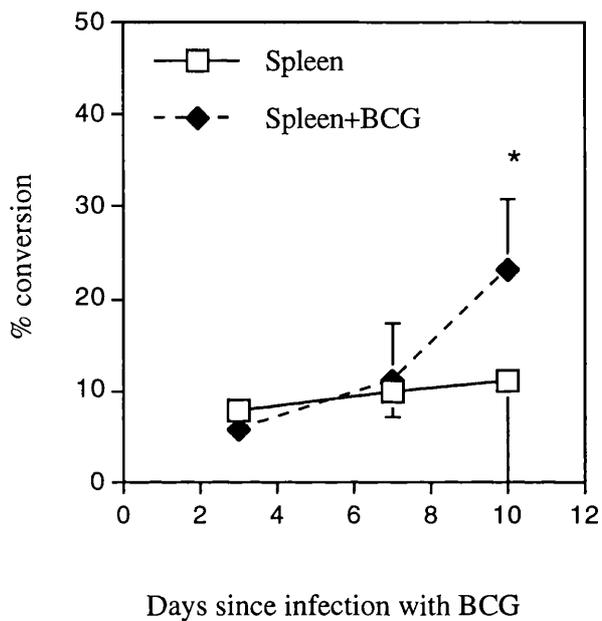


Figure 5-6D

The above figure showed that BCG vaccination increased the oxidase activity of 11beta-HSD. This was noticed on day 10 after vaccination in comparison to the non-vaccinated group. The reading on day 10 represents the mean (+/- SD) of 10 organs. * = statistically significant P = 0.01

**BCG effect on conversion rate of cortisol to cortisone by
11 β -HSD in the thymus.**

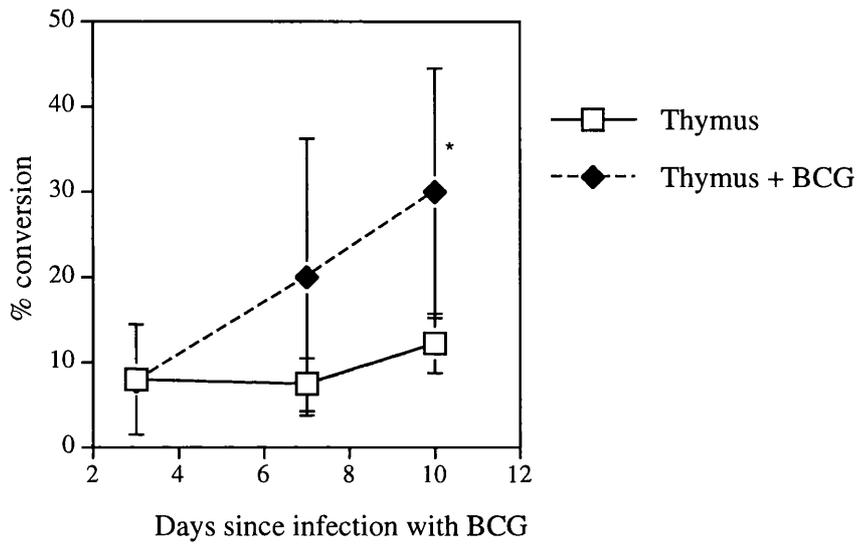


Figure 5-6E

BCG markedly increased the conversion rate of cortisol to cortisone 10 days after infection with live BCG. The 10-day group readings represent the means (+/- SD) of 10 organs compared to 7 organs in the control group. * statistically significant P= 0.0064

5.4 Discussion

The enzyme 11 β -HSD dehydrogenase is a very important enzyme to keep the balance of interconversion from cortisol to cortisone, and vice versa. Throughout human life, 11 β -HSD controls the conversion of cortisol to cortisone in many organs. This conversion occurs whenever a growth of that particular organ is needed (Beverley & Murphy 1981). The activity of 11 β HSD is very important to the immune system outcome. The activity of this enzyme in lymphoid tissues can make animals either susceptible or resistant to some infectious diseases. Also, the activity of this enzyme is reduced in the ageing animals by 70 - 80%. This decline is associated with an increase in the susceptibility of these animals to infectious disease (Hennebold *et al.* 1996). 11 β -HSD also plays a pivotal role in regulating local concentrations of immunosuppressive steroids : a high activity of 11 β -HSD-2 in the kidney is very beneficial to keep the electrolyte receptors free from occupation by cortisol. However, in case of inflammatory diseases, these enzymes can convert the immune suppressive steroid to inactive ones or *vice versa* (Escher *et al.* 1994).

