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Lymphocytic 5'-Ectonucleotidase:

A marker of psychological stress-induced immune suppression

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ABSTRACT

Lymphocytic 5'-Ectonucleotidase: A marker of psychological stress-induced immune suppression

The aim of the studies reported in this thesis was to find a mechanism to explain the well known phenomenon of stress-induced immunosuppression. Lymphocyte 5'-ectonucleotidase (NT) was selected since it is a lymphocyte differentiation marker and is low in a variety of situations of clinical immune suppression such as X-linked hypogammaglobulaemia, HIV infection and neonates exhibiting with persistent infections. NT was looked at in the following stress groups: Honours students in Psychology undergoing thesis submission and examinations, persons undergoing stressful life events, and patients with major depression and melancholia. In all of these stressed groups there was a 50% lowering of NT which normalised when the stress was resolved. There was a significant negative correlation between NT and the Total Mood Disturbance (TMD) score of the Profile of Mood States (POMS). Depressed patients on high antioxidant intake which included various combinations of ascorbate, zinc, vitamin E, and vitamin A had NT values which were normal suggesting that the reduction of NT may be mediated by oxygen radicals.

Generation of superoxide anion in vitro reduced lymphocytic NT, and this effect was reversed with 100 μmolar of ascorbate. Using NBT as an electron acceptor from the superoxide anion showed that ascorbate, zinc and glutathione at physiological levels protected NT against oxygen radical damage.

Ascorbate stores were reduced significantly in stressed individuals compared to normal healthy controls and NT correlated negatively with the TMD scores. Antioxidants, but not ascorbate exclusively, given to a depressed patient resulted in NT value increasing from 0.55 to 0.85 nmol/h/μgDNA, and protection of NT from the superoxide anion.

Using a rat model of stress, antibody response to sheep red blood cells (SRBC) and NT values were unaffected, however ascorbate stores fell significantly in rats during a learned

helplessness manipulation. The lack of effects on antibody responses and NT were discussed, and probably related to the acute nature of the stressor used in this model, as opposed to the more chronic stressors that occurred in the human model.

In conclusion, NT is a good marker of stress induced immune suppression and correlates negatively with depression scores such as the Beck Depression Inventory, and psychological distress as measured by the TMD scores, and STAI-trait. The suppression of NT appears to be mediated by reactive oxygen species (ROS), and is consistent with the finding of normal NT levels in melancholic patients with high antioxidant intake, the heightened inflammatory responses, the low serum levels of zinc, the low ascorbate stores, and the down regulation of glucocorticoid receptors reported to occur in stressed/depressed patients.

STATEMENT

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contain no material previously published or written by any other person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed Date 31·7·96

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The inspiration for my dissertation came from a study conducted by Professor David Spiegel and his colleagues at Stanford University School of Medicine (Spiegel et al., 1989). My hope is that the mechanism proposed in this dissertation may help to elucidate David Spiegel's finding of enhanced survival in terminally ill breast cancer patients, and ultimately benefit individuals and their families who suffer from cancer.

Finally, and most importantly, I would like to thank my family for their enduring love, faith and belief in me. I dedicate this work to them.

He made her melancholy, sad, and heavy;
and so she died; had she being light like you,
of such merry, nimble, stirring spirit,
she might ha' been a grandma ere she died;
and so may you, for a light heart lives long.

Love's Labour Lost (V. *ii*, 14-18)
William Shakespeare

CHAPTER ONE

GENERAL INTRODUCTION

1.1 General overview

Psychoneuroimmunology, (PNI) also known as neuroendocrinimmunology, is an interdisciplinary field that may be defined as the study of interactions between behaviour, the nervous system and the immune system, and their potential implications for health (Maier, Watkins, & Fleshner, 1994; Sheridan, Dobbs, Brown, & Zwilling, 1994). Most PNI research has focussed on the effects of stress on immunity, with the assumption that disease onset and progression can be mediated by the effects of psychological influences on immunity. Nevertheless, this assumption is still controversial. It will be argued that one of the reasons for the controversy is that we have yet to find a sensitive, stable measure of immune suppression during psychological distress. The aim of this dissertation is to seek and test an alternative measure.

Before proceeding further a definition of stress, and an overview of the historical background to the development of the field of PNI is introduced. This is followed by an overview of the immune response, and an outline of bidirectional interactions between the brain, the immune system and behaviour, and their implications for health. Finally, a critique of the methodological limitations of the current tests commonly used in psychoneuroimmunology in humans and animal research is presented. [1]

The review of the animal research focuses specifically on learned helplessness research as this is of most relevance to this thesis. [1]

1.2 Definition of stress

What is stress? Stress is an elusive concept to define (Baum, 1990). For the purpose of this review stress is conceptualised as the response of the body to internal or external threats that disturb its equilibrium or homeostasis (Chrousos & Gold, 1992; Khansari, Murgo, & Faith, 1990; Sheridan et al., 1994; Sternberg, Chrousos, Wilder, & Gold, 1992). In other words it is defined as "an adaptive response to abnormal environmental conditions or a maladaptive response to normal conditions" (Husband, 1993, p. 806). A stressor is defined as any external or internal challenge that disrupts homeostasis (Chrousos & Gold, 1992; Khansari et al. 1990; Sheridan et al., 1994; Sternberg et al., 1992)

1.3 Historical development of the field of Psychoneuroimmunology

The notion that emotional states such as melancholy and sadness may lead to physical illness and death is a prominent theme in our literature, and is part of our popular wisdom. Anecdotes of people who have suffered terminal illness and overcome the disease by the power of the mind are prevalent (Shukla, Solomon, & Doshi, 1979). This popular wisdom has its roots in philosophy and religion and dates back to antiquity (Rosch, 1995).

The writer of Proverbs in about 300 BC brought together religious teachings from different periods in history which reflect the belief that positive emotions like hope and light heartedness are beneficial to one's health (Bratton, 1961):

> *"Hope deferred maketh the heart sick, but when the desire cometh it is the tree of life"* (Proverbs 13, v.12) and,
>
> *"A merry heart doeth good like medicine: but a broken spirit drieth the bones"* (Proverbs 17, v.22).

This notion that the mind and the body are inseparable is also reflected in the philosophical teachings of antiquity. It is intrinsic to ayuvedic principles and practices in ancient India (Sanyal, 1964):

" There are two classes of disease-bodily and mental. Each arises from the other. Thus mental disorders arise from physical ones, and likewise physical disorders arise from mental ones" (Santi Parva, XVI 8-9).

A similar ethos existed in Classical Greece. Plato (428-347 B.C.) in the book Charmides, quoted Socrates as follows:

"As it is not proper to try to cure the eyes without the head, nor the head without the body, so neither is it proper to cure the body without the soul, and this is the reason why so many diseases escape Greek physicians who are ignorant of the whole" (Plato, 1942).

According to Hippocrates (460-377 B.C.) psychosomatic disorders arose from an abnormal physical reaction to stressful experiences. He observed that strong emotions such as fear and anger could result in physical disturbances. Similarly, the Greek physician Galen as early as (A.D. 129-199) maintained that strong negative emotions such as fear, anger, and grief were "diseases of the soul". He reported that melancholic women were more likely to die from cancer than sanguine women because they had an excess of black bile (Rosch, 1995). Galen believed that emotions, and thoughts were constantly circulating through the body and could impact directly on physical processes.

Galen's philosophy dominated Western medicine for 16 centuries (Rosch, 1995). Renaissance physicians firmly believed that imagination, thoughts and physical processes were constantly influencing each other, and that certain images or thoughts could directly affect specific physical functions of the body. Rosch notes that Renaissance physicians often treated patients by appealing to their imagination (1995, p. 209):

"The physician must invent and devise some spiritual pageant to fortify and help the imaginative faculty, which is corrupted and depraved; yea, he must endeavour to deceive and imprint another conceit, whether it be wise or foolish, in the patients

braine, thereby to put out all former phantasies" (In Approved Directions for Health in 1612).

However, during the 17th century the importance of the mind in influencing health and illness fell into disrepute and came to be regarded as unscientific. This was due largely to the influence of the French philosopher René Descartes who advocated that the mind is separate from the body, and that the aetiology of illness can be reduced to purely physical or mechanistic laws (Rosch, 1995). During this time the understanding of the mind and soul was largely relegated to religion and philosophy, and the body was considered to be a separate realm of physical medicine (Gatchel, Baum, & Krantz, 1989; Rosch, 1995).

Cartesian notions led to many influential discoveries in the field of medicine, such as the discovery of germs by Pasteur and Koch, the finding that scurvy was due to nutritional deficiencies, the use of vitamin therapy for deficiencies, and the astonishing success of vaccines and antibiotics. These exciting discoveries further served to establish the doctrine that all illness could be explained purely by a physical cause and effect relationship (Rosch, 1995).

Although Cartesian notions still dominate contemporary medicine, challenges from several different sources make it difficult to sustain a strictly dualistic philosophy. From a physiological perspective, the eminent scientist Claude Bernard in the 19th century proposed the notion that health was dependent on maintaining "milieu interieur", a dynamic physiological equilibrium (Chrousos & Gold, 1992; Gatchel, Baum, & Krantz, 1989). Subsequently, Walter Cannon coined the term homeostasis and extended Bernard's concept to emotional as well as physical dimensions (Chrousos & Gold, 1992). In describing the flight fight response Cannon linked the adaptive response to stress with the secretion of humoral substances which he called "sympathin" later to be known as catecholamines. His work challenged the dualistic notion that the mind and the body were separate non-interacting entities (Cannon, 1935; Cannon, 1939). A few decades later the Canadian Hans Selye (1936)

was the first to show the effects of stress on the immune response. He demonstrated that illness may result from excessive adaptational responses which he termed the General Adaptation Syndrome, or stress syndrome.

Moreover, in the early part of this century the influential work of Freud and his followers emphasised the association between personality and illness. For example, Freud was able demonstrate "a cure" of hysterical paralysis through psychodynamic techniques. Also at this time Flandar Dunbar, Franz Alexander and others developed the field of psychosomatic medicine, summarised in Dunbar's "Emotions and Bodily Changes," to explore further the intriguing relationships between illness, stressful life events, personality and emotions from a psychodynamic perspective (Dunbar, 1954). Pioneering studies by psychologists Wolf (1954), and Holmes and Rahe (1967) provided further evidence of the role of stressful life events, and psychological distress in disease progression.

Twenty years before psychoneuroimmunology became an established field Solomon and Moos (1964) in their landmark article, "Emotions, immunity and disease: A speculative theoretical integration, coined the term "psychoimmunology" and associated stress, emotions and disease with recent findings in endocrinology and immunology. Subsequently, the landmark study by Ader and Cohen (1975) showing that behavioural conditioning can modify an immune response challenged the conventional notion that the immune system operated as a closed system. Advances in immunology and the gradual realisation that the immune system does not operate autonomously but interacts with the brain, and endocrine system (described later) has resulted in the emergence of the field of psychoneuroimmunology. From an historical perspective psychoneuroimmunology appears to be an "old wine in a new bottle", and can be conceived as a re-emergence of ancient ideas (Rosch, 1995, p.208; Lloyd, 1987).

In order to conceptualise how stress may affect health it is necessary to have a basic appreciation of the immune response, and the interactions between the neuroendocrine and lymphoid systems.

1.4 The immune system: An overview

The immune system originates from the Latin word "immunis" meaning "exempt". This refers to the primary function of the immune system which is to distinguish "self" from "non-self" and to eliminate "non-self" substances or antigens. It achieves this through a dynamic process made up of a complex series of events involving innate immunity and acquired or adaptive immunity (described later) (Roitt, Brostoff, & Male, 1989).

1.4.1 Factors involved in the immune response

The types of factors involved in the immune response and their functions are summarised in Table 1.1. The main immune cells are phagocytes (monocytes, macrophages), T lymphocytes (T cells) and B lymphocytes (B cells). To mount an immune response these cells interact both physically and also chemically with one another through cytokine (or interleukin) communication (Goodwin, 1994).

1.4.2 Innate immunity: The immediate response

Innate immunity is present from birth and protects the body from disease in a non specific way by the following mechanisms: 1) The skin and mucous membranes act as a barrier to infection; 2) Cells infected with virus release interferon which activates natural killer cells (NK) and macrophages to kill the virus containing cell; 3) The macrophage interacts with bacteria which contain a mannose rich polysaccharide coat, and subsequently stimulates the liver to produce mannose binding protein (MBP) which binds to the antigen and leads to lysis through complement activation by MBP (Roitt et al., 1989); 4) Certain bacteria activate complement directly by either enhancing phagocytosis or complement-mediated lysis of the foreign pathogen (reviewed by Frank, 1994). Complement is one of the acute phase proteins released during inflammatory processes as described in Table 1.1 (Steel & Whitehead, 1994). Innate immunity is a response which occurs immediately and is completed within one to two days post-infection.