In humans lung 11 β -HSD-2 converts cortisol to cortisone. The lungs contain 11 β -HSD-1, but also a small quantity of 11 β - HSD-2 (B. Walker, personal communication). Therefore Schleimer has suggested that the actions of these enzymes may regulate the local anti-inflammatory effects of cortisol in the lung (Schleimer 1991). The results of the present study show for the first time that increased or reduced conversion of cortisol to cortisone can be caused by the inflammatory reaction following an intravenous injection of live BCG. The rate of conversion was different from one organ to another. In liver and kidney the conversion rate did not change over the

vaccination period. In the lung, spleen and thymus, percentages of conversion of cortisol to cortisone were significantly increased at 10 days.

This result shows that the activity of this enzyme can be regulated at the site of inflammation caused by infection with BCG. Changing the activity of this enzyme in this model could be due to generation of cytokines caused by such infection. These cytokines may be generated locally i.e. in the lung, thymus and spleen. IL-1 β and TNF α have been reported to increase the reductase activity of 11 β -HSD-1 (Escher *et al.* 1997). This result was obtained by incubating glomerular mesangial cells (GMC) which express 11 β -HSD. In the present study, results showed that during the first three days, after infection, cortisol was maintained at a high level in the lung compared with non-BCG animals. Hernandez-Pando, Pavon *et al.* (1998) showed that in the murine model, TNF α and IL1- β are pro-inflammatory cytokines and are abundant during the first three days of TB infection.

Pulmonary tuberculosis causes a shift in the enzyme activity towards reductase (Rook *et al.* 1996), which suggests that the 11 β -HSD activity can be regulated locally in the inflammatory site such as lung in a manner which may enable the immune system to control or eliminate BCG or TB. Rook *et al.* suggested that the activity of this enzyme, in case of TB, is regulated inside the lung, also shifts its activity toward reductase, since more cortisol metabolites were found in TB patients' urine (Rook & Hernandez-Pando 1997; Rook *et al.* 1996). The current results not only support this suggestion but also suggest that only important organs, from an immunological point of view, such as spleen thymus and lung may be sites for regulation of 11 β -HSD activity, since kidney and liver showed no changes in cortisol / cortisone conversion

ratio, whether in the BCG group or the normal group. The results described here show that, in the BCG-lung the cortisol was higher than in normal lung only during the first three days. This result is similar to Escher's result since the $\text{TNF}\alpha$ and $\text{IL}1\text{-}\beta$ would be the more likely the drive force causing more cortisol in the BCG lung than in normal mice

However, the group of mice after 7-days showed no changes in the conversion rates, either in the lung or in the spleen and thymus. This could be a transit interval where shifting from TH1 to TH2 occurs, and more cytokines such as IL-4 and IL-5 are generated. On day 10, in the lung, spleen and thymus, the interconversion of cortisol to cortisone increased. This could mean that, in murine BCG model there is a decrease in pro-inflammatory cytokines, and more IL-4 and IL-5, and therefore increased conversion of cortisol to cortisone. This hypothesis needs to be tested. In contrast, the continuing shift to cortisol in human TB is in agreement with the continuing dominance of TH1 cytokines, and continuing release of IL-1 and $\text{TNF}\alpha$ seen in the human disease.