Table 1.1: Factors involved in the immune response and their functions

Immune factor	Function
Helper T cells (TH)	Initiates the immune response in cell mediated and humoral responses.
Cytotoxic T cells (Tc)	Role in cell mediated immunity. Destroys foreign cells.
Inducer T cells (Ti)	Involved in cell mediated immunity. Receive cytokine messengers from TH cells which induce the development of T cells in the thymus
Suppressor T cells (Ts)	Dampen the immune response
B cells	Involved in the humoral immune response
Phagocytes a) neutrophils (polymorphonuclear (PMN) b) eosinophils c) basophils d) Macrophages (in tissues) / Monocytes (in serum)	Play a role in innate and acquired immunity a) phagocytosis b) engulf antigen / antibody complexes c) release histamine during inflammatory processes d) Presents processed antigen to TH cells Destroys viral containing cells through phagocytosis.
Natural killer cells	Involved in innate immunity. NK cells are a type of lymphocyte that is capable of recognising and destroying various tumour and virus infected cells by a mechanism that is not yet fully understood (Goodman, 1994).
Interferons (IFNS)	Protects non-infected cells from viral infection. Activates NK cells and macrophages to kill the infected virus.
Complement, kinin, clotting and fibrinolytic systems	Enzyme systems that interact with the immune system during inflammation

Table 1.1: Factors involved in the immune response and their functions Cont'd

Immune factor	Function
Complement (C')	Complement represents a family of serum proteins which have a role in both innate and acquired immunity. Once activated complement punches holes in the cell membrane of the target cell leading to cell death.
Acute Phase Protein	Complement forms part of the acute phase proteins released during inflammatory processes. They have a range of activities. They can directly neutralise inflammatory agents, minimise the extent of local tissue damage, and are involved in tissue repair. Major acute phase proteins (APR) such as C-reactive protein are increased 1000 fold during infection. Proteinase inhibitors such as α1-antichymotrypsin and α1-antichymotrypsin neutralise inflammatory agents. Increased serum levels of some metal binding proteins such as haptoglobin help prevent the loss of iron during infection and injury, and act as scavengers for potentially damaging free radicals (reviewed in Steel & Whitehead, 1994).
Major histocompatibility complex protein (MHC- MHC I or MHC II)	MHC proteins are self labels that are unique to each individual, and are recognised by immune receptors which use them to distinguish "self" from "non self". There are two classes. MHC I and MHC II. MHC I is present on all nucleated cells. MHC II is present on macrophages, B cells, and T4 cells.
Cytokines (or interleukins)	Factors involved in communication between cells in the immune system.

1.4.3 Acquired immunity

If innate defences fail to halt infection, acquired immunity is initiated by the activation of the T helper cell. As described below, acquired immunity is specific to a given antigen and involves a memory component in this response. Figures 1.1, 1.2 and 1.3 give a brief overview of cell mediated and humoral immune responses. Important to these responses is the cell to cell communication. This communication is carried out by a variety of cytokines or protein liberated by the immune cells. The cytokines are also called interleukins or ILS. The role of some of these cytokines in a developing immune response is summarised briefly in Table 1.2. (Goodwin, 1994; Roitt et al., 1989).

Table 1.2: The source and function of the major cytokines in the immune response

Cytokine	Source	Function
IL-1 IL-6	APC THACT	B cell activation
IL-2 IL-4 IL-5	THACT	B cell proliferation
IL-4 IL-5 IL-6 IF-α	THACT	B cell differentiation to plasma cells
IL-2	THACT	Tc cell proliferation and differentiation ⇧ IF-α production ⇧ growth of NK cells
IF-α	THACT	⇧ MHC I and II expression ⇧ antigen processing ⇧ macrophage killing
MIF	THACT	⇧ migration inhibition
IL-1 IL-6 TNF		Initiate the acute phase response

Note. APC indicates antigen presenting cell. THACT indicates activated T helper cell.

1.4.4 T helper cell activation

The first event in the immune response is antigen uptake and processing by the antigen presenting cell (APC) which is usually a macrophage or monocyte. During this process the antigen is digested by the antigen presenting cell and specific fragments of the antigen or epitopes become transported and attached to the surface the MHC II molecules of the APC (Figure 1.1). The T helper cell receptor (TCR) specific to that particular epitope binds to the class II MHC-antigen complex on the APC. This interaction, together with a release of cytokines from the APC triggers T helper cell activation. The activated T cell can then initiate either the cell mediated or humoral response, or both, depending on the epitopes (Goodwin, 1994; Roitt et al., 1989).

1.4.5 The cell mediated response

The cell mediated response as its name implies results in the death of the invading organism by direct cell to cell contact with activated cytotoxic T cells. The activated T helper cell interacts with the cytotoxic T cell, and releases interleukins which stimulate the cytotoxic T cell to differentiate into activated T cells, and cytotoxic memory T cells (Figure 1.2). The activated T cell in the presence of antigen secretes toxins that kill the target cell. In addition to cytotoxic activities against tumour cells and viruses, cytotoxic T cells also play a role in delayed hypersensitivity and transplantation reactions. Memory cytotoxic T cells provide the immune system with the ability to respond rapidly to future encounters with the same foreign cell.

In addition to its above role, the activated T helper cell also releases many factors or cytokines that result in the following functions: 1) The activation of killer cells to destroy cells with foreign antigen; 2) The secretion of macrophage inhibition factor (MIF) which attracts macrophages to the site of infection and leads to cell death by phagocytosis; 3) Increased interferon production, and the growth of natural killer cells which also enhances killing of

cells with antigen; 4) The stimulation of the T inducer cells to differentiate intrathymically into T helper, T suppressor and T cytotoxic cells.

As indicated activated T helper cells augment the immune response, but ultimately the slow induction of T suppressor cells dampens this process by decreasing the effect of the inducer cells, and decreasing cytotoxic T cell proliferation. Thus the T suppressor cells add a balance to the immune response by halting and dampening the response against a specific organism (Goodwin, 1994; Roitt et al., 1989). Without this balance the response would go on unabated, and eventually leave widespread tissue damage.

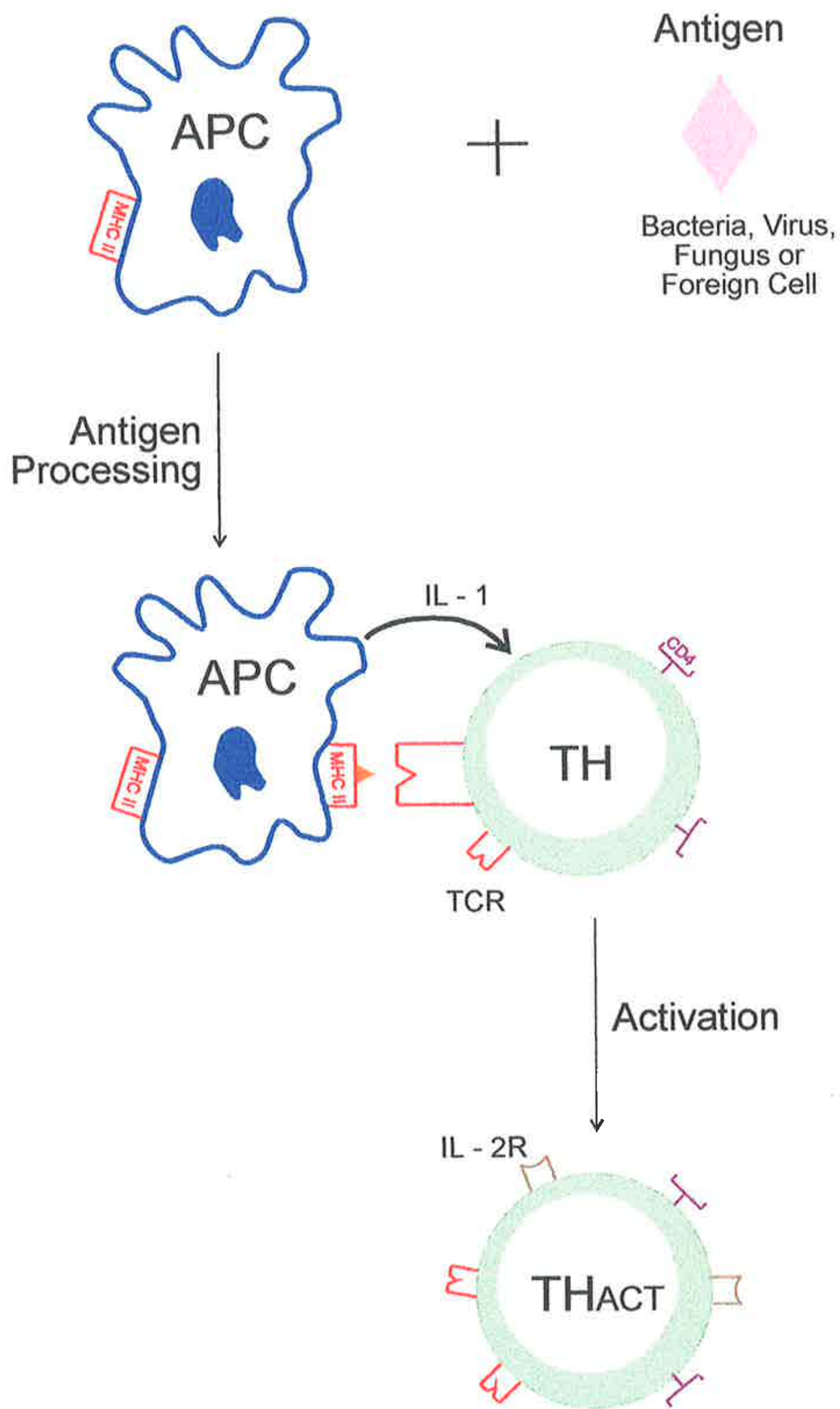
1.4.6 The humoral response

Figure 1.3 illustrates the humoral response which ultimately results in B cell activation and differentiation into antibody-producing plasma cells. The sequence of events is as follows. The activated T helper cell interacts with the B cell, binding antigen / MHC II complex on the B cells to its receptors, and releases factors such as B cell growth factor (BCGF), B cell differentiation factor (BCDF) and interleukins which induces differentiation and proliferation of B cells into antibody secreting plasma cells and B memory cells. A specific immunoglobulin or antibody binds to antigen, and forms an antigen-antibody complex. Death of the foreign invader is mediated either directly by antibody/complement mediated lysis, or by antibody enhanced phagocytosis.

To conclude, both cell mediated T cell and humoral B cell responses are characterised by both cell proliferation and differentiation. Differentiation is important for the cells to become immunocompetent or active, and also to generate memory cells for any future invasion. Proliferation is important in generating a large army of immune competent cells to fight the foreign invaders.

FIGURE 1.1

Antigen processing and T helper cell activation. Ordinary arrows (→) indicate processes involved in the activation of T helper cells (TH), whereas the thick arrows (➜) indicate the production of interleukins by the antigen presenting cell (APC) influencing T helper cell activation.



APC
MHC II
Antigen
Bacteria, Virus, Fungus or Foreign Cell
Antigen Processing
IL - 1
APC
MHC II
MHC II
CD4
TH
TCR
Activation
IL - 2R
THACT

FIGURE 1.2

The cell mediated immune response. Ordinary arrows (→) indicate the processes of activation, proliferation and differentiation, whereas the wavy line indicates inhibition. The thick arrows (➔) indicate the production of interleukins by the activated T helper cell (THACT) influencing the various processes indicated.

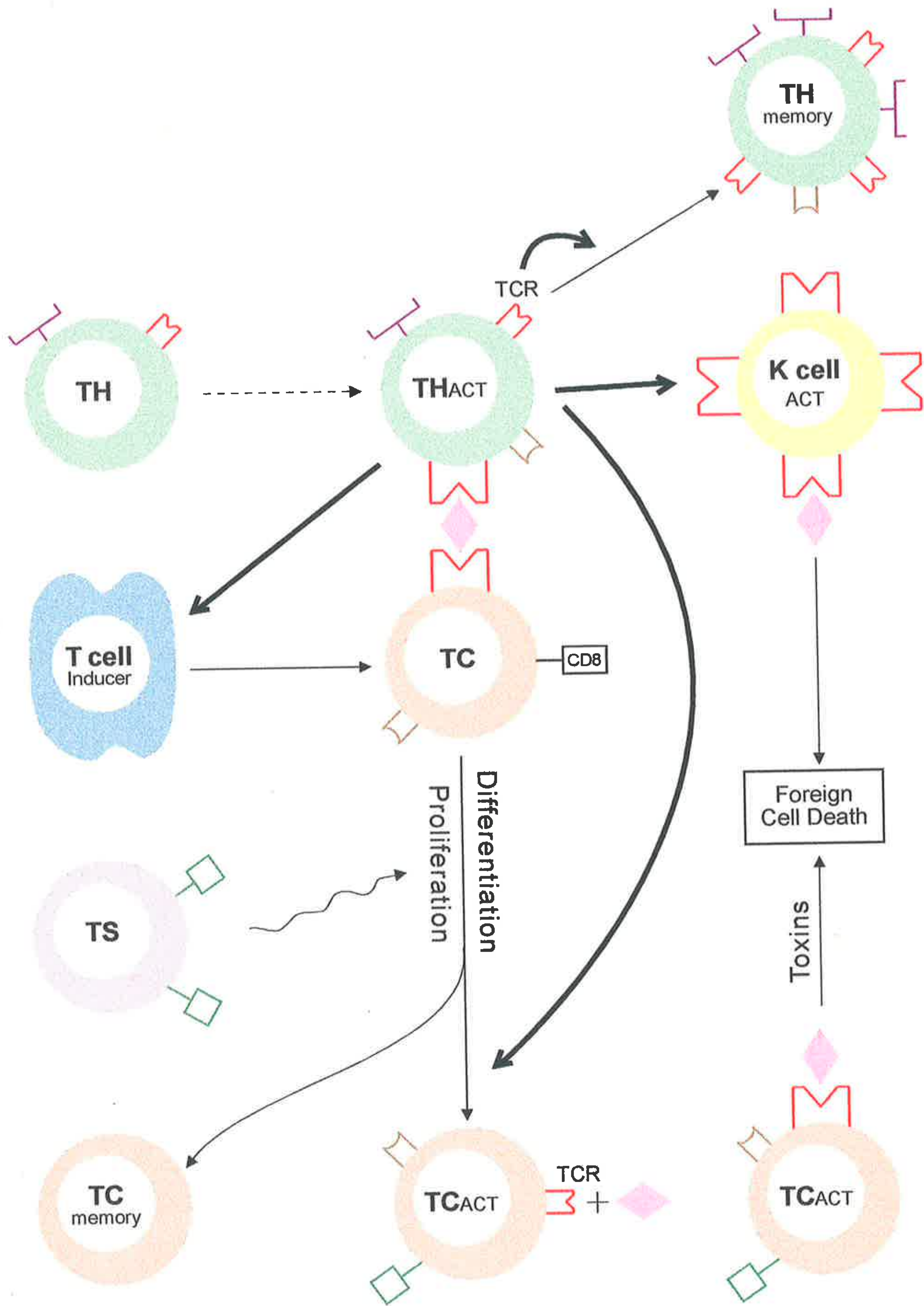
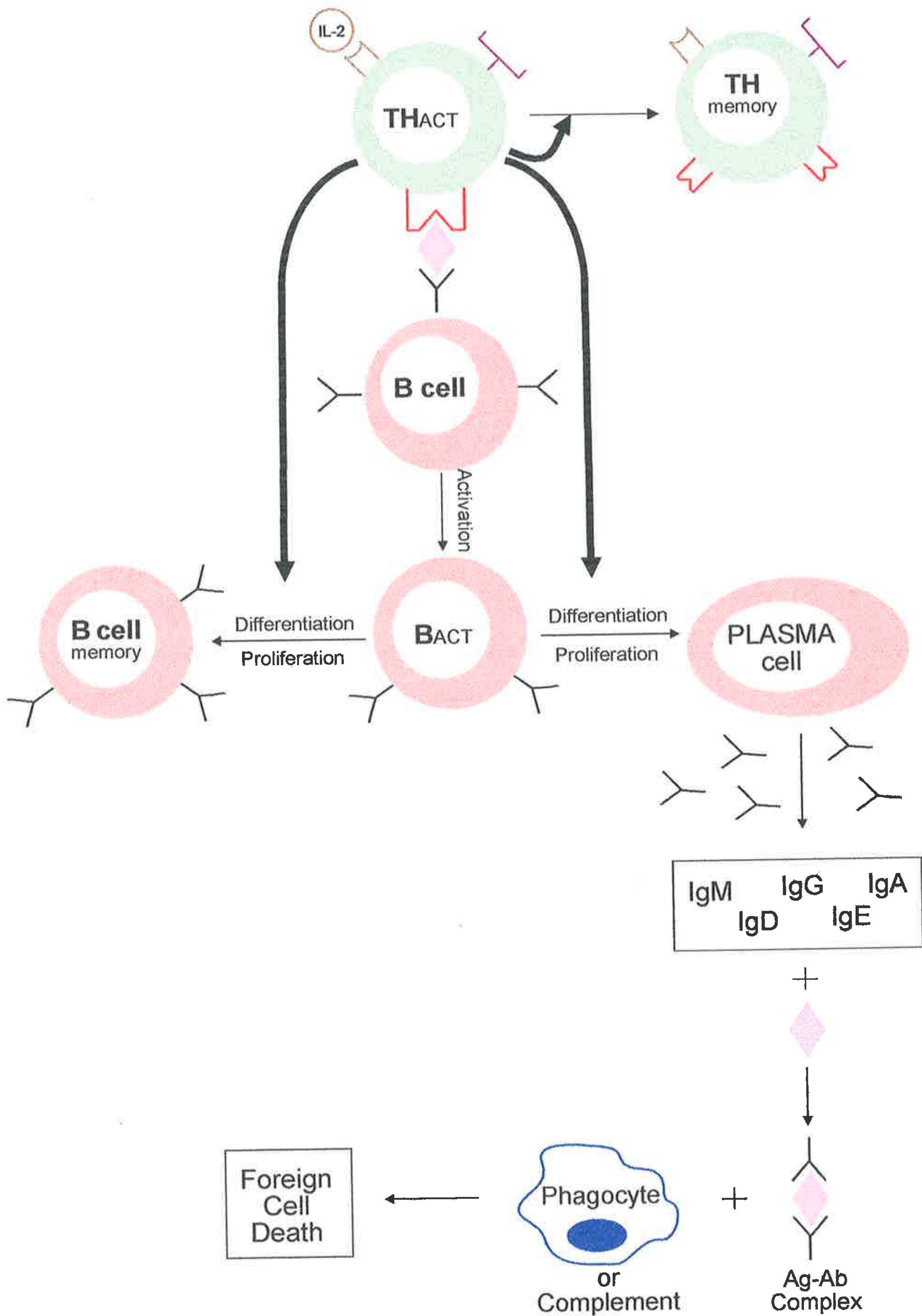

TH
memory
TCR
TH
THACT
K cell
ACT
T cell
Inducer
TC
CD8
Proliferation
Differentiation
Foreign
Cell Death
TS
Toxins
TC
memory
TCACT
TCR
TCACT

FIGURE 1.3

The humoral/antibody response. Ordinary arrows (→) indicate the processes of activation, proliferation and differentiation, whereas the thick arrows (➔) indicate the production of interleukins by the activated T helper cell (THACT) influencing the various processes indicated.



IL-2
THACT
TH
memory
B cell
Activation
B cell
memory
Differentiation
Proliferation
BACT
Differentiation
Proliferation
PLASMA
cell
IgM
IgG
IgA
IgD
IgE
Foreign
Cell
Death
Phagocyte
or
Complement
Ag-Ab
Complex

1.5 The interactions between the neuroendocrine and lymphoid systems

The pathways through which behaviour, the nervous system and the immune system interact are summarised in Figure 1.4.

Anatomical and biochemical pathways between the brain and the immune system make it possible for psychological stressors or behavioural factors to impact on immunity. For example, the sympathetic nervous system (SNS) innervates immune organs (Felten, Ackerman, Wiegand, & Felten, 1987), and thereby makes contact with and modulates the activities of lymphocytes (Felten & Felten, 1991). Also, chemical destruction of the sympathetic neural pathways in animals using 6-hydroxydopamine can reduce the primary antibody response, and suppress cytotoxic activity (Hall et al., 1982; Livnat, Felten, Carlson, Bellinger, & Felten, 1985; Livnat, Madden, Felten, & Felten, 1987).

There is now compelling evidence for bidirectional communication between the brain and the lymphoid system. That is, the immune system may influence the brain and thereby behaviour (reviewed by Chrousos & Gold, 1992; Blalock, 1994; Blalock, Smith, & Meyer, 1985; Husband, 1995; Sheridan et al., 1994, Sternberg et al., 1992). How does the immune system modulate behaviour? As has been discussed, after infection the immune cells release a host of cytokines, such as IL-1, IL-6, TNF-α, and interferons. Some of these cytokines, after interaction with the Central Nervous System (CNS) can alter behaviour. For instance, IL-1 and interferons induce general malaise, lethargy, depression, anorexia, vomiting and fever (Husband, 1995). Similarly, illness inducing agents administered to rats stimulate macrophages to release IL-1 which produces hyperalgesia, or an increased sensitivity to pain (reviewed by Maier et al., 1994). Therefore, it is possible that subtle changes in a person's mood, emotional sensitivity, and perception of pain may result from changes in the status of the immune system (Maier et al., 1994). These behavioural changes are different to those caused directly by stressors, such as reduced activity, exploration, social interaction and appetite (Maier et al., 1994).

Two way communication between the brain and immune cells serves an important role in homeostasis has been recently reviewed and is summarised in Figure 1.5 (Sternberg et al., 1992). After infection, the immune system releases cytokines which stimulate the hypothalamus to release corticotrophin releasing hormone (CRH) which in turn promotes the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland. ACTH then stimulates corticosteroids release from the adrenal gland which suppresses inflammation and dampens the immune response by suppressing antibody secretion, natural killer cell activity (NKCA), cytokine production, lymphocyte migration, macrophage activation and major histocompatility complex (MHC) expression on antigen presenting cells (Khansari et al., 1990; Sheridan et al., 1994). Thus cytokines which are used to augment the immune response initially are used indirectly to stimulate corticosteroid release to dampen the response.

The interaction between the immune cells and the brain is however, more complex, in that other factors may be released. Beside immunosuppressive glucocorticoids and catecholamines, released by the brain, there are other factors which may stimulate or inhibit the immune system depending on the nature of the stressor (Maier et al., 1994). Some of these factors which have been identified are growth hormone, prolactin and opoid peptides which are immunoenhancing, whilst others such as neuropeptide Y and α-melanocyte stimulating hormone are immunosuppressive (Husband, 1995; Sheridan et al., 1994).

Serious illnesses may result from a breakdown of adaptive responses during severe and prolonged stress (Chrousos & Gold 1992; Selye, 1956; Selye, 1976; Sheridan et al., 1994; Sternberg et al., 1992). In an emergency situation the flight fight reaction is adaptive and serves to direct energy to muscles and the brain so that the body can escape the stressor, but chronic stressors such as depression may be maladaptive, and result in a dysfunctional state. For example, depression may be defined as a stress response, leading to activation of the HPA axis and the sympathetic nervous system (Sternberg et al.,1992). Indeed, attenuated and elevated levels of glucocorticoids and catecholamines are evident in depressed people,

especially those with melancholia, and suggest a dysregulation of the HPA axis. That is, the normal homeostatic mechanisms described above for the HPA-immune cells are no longer functional (Carrol, 1978; Chrousos & Gold, 1992; Sternberg et al., 1992).

The main focus of PNI research has been on the effects of psychological factors on infection, autoimmune diseases, and cancer (Jemmott & Locke, 1984; O'Leary, 1990; Sheridan et al., 1994). There is persuasive evidence that stress increases susceptibility to bacterial, fungal and viral infections in both humans and animals (reviewed by S. Cohen & Williamson, 1991; Kiecolt-Glaser & Glaser, 1995; Sheridan et al., 1994), and that depression may result in an inflammatory-like process (Joyce et al., 1992; Maes et al., 1993). It is also of interest that autoimmune (inflammatory) diseases such as rheumatoid arthritis in humans, like major depression, result in disruptions of the HPA and Sympathetic Nervous System (SNS) (reviewed by Sternberg et al., 1992). This finding in humans has been confirmed in animal models in which surgical or chemical disruptions of the HPA axis are associated with greater susceptibility to inflammatory diseases (reviewed by Sternberg et al., 1992). Thus, the evidence would tend to suggest that chronic HPA stimulation results in desensitization of immune cells with an inflammatory-like process resulting as a consequence.