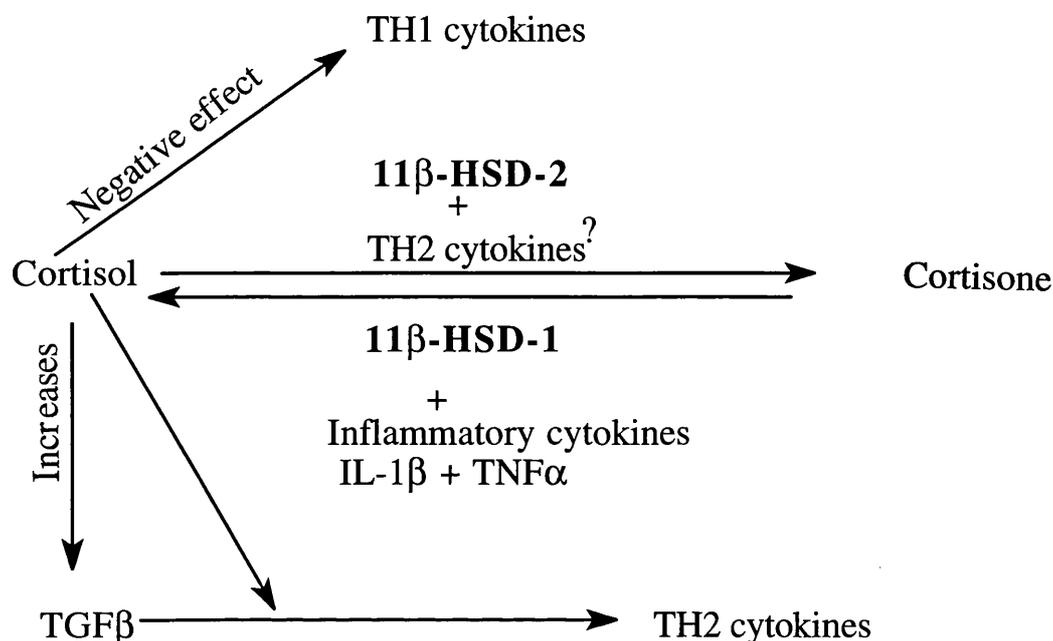
Alternatively, since cortisol inhibits TH1 cytokines, and has negative effects on IL-2 and $\text{IFN}\gamma$, this could mean that the cortisol needed to be inactivated as soon as possible. It is highly likely that in association with BCG infection there were cytokines produced which may have affected the activity of $11\beta\text{-HSD}$. However, the high level of cortisol resulting from HPA-axis activation during the first three days of infection could not be dealt with as a result of presence of $\text{TNF}\alpha$ and $\text{IL}1\text{-}\beta$, but once these cytokines had been neutralised by other cytokines such as IL-4, IL-5 and $\text{TGF}\beta$, the shuttle of cortisol toward cortisone could be observed. By comparing the three-day group with the 10-day group, it can be seen that more cortisol was maintained in the lung in the three day group, but more cortisone was seen in 10-day group. This

could mean the presence of one or more cytokines, more likely to be TH2 cytokines or TGF β , which may have increased the dehydrogenase activity of 11 β -HSD in the 10 days group as most of the BCG will already have been dealt with. It is known that cortisol generates more TGF β , which has a negative effect on the TH1 response (Ayanlar-Batuman *et al.* 1991). Therefore, TGF β is also a candidate to play a role in this scenario. However, the effects of these cytokines on 11 β -HSD-1 have not yet been investigated.

Raised corticosterone levels during the infection would push thymus and spleen cells to apoptosis. Therefore, once the corticosterone has circulated at high concentration, for a few days it would be logical for this to be converted to the inactive form in the thymus to enable thymus to re-generate more T-cells. The BCG- group which will have had high circulating corticosterone after infection, showed high dehydrogenase activity of 11 β -HSD in thymus and spleen by day 10. It is not known whether the signal for this change was the raised corticosterone itself, or accompanying cytokine changes as suggested above.

It seems that the balance of cortisol and TH1 cytokines is important in respect of BCG or *Mycobacterium tuberculosis* clearance. Cortisol drives TGF β which has a negative effect on TH1 cytokines which are needed most in these circumstances and cortisol itself drives the response toward TH2. Cortisol can be converted to cortisone but it is possible that it needs a signal from TH2 cytokines which are not good at clearing the pathogen. Therefore, TH2 cytokines may be required but in a very delicate balance.

The following diagram may explain what might be happening in a lung where too much cortisol exists.



The conclusion from these results would be that the activity of 11β-HSD may be regulated by some inflammatory cytokines.

It is very clear that BCG has changed the activity of the enzyme in lung, spleen and thymus. BCG did not change or increase the activity of the enzyme in the liver or kidney. Further studies will be required to confirm the hypothesis that the switch to increased dehydrogenase activity by day 10 was due to TH2 cytokines, and also to discover whether the dehydrogenation was due to reversal of 11b-HSD-1, or to increased activity of the recently discovered lung 11β-HSD-2

CHAPTER-6

GENERAL DISCUSSION

6.1 The balance of glucocorticoids and antiglucocorticoids

6.2 Factors which may disturb the balance of DHEA/cortisol

6.2.1 Production of cytokines

6.2.2 Sulphatase enzymes

6.2.3 Activities of 11β -HSD

6.3 Consequences of disturbing the balance of DHEA or its derivative and glucocorticoids

6.4 Future steps

6.1 The balance of glucocorticoids and antiglucocorticoid

Steroids are derived from cholesterol and are of low molecular weight. Both glucocorticoid and DHEA are produced by the adrenal gland but by different layers. Glucocorticoids generally act as immunosuppressors, whereas DHEA, which can be metabolised to different steroids e.g. androgens, may, with some of its metabolites, play a role as an immunoregulator or as an antagonist to the inhibitory effects of glucocorticoid. Cortisol particularly suppresses TH1 cytokines which are needed in addition to cytotoxic T cells, TNF α and activated macrophages to react against *M. tuberculosis*. On the other hand, DHEA and AED stimulate TH1 cytokines (Hernandez-Pando *et al* submitted). Thus cortisol and DHEA are acting against each other, but also they are controlling each other. Therefore, any factor which may lead to disturbing either the production or function of one of them may lead to serious consequences to the immune system.

During infection with *Mycobacterium tuberculosis*, there are many factors which play a role in controlling the disease. Amongst these factors is the balance between DHEA and cortisol. It is well known that DHEA declines with age. DHEA starts to rise at adrenarche at adolescence and reaches its highest level at the age of 20 - 30 years (Nestler 1995). On the other hand cortisol, remains constant despite ageing. The importance of the DHEA / cortisol balance in tuberculosis comes from its effect on immune system components.

6.2 Factors which may disturb the balance of DHEA/cortisol

6.2.1 Production of cytokines

The present study showed that AED can increase IFN γ production by lymphocytes taken from C57Bl mice. Also DHEA and AED were able to increase the production of IFN γ by human T-cells. These data are in accordance with previous studies reviewed earlier. AED not only increased IFN γ production but also protected mouse thymus against the corticosterone suppressive effect. In this there is clear evidence that AED acts as an anti-glucocorticoid, though the mechanism of action is not understood. Different steroids were examined *in vitro*, and none of them apart from AED antagonised the corticosterone action. However, AED, 3 β -7 β dihydroxy 5 α androstan-17one enhanced the proliferation of murine lymphocytes. It is very interesting to see that AED was the most active compound in systems investigated. AED enhanced the proliferation, partially antagonised the corticosterone inhibitory effect and at a very low dose stopped the suppressive effect of corticosterone on the thymus. Thus, the effect of DHEA and AED on cytokine production plays a vital role to neutralize the negative effect of glucocorticoids on TH1 cytokines. It is believed that DHEA and AED exert their effect on the immune system components via their effect on the cytokines production (Loria & Padgett 1992).