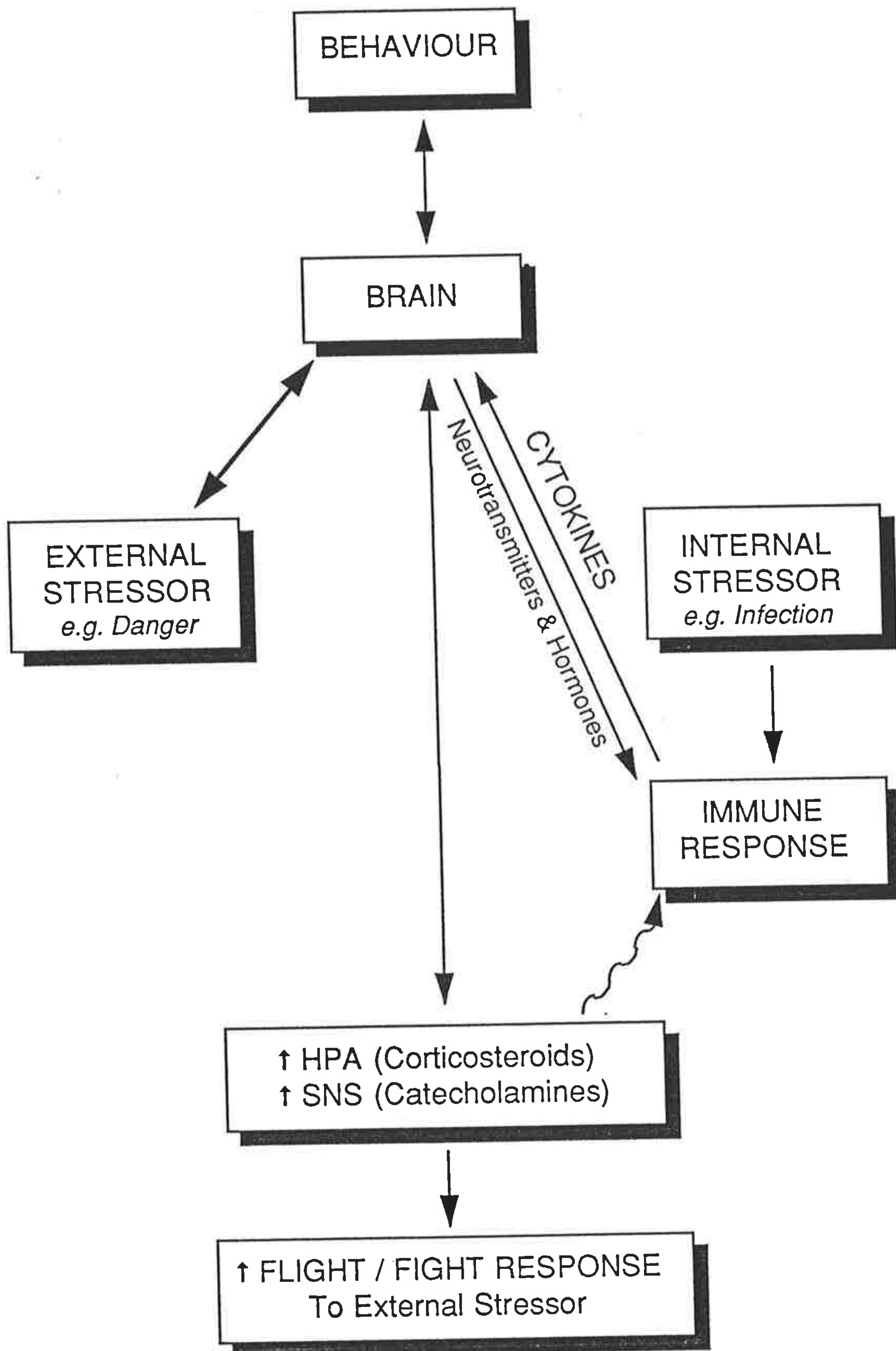
Reviews on the role of stress in cancer have been inconsistent with results indicating both inhibition as well as promotion of neoplasms in animals (eg. Justice, 1985; LaBarba, 1970; Newberry, Gordon, & Meehan, 1991; Peters & Mason, 1979, Sklar & Anisman, 1981). Attempts to relate cancer to stress in humans are even less definitive, making any generalisations about psychological factors and cancer problematic (eg. Fox, 1988; Newberry et al., 1991). We do know that patients taking immunosuppressive drugs are more prone to developing lymphomas, leukaemia and certain types of skin cancers (eg. kaposi's sarcoma). However many of the drugs used are mutagenic and so the effect may not be entirely immune response specific (Disney, 1992). Lymphocytes of depressed psychiatric in-patients have been shown to have significantly poorer ability to repair DNA damaged by irradiation suggesting a

possible mechanism interrelating psychological depression and the development of cancer (Kiecolt-Glaser, Stephens, Lipetz, Speicher, & Glaser, 1985). Perhaps the most persuasive evidence comes from well designed randomised trials showing that group therapy can reduce psychological distress, lessen recurrence and enhance survival in cancer patients, compared to a control group not receiving the psychological intervention (Fawzy et al., 1993; Spiegel, Kraemer, Bloom & Gottheil, 1989). The mechanism responsible for life prolongation in these cancer patients is very likely complex and awaits further biochemical and physiological investigations for their elucidation.

Definitive answers regarding the role that the immune system may play in the survival of people who are depressed or experiencing other forms of acute or chronic psychological distress is difficult to ascertain (e.g. Hickie, Hickie, & Bennett, 1993; Ader, Cohen, & Felten, 1995). This may be due in part to the methodological problems discussed in the following section.

FIGURE 1.4

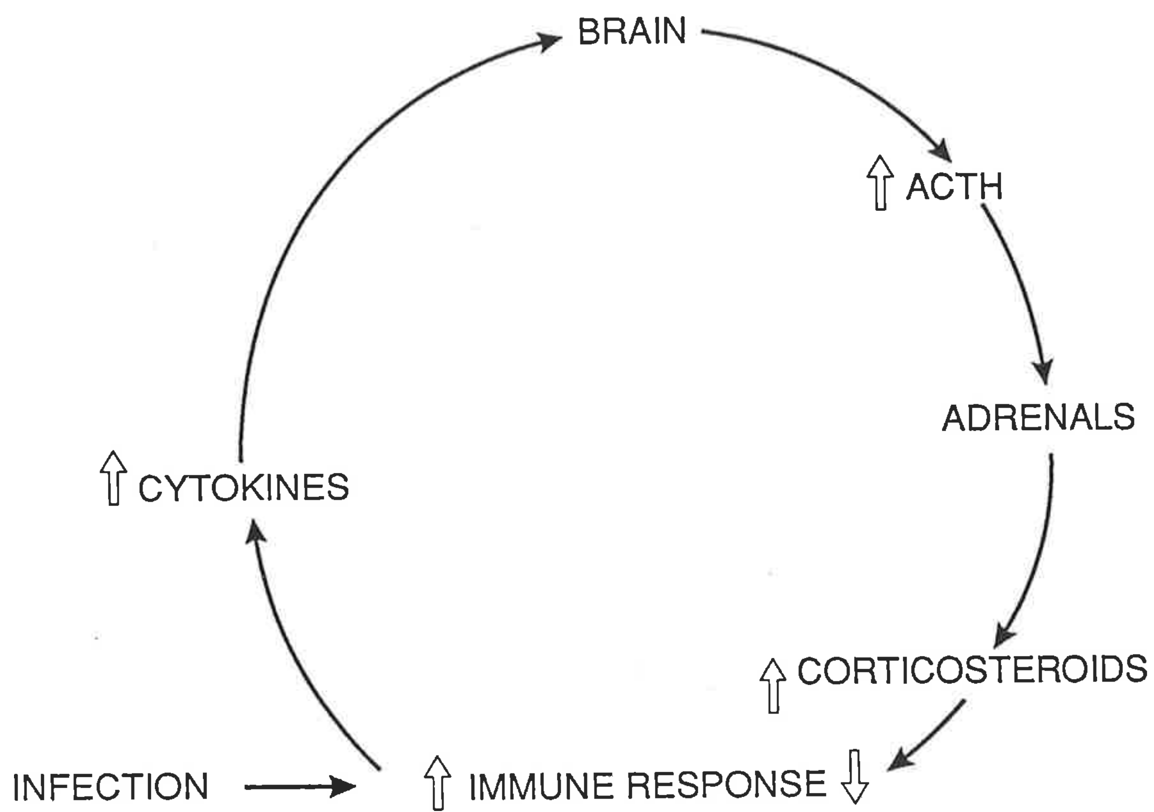
The pathways through which behaviour, the nervous system and the immune system interact and influence each other are shown by the arrows. Wavy lines indicate inhibition.



BEHAVIOUR
BRAIN
EXTERNAL STRESSOR
e.g. Danger
Neurotransmitters & Hormones
CYTOKINES
INTERNAL STRESSOR
e.g. Infection
IMMUNE RESPONSE
↑ HPA (Corticosteroids)
↑ SNS (Catecholamines)
↑ FLIGHT / FIGHT RESPONSE
To External Stressor

FIGURE 1.5

A homeostatic mechanism operating between the immune system and the hypothalamic pituitary axis. Open arrows indicate increased (⇧) or decreased (⇩) activities or concentrations. Full arrows (→) show the effect of different processes on the production and subsequent interactions of various chemicals with tissues influencing immunity.


BRAIN
ACTH
ADRENALS
CORTICOSTEROIDS
IMMUNE RESPONSE
INFECTION
CYTOKINES

1.6 Methodological and conceptual issues

1.6.1. Definition of depression

Confusion exists regarding the definition of depression because there is a lack of consensus of how it should be conceptualised. It has been portrayed as a mood state, a set of symptoms, and a clinical syndrome (Coyne, 1986; Weisse, 1992).

In general terms, depression may include feelings of worthlessness, excessive guilt, difficulty concentrating, preoccupation with death, sleep disturbances, loss of weight, and appetite (Weisse, 1992). However, the main diagnostic tools used in PNI studies of depression are the Diagnostic and Statistical Manual of Mental Disorders, (DSM-III; DSM-III-R, DSM-IV, American Psychiatric Association, 1980; 1987; 1994; respectively), and the Research Diagnostic Criteria (Spitzer, Endicott, & Robins, 1978). Criteria differ slightly depending on which classification system is used. According to the DSM criteria, for example, a major depressive episode (MDE) is characterised by depressed mood that has persisted for at least two weeks. The Research Diagnostic Criteria, on the other hand, recommends that a mood disturbance should persist for a week before assigning a diagnosis of depressive episode. Major depression (DSM), or major depressive disorder (RDC), is characterised by one or more depressive episodes without evidence of mania (e.g., excessive euphoria, inflated self esteem, hyper-activity, irritability).

One important distinction in diagnostic classifications is that of bipolar (endogenous) versus unipolar (non-endogenous) depression. Bipolar subtype is characterised by one or more episodes of mania, and may be associated with psychosis. Bipolar subtype is usually accompanied by one or more MDEs. Unipolar subtype, on the other hand, consists of a major depression which is not associated with mania. Another important criterion is whether the major depressive episode is of melancholic type. This is a particularly serious feature of depression and consists of the following types of symptoms as specified by the latest version of the DSM, the DSM-IV: 1) Ahedonia, or loss of pleasure in all activities, 2) lack of

response to pleasurable activities; 3) depression that is regularly worse in mornings; 4) early morning awakening; 5) significant weight loss or anorexia 6) pronounced psychomotor retardation or restlessness; 7) excessive or inappropriate guilt.

1.6.2 Characteristics of studies that have been conducted to investigate the relationship between depression and immunity.

The table in Appendix A summarises the characteristics of a range of studies that have been conducted to investigate the relationship between depression and immunity. A range of methodological flaws make it difficult to interpret the findings as elaborated below.

(i) Experimental design

Experimental designs of human PNI studies can be divided into cross-sectional or prospective studies. Cross-sectional studies assess immune status at a single moment in time. In contrast, prospective studies assess immune status across more than one observation point set apart by weeks or months. The majority of studies examining depression and immunity are cross-sectional (Appendix A). With cross-sectional studies it is difficult to assess the directional relationship between depression and immunity especially since most of the immune measures currently used in PNI studies are volatile (discussed later). For this reason there is a need for more prospective studies using immune measures that are physiologically and analytically stable to assess the long term implications of the effects of depression on the immune system and health.

(ii) Heterogeneity of diagnostic classification

Another major concern relates to the heterogeneity of diagnostic classification. Although standardised diagnostic tools ensure greater reliability in diagnostic classification, there are several conceptual and methodological difficulties. Few studies have carefully considered diagnostic subtypes of major depression, and differences in results across studies may be partially due to differences in the specific subtypes examined (Andreoli et al., 1992; Stein, Miller, & Trestman, 1991; Weisse, 1992). For example, co-morbidity of major

depression with other conditions such as panic disorder complicate interpretation of enumerative and lectin response assays (e.g. Andreoli et al., 1992; Marazzitti et al., 1992). Also, most of the studies, with the exception of those by Maes and coworkers, (eg. Maes et al., 1992a; Maes et al., 1992b; Maes et al., 1992c; Maes et al., 1992d; Maes et al., 1993; Maes et al., 1994a; Maes et al., 1994b) fail to distinguish those patients who suffer major depression and melancholia. This may be important because major depression that is associated with melancholia is considered to be a more severe form of depression (Maes, Cosyns, Maes, D'Hondt, & Schotte, 1990a; Maes, Schotte, Maes, & Cosyns, 1990b). Furthermore, some studies do not distinguish between unipolar and bipolar subtypes of depression (eg. Alshuler, et al., 1989). Moreover, there is evidence that subtypes of depression such as melancholic, psychotic, unipolar or bipolar disorders represent quite separate biological entities (Maes et al., 1990a; Maes et al., 1990b; Roy, Pickar, Linnoila, & Potter, 1985; Stein et al., 1991).