6.2.1 sulphatase enzymes

There are factors which contribute to the disturbance of the DHEA / cortisol ratio. It has been noticed that DHEA declines with the advance in HIV symptoms and declining CD⁴ counts (Wisniewski *et al.* 1993). In addition, it has been found that DHEA levels in HIV⁺ infected patients, at the advanced stage, drop below the level of normal individuals, at the same time cortisol level increases sharply, which has been considered a reason behind CD⁴ cell counts declining (Christeff *et al.* 1997). Also, the metabolism of DHEA in TB patients is not normal (Rook *et al.* 1996). The activity of sulphatase is a key factor in controlling the shuttle between DHEA and DHEA-S. Moreover, the metabolism of DHEA-S not only to DHEA but to AED is another key point, since it was found in this study and other studies that AED is more active than DHEA itself (Loria & Padgett 1992). Thus sulphatase and 17 β -hydroxy steroid reductase are considered control points.

The present study showed that 16 α 0H-DHEA was an inactive compound. This result is in accordance with the observation that during normal pregnancy DHEA is converted to such compounds. This could mean that since TH1 response is hostile to the foetus, TH2 response should be mounted during pregnancy. Therefore, DHEA has to be converted to inactive compounds (Hill *et al.* 1995).

In *vivo* and in *vitro* treatment showed that too much DHEA may have a negative effect on the immune system components. When DHEA was given in a very high doses, the thymus atrophies. This may be due to the fact that DHEA was converted to sex hormones. Sex hormones (androgens and oestrogen) have a negative effect on

the thymus as they decrease thymus weight, possibly due to increasing TGF β expression by testosterone (Olsen *et al.* 1993). Also, data from humans seem to confirm the animal data. When 200 mg of DHEA was given to patients with complete panhypopituitarism, an increased level of testosterone, similar to the testosterone level in normal men, has been noticed. On the other hand, when 50 mg of DHEA was given to another group a similar level to the testosterone level in women was detected (Young *et al.* 1997). These data suggest that the higher the doses the more testosterone produced. Therefore, all previous data obtained with high doses should be re-evaluated. Also, this highlights the importance of determining appropriate doses in this study, close to the physiological level of circulating DHEA in animals. In the study described here 15 μ g was sufficient to protect the thymus against corticosterone (1.6mg). Also based on these data, it has been shown that in the TB murine model the animals were protected against TB by using very small doses similar to those used in this study starting on the day of infection (submitted to Infection and Immunity). Therefore, the presence of DHEA or its derivative is essential to counter the cortisol immunosuppressive effect, but it has to be well balanced. The evidence for this came from further data obtained by Hernandez-Pando *et al.* His data showed that the AED / cortisol ratio is very critical. In the murine-TB model if AED was given in a very advanced stage of TB (day 60) when the adrenal is atrophic, and corticosterone levels very low, the AED did not protect the animal. The mice died from huge inflammation which results from migration of inflammatory cells to the region. However, if AED was given concomitantly with corticosterone, the mice were protected. Finally corticosterone on its own accelerated the death of the mice, when given at this late stage of the disease, so the adrenal

atrophy may be a necessary response to keep corticosterone low. Thus both AED and corticosterone are needed, the first to promote TH1 response and the latter to counter any over reaction, but the ratio is critical. These data are similar to (Araneo *et al.* 1993) data when she showed that the competence of the immune system can be restored in aged mice by treatment with DHEA. The incompetence may have resulted from a decline in DHEA level though these levels are always low in mice, and were not measured in this study (Araneo *et al.* 1993).

6.2.2 Activities of 11 β -HSD

Another factor that contributes to controlling the balance of DHEA/cortisol is that of 11 β -HSD. The action of glucocorticoid is regulated at many levels inside human body. Amongst these is its metabolism in liver, kidney and other parts of the body including the lung. The metabolism of cortisol to corticosterone or *vice versa* is regulated by 11 β -HSD. In the kidney, cortisol and aldosterone have approximately equal affinities for the receptor that regulates sodium uptake; yet aldosterone, not cortisol, regulates sodium uptake, despite being found at one thousandth the concentration of cortisol in the circulation (Walker 1997)

In the lung, 11 β HSD possesses both dehydrogenase and reductase activity, but these activities are regulated according to need, see chapter-5. The activity of 11 β -HSD determines the anti-inflammatory effect of 11 β -hydroxy glucocorticoids (Escher *et al.* 1997). The pro-inflammatory cytokines IL- β and TNF α upregulate the reductase activity of 11 β -HSD which results in high tissue

concentration of cortisol (Escher *et al.* 1997). Too much cortisol is bad for TB since it shifts the balance of DHEA/cortisol and therefore, promotes TH2 cytokine production.