(iii) Sampling

Small sample sizes sometimes make it difficult to control for the many extraneous factors which may affect the status of immune function (reviewed by Kiecolt-Glaser, 1988a). Another potential problem is that age and sex differences may complicate interpretation in some studies (eg. Kronfol & House, 1989; Maes et al., 1992b; Maes et al., 1992c; Maes et al., 1993; Maes et al., 1994a; Maes et al., 1994b; Appendix A). Age and gender associated differences in immune function are well established (Darko et al., 1988b; Oyeyinka, 1984). The majority of PNI studies investigating the relationship between depression and immunity attempt to control for this difficulty by including control groups that do not differ statistically in age or sex ratios from the experimental group (see Appendix A).

Another concern is that most of the research has been conducted on healthy subjects under 50 years of mean age (see Appendix A). J. J. Cohen (1987a) suggests that the immune system is robust enough in healthy, young subjects to tolerate substantial fluctuations without

affecting susceptibility to illness. Furthermore, few studies have included health data measures (O'Leary, 1990). Moreover, with the exception of Targum et al. (1989), the majority of studies do not include elderly patients, and one needs to be cautious in attempting to generalise findings from younger people to the elderly.

(iv) Severity of depression and hospitalisation status

Most studies in this review of the effects of depression on immunity used the Hamilton Rating Depression Scale (HRDS) to assess the severity of the patient's depression which makes it easier to compare studies in relation to this variable. Hospitalisation status is also reported in most studies, and may be conceived as another index of the severity of depression. It is assumed that patients who are hospitalised are generally more seriously depressed than outpatients (Appendix A).

1.6.3 Characteristics of studies that have been conducted to investigate the relationship between psychological stress and immunity.

(i) Definition of stress and conceptual problems

There are conceptual difficulties in defining stress. Baum (1990) aptly talks of the "fuzziness" of this imprecise construct. The concept has evolved more or less independently in biological and the psychological sciences, and attempts to integrate these disparate positions have been difficult (Baum, Singer, & Baum, 1981; Cannon, 1935; Mason, 1975, Selye, 1956; Selye, 1976).

How does one define what is acute and what is chronic stress? It is frequently assumed that stress may be defined in terms of the time of exposure to the stressor. However this fails to take into account that certain stressors can exert effects when they are no longer present, i.e. after-effects, or recollections of the trauma can sustain chronic stress (Baum, 1990). Manipulations of stressors in both animal and human research are inconsistent, and this makes it difficult to compare across studies (Baum, 1990). Nevertheless despite these

conceptual difficulties, and for practical reasons research in PNI often does define stress in terms of its duration (Herbert & Cohen, 1993).

In respect to PNI research there is a need to develop stress models in humans and animals that produce a reliable immunological effect. Current models produce inconsistent findings (discussed later).

(ii) Experimental design

The characteristics of studies investigating the effects of acute and chronic stressors on immune function are summarised in Appendices B-D. The advantage of laboratory studies (Appendix D) is that they allow for careful control of experimental setting. However, they may not be the ideal stressors for PNI research. Although most laboratory studies assess immune status at baseline, during or after exposure to the stressor, they are essentially cross-sectional in design, generally time limited to minutes or hours. The relevance of short term immunological changes to health has yet to be established (Biondi & Pancheri, 1995; J. J. Cohen, 1987a; J. J. Cohen, 1987b; O'Leary, 1990).

A greater proportion of the studies investigating the effects of chronic and acute stressors on immune function use prospective designs (Appendices B-C, respectively). Theoretically, this within-subjects design makes it easier to establish the direction of the relationship between stress and immune function.

(iii) Sampling

There are two main populations that are recruited in PNI studies. There is the younger student population, whose stressors are 1) related to academic examinations, or 2) manipulated by the experimenter in acute laboratory tasks. The majority of these subjects are under 30 years of age (Appendices C-D). There are limitations in generalising findings from such samples to the general population. In particular, it is difficult to generalise the findings derived from the laboratory setting to field studies (Dimsdale, 1984).

The second population consists of those who are exposed to what may be considered to be a more severe psychological stressors such as bereavement, marital or employment problems, life-threatening illnesses (or the threat of illness) and hospitalisation. A greater proportion of the population is represented in studies of chronic stress and immunity. Approximately two thirds of these subjects are under 50 average years of age, and about one third are over 50 years mean age (Appendix B). As in the depression research, quite a few studies suffer from small sample sizes.

(iv) Lack of standardisation in the stressors used and psychological measures

Although standardised psychological tests are frequently used in PNI research, few studies use the same tests which makes it difficult to compare studies in relation to the severity of the emotional disturbance. The situation is further complicated by the use of idiosyncratic assessment methods without providing information regarding the reliability or validity of the measures used.

Another problem relates to life event research in PNI studies. How an individual perceives the stressful event is crucial (Lazarus, 1993). Most studies measure an individual's stress associated with life events based on recommendations by Holmes and Rahe (1967) (eg. the Social Readjustment Rating Scale). Such scales may be of limited use because they assign the same value to an event whether the subject perceives it as good or bad (J. J. Cohen, 1987b).

(v) Methodological limitations of PNI tests

A primary concern is the methodological limitations of the current tests commonly used in psychoneuroimmunology in humans. The following section presents a critique of these measures.

1.7 A critique of the current tests commonly used in psychoneuroimmunology in clinical depression, and non-clinical stressors in humans.

1.7.1 Enumeration studies

Enumeration studies are measured by flow cytometry or FACS. The florescent activated cell sorter (FACS) measures by florescence different cell populations in the blood such as T and B cells, macrophages, CD4 helper cells, CD8 suppressor cells and many others.

The significance and interpretation of the numbers of lymphocyte sub-populations is controversial. The prevailing hypothesis is that major depression, particularly melancholia is characterised by a) leukocytosis, an increase in the percentage or number of white blood cells b) neutrophilia, an increase in the number of neutrophils c) monocytosis, an increase of monocytes d) an increased T helper / T suppressor ratio e) increased proportion of activated T cells in the blood [eg. CD25+ (IL-2R) and HLA-DR+ T cells]. The thirty two studies cited in this review which included enumeration measures in major depression are summarised in Table 1.3.

Results are highly inconsistent. Eleven studies show evidence of leukocytosis, while 6 studies show no elevation of wbc count. Nine studies show evidence of neutrophilia, while 5 studies show no changes in the number of neutrophils. 4 studies showed evidence of monocytosis, while 6 studies find no changes in monocytes, and 1 study shows a decrease in monocytes. Only 3 studies show an increased T helper / T suppressor ratio, 9 studies show no changes, and 3 studies show a decreased ratio. The 3 studies that have assessed the proportion of interleukin-2 receptors [CD25+ , (IL-2R)] show an increase. Of the 4 studies that have assessed the proportion of activated T cells in the blood (HLA-DR+), 3 studies show an increase of HLA-DR+ and 1 study shows no changes in HLA-DR+.

The severity of the depression does not appear to account for the discrepancy in results (Appendix A). The studies by Maes and colleagues (eg. Maes et al., 1990; Maes et al., 1992a; Maes et al., 1992b; Maes et al., 1994a; Maes et al., 1994b) which included severely

depressed melancholic patients with HDRS of greater than 25 generally show evidence of leukocytosis, neutrophilia, and monocytosis, an increased T helper / T suppressor ratio and increased proportion of activated T cells in the blood [eg. CD25+ (IL-2R); HLA-DR+ Tcells]. Nevertheless other studies that have included severely depressed patients show no changes (eg. Calabrese, et al., 1986; Schleifer et al, 1989). Similar discrepancies are found in enumeration studies of chronic and acute stressors as summarised on Tables 1.4, 1.5, and 1.6.

There are also difficulties in interpreting what the reported differences between non-stressed healthy controls and depressed patients in enumeration measures actually means. It is often assumed that a reduced number of lymphocytes reflects reduced immunocompetence. However, the number of cells gives no indication of degree of lymphocyte differentiation, i.e. the level of functioning of the cells. A further complication is the possibility that alterations in cell numbers may reflect redistribution of cells to (or from) lymphoid organs, rather than reduced immune competence per se (J. J. Cohen, 1987a; J. J. Cohen, 1987b; O'Leary, 1990).

Abbreviations for Tables 1.3, 1.4, 1.5 and 1.6	
wbc	White blood cell count
neut	Neutrophil count
mono	Monocytes
lymph no	Lymphocyte number
CD3	Total T cell
CD4	T helper cells
CD8	T suppressor cells
NK cells	Natural killer cells
CD4/CD8 ratio	T helper/T suppressor cell ratio
CD25+9 (IL-2R)	Activated T cells
HLADR+	Activated T cells
↑	Indicates a significant increase in number or percentage relative to a control group
↓	Indicates a significant decrease in number or percentage relative to a control group
→	Indicates no difference in number or percentage relative to a control group

Table 1.3: Summary of studies of enumeration and major depression

Study	wbc count	neut	mono	lymph no	Total T cells	CD4	CD8	CD4/ CD8 ratio	B cells	NK cells	CD25+ (IL-2R)	HLA-DR+
Albrecht et al. (1985)	→	→		→								
Andreoli et al. (1993)				→	→	→	→	→	→	↓		→
Anesi et al. (1994)	→	→	→		→	→	→	→				
Calabrese et al. (1986)					→	→	→	→				
Darko et al. (1986)					→				→			
Darko et al. (1988a)					→	→	→	↑	→	→		
Darko et al. (1988b)					→				→	→		
Darko et al. (1988c)				→	→	→	→	↑	→	→		
Darko et al. (1988d)	↑	↑	→	→								
Evans et al. (1988)	→			→	→				→	↓		
Evans et al. (1992)										↓		
Kronfol & House (1984)	↑					→	→	→				
Kronfol & House (1989)	↑	↑	↓	↓								
Krueger et al. (1984)					↓	↓	→	↓	→	→		
Levy et al. (1991)						→	→					
Irwin et al. (1987)	↑	↑	→	→								
Caldwell et al. (1991)	↑	↑	→	↑								
Maes et al. (1990)	↑			→		↑	→	→			↑	
Maes et al. (1992a)	↑		↑	↑	↑	→	→	→	↑			
Maes et al. (1992b)	↑	↑	↑	→								
Maes et al. (1992c)												↑
Maes et al. (1993)	↑	↑	→	→							↑	↑
Maes et al. (1994a)	↑	↑	↑	↓						→	↑	↑
Maes et al. (1994b)	↑	↑	↑	↑						→		
Schleifer et al. (1984)	→	→		↓	↓				↓			
Schleifer et al. (1985)	→	→		→	↓							
Schleifer et al. (1989)	→	→	→	→	→	→	→	→	→			
Syvalahti et al. (1985)					→	→	→	→				
Targum et al. (1989)		↑		↓	↓	↓	→	↓				
Tondo et al. (1988)					→	↑	↓	↑				
Wilson et al. (1990)						→	↓	↓				
Zisook et al. (1994)						→	↓	→				

Table 1.4: Summary of studies of enumeration and chronic stress

Study	Type of stressor	wbc count	neut	mono	lymph no	Total T cells	CD4	CD8	CD4/ CD8 ratio	B cells	NK cells
Bartrop et al. (1977)	Bereavement					→				→	
Spratt & Denney (1991)	Bereavement	→		→		→	↑	↓	↑	→	
Zisook et al. (1994)	Bereavement				→		→	→	→		
Schleifer et al. (1983)	Anticipatory bereavement				→	→				→	
Arnetz et al. (1987)	Unemployment			→	→	→	→	→	→	→	→
Kiecolt-Glaser et al. (1987a)	Divorced /separated vs married						↓	→	→		↓
Kiecolt-Glaser et al. (1988)	Divorced /separated vs married					↓	↓	→	↓		→
Kiecolt-Glaser et al. (1987b)	Alzheimer caregivers					↓	↓	→	↓		
Kiecolt-Glaser et al. (1991)	Alzheimer care-givers					→	→	→	→	→	→
Schaeffer et al. (1985)	Three Mile Island					↓	↓	↓		↓	
Jamner et al. (1989)	Anxiety/depression			↓							
McKinnon et al. (1989)	Three Mile Island	→	↑	→	→	→	→	↓			
Irwin et al. (1986)	Stressful life events				→		→	→	→		
Irwin et al. (1990)	Stressful life events	→	→	→	→						
Kemeny et al. (1989)	Stressful life events					↓	↓				