11 β -HSD activity is also reduced (by 70-80%), in ageing animals. This could be associated with immunosenescence, since the inhibition of the enzyme activity in mature adult animals results in a reduced T-cell production of IL-2, with an increase in the synthesis of inducible IL-4 and IL-10. Animals with compromised activity of 11 β -HSD are more susceptible to agents which are cleared easily by animals with normal 11 β -HSD activity (Hennebold *et al.* 1996).

This study showed that the activity of 11 β HSD is a target for cytokine regulation which may be generated as a results of infection with live BCG. When BCG was given intravenously to animals, 11 β -reductase increased during the first three days. However, 10 days after vaccination, 11 β dehydrogenase activity had increased. This result also suggests that, the activity of 11 β -HSD, dehydrogenase or reductase, being promoted depends on the type of cytokines which are circulating. However, the cytokines concerned were not identified.

As was mentioned earlier, the inflammatory cytokines are the first cytokines to circulate, in an attempt to activate the cellular response in order to control the disease. It appears that these cytokines (IL-1 β and TNF α) increase reductase activity. Our hypothesis is that TH2 cytokines, which circulate later, may increase dehydrogenase activity. TGF β might play a role in shifting the response towards TH2, since TGF β suppresses TH1 cytokines. TGF β mediates shifting towards TH2 cytokines either directly or via the induction of IL-10 (Hiroaki & Akio 1996). TGF β production increases in the presence of

cortisol. Induction of TGF β by cortisol could be another mechanism by which cortisol mediates its effect on TH1 cytokines, in addition to its direct effect on T-cells. The excess amount of cortisol has to be converted to the inactive form. However, it is possible that cortisol cannot be converted unless a mixture of TH2 cytokines appears, yet TH2 cytokines are not desirable in controlling TB. Therefore, a delicate balance has to be struck between conversion of excessive amounts of cortisol and generation of TH2 cytokines. Thus the activity of 11 β -HSD is extremely important and a major factor in keeping the DHEA/cortisol ratio balanced.

6.3 Consequences of disturbing the balance of DHEA or its derivative and glucocorticoids

Not only in TB DHEA/cortisol balance is important, but also in HIV patients. DHEA declines is used nowadays as a marker of disease progression and appearance of the symptoms. In such patients, the cortisol is elevated whereas DHEA declines throughout the progress of the disease (Christeff *et al.* 1997). This study shows how close are the data obtained from the murine model and from humans. Both studies confirm that these two steroids (DHEA or its derivative and glucocorticoid) should be in a balance. The ratio of DHEA/ cortisol is important in the immunosenescence and ageing process. DHEA levels decline with advancing age whilst the cortisol level is maintained. This reflects the deteriorating immune system in the elderly. A replacement therapy has shown that people can regain their immune system competence. However, high doses may lead to unpleasant

results such as liver cancer. Therefore, great caution should be taken when people are treated with DHEA.

Glucocorticoids (GC) are immunosuppressive and can induce lympholysis *in vitro* and *in vivo* (Ayanlar-Batuman *et al.* 1991). However, the mechanisms by which GC modulate immune system functions are unclear. One possibility, supported by accumulating experimental evidence, is that GC inhibit the synthesis and release of immunoregulatory molecules, such as IL-2 and IFN γ , by activated cells (Arya *et al.* 1984; Blauer *et al.* 1991; Surh *et al.* 1993). These cytokines promote the expansion of antigen-specific helper/inducer or cytotoxic/suppressor T cell clones as well as natural killer cells that are involved in immune regulation and surveillance (Smith 1988). The inhibitory effects of GC on immunoregulatory T cell functions *in vivo* was demonstrated in studies that showed inhibition of both IL-2 and IL-2 receptor (IL-2R) gene expression in activated T cells from GC-treated patients with pulmonary sarcoidosis (Konishi *et al.* 1988; Pinkston *et al.* 1987).