Table 1.5: Summary of studies of enumeration and acute stress

Study	Type of Stressor	wbc count	neut	mono	lymph no	Total T cells	CD4	CD8	CD4/ CD8 ratio	B cells	NK cells	CD25+ (IL-2R)	HLA-DR+
Fischer et al. (1972)	Space flight				→								
Taylor et al. (1986)	Space flight			↓	↓	↓	↑	→	↑	↓			
Schedlowski et al. (1995)	Parachuting stress			↑	↑	↑	↑				↑		
Dorian et al. (1982)	Academic stress	↑		↑	↑					↑			
Baker et al. (1984)	Academic stress						↑						
Kiecolt-Glaser et al. (1985)	Academic stress				↓	↓	↓	↓	→				
Kiecolt-Glaser et al. (1986)	Academic stress						↓	→	↓				
Halversen & Vassend (1987)	Academic stress			↑			→	→	→			↓	
Baker et al. (1985)	State anxiety	→			→		↑	→					
Ironson et al. (1990)	Disease notification						→				→		

Table 1.6: Enumerative studies and acute laboratory stress

Study	Group/condition	wbc count	mono	lymph no	Total T cells	CD4	CD8	CD4/ CD8 ratio	B cells	NK cells	CD25+ (IL-2R)	HLA-DR+
Landmann et al. (1984)	Stress group	→	↑		→	→	→		↑	↑		
Manuck et al. (1981)	High reactors vs. low reactors vs. no stress controls					→	↑		→			
Bachen et al. (1992)	Stress group vs. controls				→	↓			→	↑		
Herbert et al. (1994)	Stress group vs. controls				→	→	↑		→	↑		
Naliboff et al. (1991)	a) Young				→	→	↑		→	↑		
	b) Old				→	→	↑		→	↑		
Brosshot et al. (1992)	Stress group vs. controls	→	→		→	→	↑			↑		→
Weisse et al. (1990)	Controllable vs. uncontrollable shock		↓		→	→	→		↓			
Sieber et al. (1992)	Controllable vs. uncontrollable noise				→	→	→		→	→	→	
Knapp et al. (1992)	Experimental group: recall of negative and positive experiences		→	→	→	→	→					
Kiecolt-Glaser et al. (1993)	Negative behaviour during marital conflict in laboratory context		↑		↑	↑	→	↑				

1.7.2 Lectin or mitogenic responses

Mitogenic assays as the name implies, assess how well lymphocytes divide or proliferate. In the presence of lectins which behave like non-specific antigens or foreign bodies commonly used lectins, phytohemagglutinin (PHA), concavalin A (con A), and pokeweed mitogen (PWM) stimulate lymphocyte cell division which is measured by radioactive thymidine uptake into DNA. The basic assumption is that the higher the degree of proliferation, or thymidine uptake into DNA, then the higher the level of immune competence.

The 20 studies cited in this review which included lectin response measures in major depression are summarised in Table 1.7. As with enumerative measures, studies employing lectin responses in major depression are highly inconsistent. The prevalent hypothesis is that people who are clinically depressed will have lower lectin responses relative to healthy controls. Evidence for this hypothesis is far from convincing. Seven studies of lectin responses in major depression show a decrease in PHA, 11 studies show no changes, and 1 study even shows an increase in PHA. Nine studies show a decrease in Con A, while 9 studies show no changes in Con A. Six studies show a decrease in PWM, while 8 studies show no changes. Severity of depression does not appear to account for the findings. Some investigators have found lower lectin responses in patients with scores of greater than 25 on the HDRS (e.g. Schleiffer et al., 1984), while other studies which have included severely depressed subjects show no changes in lectin response (e.g. Krueger et al., 1984; Schleiffer et al., 1989). The relevance of dose response curves is discussed later. Similar discrepancies are found in enumeration studies of chronic stressors and acute stressors as summarised in Tables 1.8 and 1.9 respectively.

Abbreviations for Tables 1.7, 1.8, and 1.9	
PHA	Phytohemagglutin
Con A	Concanavalin A
PWM	Poke weed mitogen
↑	Indicates a significant increase in lectin response of the experimental group relative to the control group
↓	Indicates a significant decrease lectin response of the experimental group relative to the control group
→	Indicates no difference in lectin response of the experimental group relative to the control group

Table 1.7: Summary of studies of lectin responses and major depression

Study	Dose Response Curve (DRC)	PHA	Con A	PWM
Albrecht et al. (1985)	NO	→	→	→
Alshuler et al. (1989)	NO	↑		
Andreoli et al. (1993)	YES	→	→	→
Anesi et al. (1994)	YES	↓		
Calabrese et al. (1986)	NO	↓	↓	↓
Darko et al. (1986)	YES	→	↓	→
Darko et al. (1988a)	YES	→	→	
Darko et al. (1988b)	YES	→	→	
Darko et al. (1989)	YES	→	→	
Kronfol et al. (1983)	NO	↓	↓	↓
Kronfol & House (1984)	NO	↓	↓	↓
Kronfol et al. (1986)	NO	↓	↓	↓
Kronfol & House (1989)	NO	→	↓	↓
Krueger et al. (1984)	NO	→	→	→
Levy et al. (1991)	NO	→	↓	→
Schleifer et al. (1984)	YES	↓	↓	↓
Schleifer et al. (1985)	YES	→	→	→
Schleifer et al. (1989)	YES	→	→	→
Syvalahti et al. (1985)	NO	↓	→	→
Zisook et al. (1994)	YES		↓	

Table 1.8: Summary of studies of lectin responses and chronic stress

Study	Stressor	PHA	CON A	PWM
Bartrop et al. (1977)	Bereavement	↓	↓	
Arnetz et al. (1987)	Unemployment	↓		
Kiecolt-Glaser et al. (1984)	Loneliness	↓		
Kiecolt-Glaser et al. (1987a)	Divorced/separated women	↓		
Kiecolt-Glaser et al. (1988)	Divorced/separated men	↓	→	
Kiecolt-Glaser et al. (1991)	Alzheimer spousal caregivers	↓	↓	
Schleiffer et al. (1983)	Anticipatory bereavement	↓	↓	↓
Spratt & Denney (1991)	Bereavement	→		
Zisook et al. (1994)	Bereavement		→	
Linn et al. (1984)	Bereavement/ family illness	↓	→	→

Table 1.9: Summary of studies of lectin responses and acute stress

Study	Stressor	PHA	CON A	PWM
Manuck et al. (1981)	Laboratory stressor	↓		
Bachen et al. (1992)	Laboratory stressor	↓		
Herbert et al. (1994)	Laboratory stressor	↓		
Brosshot et al. (1992)	Laboratory stressor	→		→
Weisse et al. (1990)	Laboratory stressor	↓	↓	
Sieber et al. (1992)	Laboratory stressor	↓	↓	
Knapp et al. (1992)	Laboratory stressor	↓	↓	
Zakowski et al. (1992)	Laboratory stressor	→	↓	
Futterman et al. (1994)	Laboratory setting			
	Positive mood	↑		
	Negative mood	↓		
Fischer et al. (1972)	Space flight	→		
Taylor et al. (1986)	Space flight	↓		
Dorian et al. (1982)	Academic stress	↓	↓	
Halvorsen & Vassend (1987)	Academic stress	→		
Linn et al. (1981)	Elective hospitalisation	↓	↓	→
Schleiffer et al. (1985)	Elective hospitalisation	→	→	→
Linn & Linn (1987)	Pre-surgery stress	↓	→	↓
Tonnesen et al. (1987)	Pre-surgery stress	↓		
Palmblad et al. (1979)	Sleep deprivation	↓		
Ironson et al. (1990)	Disease notification			
	seropositive status	→		→
	seronegative status	↓		↓

Kiecolt-Glaser and colleagues (1992) in their recent review suggest that immunological changes that are reported following short term laboratory stressors are similar to changes that have been described after epinephrine injections: increased percentage of NK cells, decreased lectin responses, and decreased percentages of CD4 cells (Kiecolt-Glaser, Cacioppo, Malarkey, & Glaser, 1992). Immunological changes after exposure to a short term laboratory stressor are more consistent than those found in relation to depression or chronic stressors (Table 1.9). Six studies find a reduction in PHA responses following an acute laboratory stressor, while 2 studies show no changes. Four out of 4 studies found a reduction in Con A responses. Six studies found an increase of NK cells, 1 study shows no changes (Table 1.5). In contrast, only 1 study out of 9 shows a reduction in CD4 (Table 1.5). Changes may partially reflect transient alterations in lymphocyte migration, and the release of epinephrine during stress provides one mechanism to explain short term immunological changes (Kiecolt-Glaser et al., 1992). Other possible mechanisms by which the central nervous system may influence lymphocyte migration are reviewed in Ottaway & Husband (1992). Immunological measures after acute laboratory stressors are volatile and may be affected by transient changes in blood pressure, heart rate, and subtle changes in physical movement (Futterman et al., 1994; Kiecolt-Glaser et al., 1992). This may be of little clinical significance since these transitory changes in healthy subjects returned rapidly to baseline.

In summary, lectin assays are too variable to be a sensitive index of immune suppression during psychological stress. Several confounding factors complicate the interpretation of this measure. 1) There is a lack of standardisation in measurement. What concentration of the lectin does one use? Stein et al. (1991) recommend the use of a dose response curve with 3 or more points to minimise interassay variability, rather than a single optimal dose. Given the hyper-variability of lectin responses, it is questionable that a dose response curve yields considerably more valuable information than a single measure, and it has the disadvantage of being extremely labour intensive (Table 1.7). The use of different

medium supplementation for lectin assays such as fetal bovine serum, autologous plasma, or horse serum, yield different results (Darko, Wilson, Gillin, & Golshan, 1991); 2) Results vary depending on the time of day they are measured (Darko et al.,1991; Stein et al.,1991); 3) The measure lacks concurrent validity as it does not correlate well with other measures of immune function (Darko et al.,1991); 4) Low levels of lectin response does not correlate with increased incidence of infection, except in extremely severe illnesses such as AIDS (Darko et al.,1991); 5) This assay is cumbersome as it requires culturing under germ free conditions. In conclusion, it is likely that changes can occur to the cells under these conditions which relate more to the conditions of culturing than to a disease process (J. J. Cohen, 1987a; Darko et al.,1991; Stein et al.,1991).

1.7.3 Natural killer cell activity

The 18 studies in this review which included NKCA measures in major depression are summarised in Table 1.10.

Results are inconsistent. Thirteen studies showed a reduction of NKCA during major depression, relative to 5 studies that showed no changes. Studies examining chronic stressors and NKCA activity are summarised in Table 1.11.

Stressful life events such as bereavement are not consistently related to lower NKCA, although some studies report a reduction of NKCA correlates negatively with high depression scores during stressful life events. Moreover, social support and personality factors such as an internal locus of control correlate with an increase in NKCA in some studies (e.g. Baron et al., 1990; Jemmott et al.,1983; Jemmott & Magloire, 1988; . Levy et al., 1990; Reynaert et al., 1995).

Thirteen studies in this review included NKCA measures during acute stressors and are summarised in Table 1.12. Results are inconsistent. Even during tightly controlled laboratory conditions, 2 studies show an increase, 1 study shows a decrease, and 1 study shows no changes. The Kiecolt-Glaser group tend to find a decrease in NKCA with academic stress, but results of patients awaiting surgery or disease notification results, are inconsistent. Moss and colleagues (Moss, Moss, & Peterson, 1989) found no association between mood ratings and NKCA. Moreover some individuals in this study showed marked variability in NKCA on a week to week basis. This has led Moss to suggest that cross-sectional studies may not be suitable for studying psychosocial factors and NKCA.

Table 1.10: Summary of studies of NKCA and major depression

Study	Dose Response Curve	NKCA
Andreoli et al. (1993)	YES	↓
Caldwell et al. (1991)	YES	↓
Darko et al. (1992)	YES	↓
Irwin et al. (1987)	YES	↓
Irwin & Gillin (1987)	YES	↓
Irwin et al. (1990)	YES	↓
Evans et al. (1992)	YES	↓
Kronfol et al. (1989)	YES	↓
Levy et al. (1991)	YES	→
Maes et al. (1992d)	NO	↓
Maes et al. (1994a)	NO	↓
Miller et al. (1991)	YES	→
Mohl et al. (1987)	YES	→
Nerozzi et al. (1989)	YES	↓
Schleifer et al. (1989)	YES	→
Shain et al. (1991)	YES	→
Urch et al. (1988)	YES	↓
Zisook et al. (1994)	YES	↓

NKCA=Natural killer cell activity, ↑ indicates a significant increase, ↓ decrease, or → no change in NKCA relative to the control group.

Table 1.11: Summary of studies of NKCA and chronic stress

Study	Type of stressor	NKCA
Irwin et al. (1986)	Bereavement / anticipatory bereavement	→
	Bereavement with high depression scores	↓
Irwin et al. (1987)	Study 1: Bereavement with high depression scores	↓
	Study 2: Life events with high depression scores	↓
Irwin et al. (1988)	Bereavement and anticipatory bereavement	↓
Zisook et al. (1994)	Bereavement	→
Irwin et al. (1990)	Severe life stress	↓
	Major depression	↓
Irwin et al. (1991)	Alzheimer spousal caregivers /	→
	High depression scores	↓
Locke et al. (1984)	Life change stress	→
	High depression scores	↓
Kiecolt-Glaser et al. (1984b)	Loneliness	↓
	Stressful life events	→
Esterling et al. (1994)	Alzheimer caregivers	↓
	Former alzheimer caregivers	↓

NKCA=Natural killer cell activity, ↑ indicates a significant increase, ↓ decrease, or → no change in NKCA relative to the control group.

Table 1.12: Summary of studies of NKCA and acute stress

STUDY	Type of stressor	NKCA
Naliboff et al. (1991)	Laboratory stressor	↑
Sieber et al. (1992)	Laboratory stressor	↓
Knapp et al. (1992)	Laboratory stressor	→
Futterman et al. (1994)	Laboratory stressor: Induced mood state negative and positive	↑
Schedlowski et al. (1993)	Parachuting stress	
	Immediately after jump	↑
	One hour after jump	↓
Dorian et al. (1982)	Academic stress	→
Glaser et al. (1986a)	Academic stress	↓
Kiecolt-Glaser et al. (1984a)	Academic stress	↓
Kiecolt-Glaser et al. (1986)	Academic stress	↓
Tonnesen et al. (1987)	Pre-operative stress	↑
	Post-operative stress	↓
Ironson et al. (1990)	Disease notification	
	Seropositive status	↓
	Seronegative status	→
Antoni et al. (1990)	Anticipation of AIDS diagnosis	↑
Moss et al. (1989)	Microstressors/mood rating	→

NKCA=Natural killer cell activity, ↑ indicates a significant increase, ↓ decrease, or → no change in NKCA in an experimental group relative to the control group.

1.7.4 Interleukins, enzymes, proteinases, acute phase proteins, DNA expression in major depression

Work on interleukin-2 and interleukin-6 production with lectin treated lymphocytes in culture has shown changes, but only in severely depressed patients with melancholia (Maes et al., 1993). Similarly, decreased levels of dipeptidyl peptidase, a membrane bound enzyme, and serum prolylendopeptidase, a serine proteinase have been found in severely depressed patients (Maes et al., 1991b; Maes et al., 1994c). Also, increased levels of acute phase proteins especially haptoglobin and alpha-1-antichymotrypsin have been reported in severely depressed patients, suggesting a non-specific inflammatory response occurs during major depression (Joyce et al., 1992; Maes et al., 1993).

1.7.5 Antibody (immunoglobulin) measures

Studies examining the relationship between psychological stress, personality characteristics and the levels of antibody in serum are summarised in Table 1.13.

Results are contradictory. In relation to acute stressors such as academic stress 2 studies show an increase in IgA and 1 study shows no change. Similarly, 2 studies show no changes in IgG during academic stress, and 1 study shows an increase. Moreover, 1 study shows an increase in IgM during academic stress, 1 study shows a decrease and 1 study shows no changes.

In relation to chronic stressors such as bereavement and job stress, results are also inconsistent (Table 1.13). Similarly, findings regarding the relationship between personality characteristics such as neurosis, negative mood states such as anxiety and depression, and somatic complaints are also inconsistent (Table 1.13). No changes in IgE are found in any of the studies cited in Table 1.13.

Salivary IgA has also been measured in PNI research because it provides a first line of defence against infection especially of the upper respiratory tract (Jemmott, Alexander & Marks, 1982; Jemmott & Magloire, 1988; O'Leary, 1990). The relationship between psychological stress, personality characteristics and salivary IgA is summarised in Table 1.14.

This relationship is controversial. Disagreement exists regarding which is the best method to assess IgA (see Graham et al., 1988; Jemmott et al., 1983; Jemmott et al., 1988; Jemmott & Magloire, 1988; Stone, Cox, Valdimarsdottir, & Neale, 1987). One study reported an increase in salivary IgA during acute academic stress, one study found no change, and one found an increase. Studies examining the relationship between negative mood states such as anxiety, psychological distress and salivary IgA are also inconsistent. Two studies examine the effect of daily hassles on salivary IgA and find no changes. Of the 4 studies examining chronic stressors such as stressful life events and living on Three Mile Island 3 studies report an associated decrease and 1 study found no change. McClelland and colleagues have found that personality characteristics such as a need for power when associated with a threat to the individuals capacity to perform powerfully or impress others is correlated with lower levels of salivary IgA (Table 1.14).

A more promising measure is that of antibody titres to herpes simplex virus, epstein barr virus and cytomegalovirus. Higher antibody titres are interpreted by Glaser and colleagues to indicate poorer control of the latent virus by the immune system, although this interpretation may be disputed. It is conceivable that higher antibody titres may be due to an overall activation of the humoral response (McKinnon, Weisse, Reynolds, Bowles, & Baum, 1989).

Table 1.15 summarises the studies conducted investigating the relationship between psychological stress and antibody titres. Results are relatively consistent across studies. This appears to be a promising measure, but awaits further replication by other groups.

Table 1.13: Summary of studies of psychological stress, personality characteristics and serum antibody (immunoglobulin) levels

Study	Type of stressor	IgA	IgG	IgM	Ig E
Vassend & Halvorsen (1987)	Academic stress	→	→	↓	→
Kiecolt-Glaser et al. (1984)	Academic stress	↑	→	→	
Glaser et al. (1986b)	Academic stress	↑	↑	↑	
Vassend & Halvorsen (1987)	High neuroticism	→	→	↑	→
	High social alienation	→	→	↑	→
	High anxiety / tension	→	→	↑	→
	High depressive mood	→	→	↑	→
	High bodily complaints	→	→	↑	→
Spittle & Sears (1984)	Personality characteristics and psychiatric symptoms in adult asthmatics				→
Linn et al. (1981)	High anxiety in seriously ill patients	→	→	→	
Pettingale et al. (1977)	Anger suppression	↑	→	→	
Ursin et al. (1984)	High neuroticism (Study 1: teacher job stress)	↓	↓	→	
	High psychological defence	→	↓	→	
	Somatic and psychological complaints	↓	→	↓	
	High internal locus of control	↓	→	→	
Bartrop et al. (1977)	Bereavement	→	→	→	
Linn et al. (1984)	Bereavement / Serious family illness	↓	↓	↓	
McKinnon et al. (1989)			→	→	
Ursin et al. (1984)	Study 1: High job stress rating in female teachers (chronic condition)	→	→	↓	
	Study 2: Acute job stress and personality traits in merchant navy students (acute condition)	→	→	→	
Endresen et al. (1987)	Job stress	↑	→	↓	
	High STAI-Trait	→	→	↓	
Theorell (1990)	High perceived job stress		↑		

IgA=Immunoglobulin A, IgG=Immunoglobulin G, IgM=Immunoglobulin M, Ig E= Immunoglobulin E. ↑ indicates a significant increase, ↓ decrease, or → no change in serum immunoglobulin concentration relative to the control group.

Table 1.14: Summary of studies of psychological stress, personality characteristics and salivary IgA

STUDY	TYPE OF STRESSOR	SIgA
Jemmott et al. (1983)	Academic stress	↑
Kiecolt-Glaser et al. (1984)	Academic stress	→
McClelland et al. (1985)	Academic stress	↓
Kubitz et al. (1986)	Daily hassles	→
	Stress symptoms	→
	POMS tension / anxiety	→
	High internal locus of control	↓
	Stress coping rating scale	→
Stone et al. (1987)	High negative mood	↓
	High positive mood	↑
Graham et al. (1988)	High anxiety	↓
	Daily hassles	→
	Stressful life events	→
	Psychological distress	→
McClelland et al. (1980; 1982)	Inhibited Power Motivation	↓
McClelland et al. (1980; 1982)	Stressful life events	↓
McKinnon et al. (1989)	Three Mile Island	↓
Schaeffer et al. (1985)	Three Mile Island	↓

SIgA=salivary Immunoglobulin A, ↑ indicates a significant increase, ↓ decrease, or → no change in salivary Immunoglobulin A concentration relative to the control group.

Table 1.15: Summary of studies of psychological stress and antibody titres

Study	Type of stressor	EBV	HSV	CMV
Glaser et al. (1985)	Academic stress	↑	↑	↑
	High Loneliness vs. Low loneiless	↑	↑	↑
Glaser et al. (1993)	Academic stress	↑		
Fittchen et al. (1990)	Academic stress		→	
	Perceived anxiety		↑	
Esterling et al. (1994)	High anxiety	↑		
	High defensiveness	↑		
Kiecolt-Glaser et al. (1984)	Loneliness	↑		
	Stressful life events	↑		
Kiecolt-Glaser et al. (1987a)	Marital quality	↑		
	Separation	↑		
	Divorce	↑		
Kiecolt-Glaser et al. (1987b)	Alzheimer caregivers	↑		
Kiecolt-Glaser et al. (1991)	Alzheimer caregivers	↑		
	Depression	↑		
Kiecolt-Glaser et al. (1993)	Negative behaviour during marital conflict	↑		
McKinnon et al. (1989)	Three Mile Island	↑		

EBV=epstein barr virus, HSV=herpes simplex virus, CMV=cytomegalovirus, ↑ indicates a significant increase, ↓ decrease, or → no change in antibody titre relative to the control group.

1.8 The effects of stress on humoral and cell-mediated immunity in animals

It is beyond the scope of this thesis to review in detail the effects of stress on humoral and cell-mediated immunity in animals. This topic has been extensively reviewed (see Ader & Cohen, 1993; Dantzer & Mormede, 1995; Koolhaas & Bohus, 1995; Weiss & Sundar, 1992). Briefly, experimental stressors have included electric shock, physical restraint, forced exercise, exposure to loud noises and cold temperatures, and social stress models such as dominance-submission relationships and separation studies. Of these stressors electric shock is the most commonly used because it has the advantage that the timing, duration and frequency of the stressor can be controlled by the experimenter (Dantzer & Mormede, 1995). An animal model of depression that provides a standardised model of studying the effects of stress on immunity is that of Seligman's learned helplessness model, discussed below (Seligman 1975).

1.8.1 The learned helplessness model of depression in animals

The phenomenon of learned helplessness refers to the interference in learning found in animals after exposure to uncontrollable, aversive events (Overmier & Seligman, 1967; Seligman & Maier, 1967). It was developed into a model of reactive depression because of observed similarities between learned helplessness and depressive symptoms such as learning deficits, slowed responding, and passivity (Seligman 1975; Miller, Rosellini & Seligman, 1986). Learned helplessness theory predicts that animals exposed to uncontrollable conditions (i.e. inescapable shock, IE) would have lower immunity than those who could control (i.e.escape, E) the shock.

Preliminary studies of learned helplessness and immunity in animals which employ commonly used measures in psychoneuroimmunology are inconsistent. For example, in accordance with learned helplessness theory, some investigators have found that inescapable or unsignalled shock suppressed lectin responses in rats (Laudenslager, Ryan, Drugan, Hyson, & Maier, 1983; Mormede, Dantzer, Michaud, Kelley, & Le Moal, 1988). However, Maier and Laudenslager were unable to replicate these findings (Maier & Laudenslager, 1988). A similar

inconsistency is evident using natural killer cells as a measure of immunocompetence. In support of learned helplessness theory Shavit and colleagues found that inescapable but not escapable shock suppressed splenic NKCA in rats (Shavit et al., 1983). Other studies, however do not support the theory of learned helplessness. For example, Irwin & Custeau (1989) found that signalled (i.e. predictable) electric shock resulted in significantly more suppression of NKCA than unsignalled stimuli.

Measurement of antibody responses also yields inconsistent findings. Consistent with learned helplessness theory some investigators report that primary and secondary antibody responses are suppressed if animals are given inescapable shock or are unable to avoid the shock (eg. Laudenslager et al., 1988; Mormède et al., 1988). Contrary to learned helplessness theory, however, Zalcman and colleagues found no differences between mice who could escape the shock and those who could not (Zalcman, Minkiewicz-Janda, Richter, & Anisman, 1988). Similar contrasting results are evident in studies of learned helplessness and immunity in humans (Sieber et al.,1992; Weisse et al., 1990).