In a very similar manner to glucocorticoids, TGF β functions to inhibit certain components of the immune system. It has been shown that transforming growth factor- β 1 (TGF- β 1) a cytokine produced by mitogen-activated T cells, B cells, monocytes, and fibroblasts, is a potent inhibitor of multiple T cell functions *in vitro* (Espevik *et al.* 1988; Kehrl *et al.* 1986 a; Kehrl *et al.* 1986 b; Lucas *et al.* 1990; Stoeck *et al.* 1989). TGF- β 1 has profound stimulatory effects on fibroblast chemotaxis and on expression of the genes for the extracellular matrix proteins collagen and fibronectin. Furthermore, TGF- β 1 can upregulate its own production by these cells (Obberghen-Schilling *et al.* 1988).

The immunosuppressive effects of GC and TGF- β 1 on T lymphocytes share important similarities. Both inhibit IL-2-mediated T cell functions, such as lectin- and IL-2-induced cell proliferation and generation of regulatory T cells *in vitro* and *in vivo* (Bodomler *et al.* 1989; Grayson *et al.* 1981; Saxon *et al.* 1977). It has been shown that dexamethasone significantly increases the expression of the TGF- β 1 gene and its protein product in T-lymphocytes (Bodomler *et al.* 1989). TGF β is considered, if produced in excess amounts, one of the key cytokines which are responsible for tissue destruction in the lungs of TB patients, since this cytokine is responsible for recruiting immature monocytes and macrophages. Also, TGF β enhances the fibrosis process. TGF β is abundant in the lesion site of TB patients, and at the same time, cortisol metabolites have been found to be higher in the urine of TB patients compared with normal individuals.

In conclusion, to enable the immune system to mount the right response, the DHEA/ cortisol ratio has to be balanced. Too much cortisol or DHEA is bad for the immune system. The data described here in conjunction with other data, suggest that the balance between DHEA/cortisol is controlled by many factors. Enzymes control the shuttle from cortisol to cortisone, and the conversion of DHEA-S to DHEA and AED seems to play a major role. Also, the kind of cytokines available in the lesion site seem to play a role in determining the direction of enzyme such as 11 β -HSD. This study has shown for the first time that 11 β -HSD activity is regulated during a course of infection. Furthermore, this activity is regulated independently in each organ. Therefore, in human TB, the activity of

11 β -HSD may play major role in either control or exacerbation of the infection.

6.4 Future steps

It is sad there is no time to continue this project which shows very interesting data. This study demonstrated that 11 β -HSD can be regulated during the course of infection with live BCG and it is hypothesised that this regulation could be due to cytokine production. Therefore, it would be very interesting to determine which cytokines are involved. This could be done by taking tissues and investigating them for the expression of cytokines at different intervals, e.g days 3, 7 and 10. mRNA could be extracted to perform reverse transcriptase polymerase chain reaction (RT-PCR). Also, it would be of great interest to determine which is enzyme responsible for such activity. Is it 11 β -HSD-1, 11 β -HSD-2 or a novel enzyme? This could be determined by carrying out in situ hybridization. In addition, cells harbouring these enzymes could be identified by in situ hybridization. The ultimate interest would be in giving tuberculous mice labelled cortisol at different stages of disease to see what happens to it. This could be done by following the protocol designed in this study.

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publication from this thesis

Rook, G; Baker, R.; Alhajoj, S.; Zumla, A. (1998) Endocrine regulation of T lymphocyte subpopulations. Kimber, I. Selgrade, M. Wiley and Sons Ltd.

Submitted to the infection and immunity

Henande-Pado R, de la Luz Streber M, Orozco H, Arriaga K, Pavon L, Al hajoj SA, Rook GAW. The effects of androstenediol and dehydroepiandrosterone on the course of tuberculosis in Balb/c mice (1998).