1.9 Summary

In summary, current measures used in PNI in human and animal studies have shown only minor changes with psychological stress and depression, leading to disagreement as to the significance of the changes by various groups (Weisse, 1992; Ader, & Cohen, 1993). In addition, these parameters are difficult to measure and also volatile since they may be affected by a host of factors including diurnal variation, modest sleep disturbances, physical activity, nutritional status, transient mood changes, smoking, medications, caffeine intake, alcohol or drug use in humans (Brahmi et al., 1985; Kanonchoff et al., 1984; Kiecolt-Glaser, 1988a; Kiecolt-Glaser & Glaser, 1992; Hersey, Prendergast, & Dwards, 1983; Hickson & Boone, 1993; Irwin, et al., 1994; Irwin, Smith & Gillin, 1992; Knapp et al., 1992; Pederson, 1991; Weisse, 1992).

CHAPTER 2

LYMPHOCYTIC 5'-ECTONUCLEOTIDASE: A MARKER OF STRESS-INDUCED IMMUNE SUPPRESSION IN HUMANS[2]

2.1 Introduction: Rationale for selecting lymphocytic 5'-ectonucleotidase

The primary aim of the experiments reported in this Chapter is to obtain a sensitive yet stable measure of immune suppression during psychological distress in a human model. The ideal parameter should be analytically and biologically stable, easy to measure, have significant changes associated with stress, and have a function related to immunity. One possibility is 5'-ectonucleotidase (NT), an ectoenzyme which is located on the external surface of the cell membrane. This enzyme converts monoribonucleotides to ribonucleosides and therefore one of its roles may be to supply nucleosides to the cell as nucleotides do not cross the external cell membrane readily. The most persuasive evidence pointing to the use of NT as a marker for stress induced immune suppression is the report of a 50 % reduction of NT activity in recently diagnosed yet otherwise healthy HIV-positive patients who were not immunocomprised (Chalmers, Hare, Wooley, & Frazer, 1990). Since there is considerable psychological stress associated with a positive HIV status (Ironson et al., 1990; Antoni et al., 1990) it is possible that the low levels of this enzyme may be influenced by stress.

NT has been shown to be present on most human lymphocytes in approximately the following distribution: 32% of CD3+, 19% of CD4+, 50% of CD8+ and 81% of peripheral B lymphoctyes (Edwards, Gelfand, Burk, Dosh, & Fox, 1979). B cell activity is about 3 times higher than T cell activity (Murray et al., 1984). More importantly, NT has a significant role in both T and B lymphocyte differentiation since adult peripheral B cells have about 10 times higher NT activity than neonate B cells, and mature peripheral blood T cells have about 5 times higher NT activity than thymocytes (Edwards et al., 1979; Thompson, Ruedi, O'Connor, & Bastian, 1986). Also infants born with persistently low NT levels are prone to many infections which

[2] The experimental work cited in this Chapter has been accepted for publication in the International Journal of Stress Management.

resolve when their NT levels return to normal (Bastian et al., 1984). Both acquired and congenitally low levels of this enzyme are associated with lower clinical immunity (Thompson, Ruedi, Low, & Clement, 1987). Clinical immunodeficiencies such as common variable and X-linked gammaglobulinaemia, acquired immunodeficiency syndrome (AIDS), and numerous immunodeficiencies of the neonate are associated with reduced NT activities (Bastian, et al., 1984; Shah et al., 1983).

Although the role of ectoenzymes in cellular function is poorly defined they are thought to play a role in cell protection and nutrient absorption among other functions (reviewed by Stanley, Newby, & Luzio, 1982). NT, in addition to its enzyme function, has been shown to be important in regulating T cell activation (Massaia et al., 1991), and lymphoid cell differentiation already outlined.

To summarise, NT was selected as a possible marker of stress-induced immune suppression because of its role in lymphocyte differentiation, and its association with both acquired and congenital immune deficiencies. Also, NT can easily be measured using an automated spectrophotometric method (Chalmers & Hare, 1990), thereby avoiding complex procedures such as cell culturing or use of radioisotopes which can complicate data interpretation.

In this Chapter, two experiments in humans are described, one involving non-clinical groups (Experiment 1) and the other involving clinical and non-clinical control groups (Experiment 2). The studies are described separately, and the implications of both studies are then outlined in a joint discussion section.

2.2 Experiment 1

Experiment 1 was conducted in order to test whether NT is a sensitive measure of immune suppression during psychological stress in a non-clinical population. It was predicted that NT would decrease significantly during times of psychological distress because low NT is associated with impaired immunity, as is also psychological stress. Psychological distress was measured by the Profile of Mood States (McNair, Lorr, & Drappelman, 1992). Dispositional or general

proneness to anxiety was measured by the State Trait Anxiety Inventory (STAI-trait) (Spielberger, Gorsuch, Lushene, Vagg, & Jacobs,1983).

2.2.1 Method

(i) Subjects

This study used 49 subjects from three non-clinical populations in the Adelaide metropolitan area. See Table 2.1 for demographic details, and Table 2.2 and Table 2.3, respectively, for mean test scores of POMS and STAI-trait at Time 1 for all groups at the commencement of the study. The three samples were as follows.

1. Honours students: 21 students undertaking an honours psychology course were recruited from The University of Adelaide. At the beginning of the study they had a mean score and standard deviation (SD) of 40.62 (10.22) on the STAI-trait. The honours course is a most demanding academic challenge for students. It encompasses a research / thesis component, formal course work and related end of year examination period (November). It was assumed that the stress levels would vary depending on the time of the year.

2. Low stress controls: Academic staff, general staff members and post-graduate students were recruited from the University of Adelaide. This group consisted of 10 subjects with a mean score (SD) of 33.60 (8.77) on the STAI-trait, and mean scores of less than 5 on the the Tension-Anxiety (T) and Depression-Dejection (D) scales of the (POMS). These mean scores were approximately half a standard deviation below those of the standardisation samples (McNair et al., 1992; Spielberger et al.,1983).

3. High stress group: 18 of the subjects, originally intended for the low stress control group, experienced stress during the course of the study and were reassigned to a high stress group. This group had a mean score (SD) of 35.22 (8.17) on the STAI-trait at Time 1. The criteria for inclusion in this group were as follows. The first criterion was a mean score of 20 or more on either the T or D scales of the POMS in any of the three testing times during the year. This criterion was selected because it is approximately one standard deviation above that of the

standardisation sample (McNair et al., 1992). The second criterion required for inclusion into this group was that subjects report currently experiencing "extremely" stressful events that had persisted for one month or longer. The high stress period was always defined as Time 2 for all subjects regardless of when the stress occurred. Thus, the first low stress period was defined chronologically as Time 1 and the subsequent or following second low stress period as Time 3. Seven subjects experienced such stress at Time 1. Eleven subjects experienced stress at Time 2. Their scores on the POMS done at each assessment time confirmed their stress status at the time of measurement. The types of stress experienced by the members of this group included bereavements, the diagnosis (or possible diagnosis) of serious illness, and assault, or the threat of assault (i.e. stalking).

(ii) Materials

Apparatus for the NT assay is described in Chalmers & Hare (1990).

The following psychological tests were administered: the State Trait Anxiety Inventory-trait (STAI-trait), a measure of dispositional or trait anxiety (Spielberger et al., 1983), and the POMS, a measure of mood disturbance (McNair et al., 1992).

(iii) Procedures

Data collection for both studies occurred from March 1994 to December 1994. All psychological and demographic assessments were done and analysed by clinical psychologist J. S. Blake-Mortimer for both Experiment 1 and Experiment 2. Biochemical and immunological assays were done by Dr. A. H. Chalmers, Department of Haematology, Flinders Medical Centre. The study was done as a blind trial in that the analyst Dr. Chalmers was unable to identify which patient was in each group. That is, which subject was an honours student, a high stress subject, or a low stress control. All subjects were healthy and free of acute infections for at least two weeks prior to biochemical and psychological assessments. During the course of this study, all participants also had their complete blood picture (CBP) assessed by a Technicon H2 analyzer by the Haematology Department at Flinders Medical Centre and were found not to be anaemic or to

have abnormal blood cell profiles. Potential subjects were asked their age, sex, number of years of school and tertiary education completed, smoking habits, alcohol consumption[3], any other recent drug intake, and given the psychological tests specified above for stress evaluations. None of the subjects in this study were taking immunosuppressive or psychotropic drugs.

Assessments were done at the time of blood collection for all groups. Assessments of honours students, the high stress group and low stress controls were taken three times during the year from March 1994 to December 1994. Honours students were assessed at the initial phase of their course (low stress), shortly before the examinations and thesis submission (high stress) and after the examinations (low stress). Thus all honours students experienced their low and high stress at about the same time due to the structure of the honours year. The low stress controls were assessed at the same time of year as the honours students, to control for any seasonal variation in NT activities. The high stress group was assessed at the same time as the other groups as specified above. The STAI-trait was administered once, at the initial assessment to assess each person's dispositional anxiety level. The POMS was given at each assessment. At each assessment time subjects were also asked 1) "Have you experienced stressful events in the past month?" (or "since the last assessment?"), 2) "Are you currently experiencing events that you feel are extremely stressful?", and if so, "Has this stressor continued for a period of one month or longer?" They were required to specify the type of stressor. If subjects were currently experiencing a major stressor which had continued for a duration of at least a month and their scores on the POMS reached the criterion specified above they were placed in a high stress group.

All blood specimens were processed within four hours of collection. NT was stable in blood maintained at room temperature (23^0 C) for up to 24 hours after collection. As indicated the study was done as a blind trial with the analyst unaware of the subject's grouping.

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[3] Alcohol consumption was assessed by the method of Armor (1978).

2.2.2 Results

(i) Demographic data

Table 2.1 shows the demographic data for the 49 subjects in this study. All groups were compared to each other in relation to male/female and smoker/non-smoker ratios using the Fisher Exact test. There were no significant differences between the groups in the male/female: in all groups females predominated (86% overall). The groups did not differ significantly in mean age, $F(2, 46) < 1.00$, or educational status in terms of the numbers of years of education completed, $F(2, 46) = 2.01$, $p > .05$. Also groups did not differ significantly in the amount of alcohol consumed per day (overall mean = .46g) in the past month, $F(2, 46) < 1.00$.

(ii) Psychological variables

Table 2.2 shows the means and standard deviations of the POMS variables for the honours students, the high stress group and the low stress controls at three separate assessment times during the year. Since the design is unbalanced (numbers are unequal in each group) the regression approach (unweighted means) that is recommended by Overall and Spiegel (1969) was used to overcome the problem of nonorthogonality.[4] To protect against the inflation of alpha we have performed two (i.e. k-1) planned contrasts (non-orthogonal) on the Total Mood Disturbance (TMD) scores. At Time 3, scores for two cases were not available, as shown in Table 2.2. A repeated measures analysis of variance (3 groups, tested three times) for the TMD scores resulted in a significant group by time interaction, $F(4, 88) = 5.14$, $p < .001$. This interaction is shown in Figure 2.1. Subsequent analyses of simple main effects revealed that TMD scores for honours and the high stress groups increased significantly during stress, $t(46) = 4.39$, $p < .05$; $t(46) = 6.23$, $p < .001$; respectively, and reduced again after stress, $t(46) = 3.96$, $p < .001$; $t(46) = 5.44$, $p < .001$; respectively. In contrast, the low stress controls were relatively unstressed on each occasion.

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[4] In this thesis all multifactorial ANOVA's with unequal sample sizes use the regression approach (unweighted means) recommended by Overall & Spiegel (1969) to overcome the problem of nonorthogonality.

Significant main effects for both group and time were also found, $F(2, 44) = 15.54$, $p < .001$; $F(2,88) = 20.39$, $p < .001$; respectively. Thus it may be concluded that: (1) the low stress group had significantly lower TMD scores (mean = 6.70), than the honours students (mean = 71.80), and the high stress group (mean = 117.57); (2) TMD scores increased significantly in Time 2 (mean = 48.12), compared to Time 1 (mean = 21.41) and Time 3 (mean = 22.00). This difference was due to the higher TMD scores reported by the honours students and the high stress group. There were significant main effects for time and group for all the POMS factors, except for Anger which showed only a main effect for time. A similar pattern of results to that found for the TMD scores was seen on each subscale as shown in Table 2.2.

Table 2.3 shows the means and standard deviations of the STAI-trait variables for the honours students, the high stress group and the low stress controls at Time 1. A one-way analysis of variance between the honours students, the high stress group and the low stress controls for scores on the STAI-trait (assessed at Time 1) show that the three groups did not differ significantly in trait anxiety, $F(2, 44) = 2.20$, $p > .05$.

Table 2.1
Experiment 1: Demographic data for the three samples

Group		Male /Female ratio	Age		Education (years)		Alcohol Consumption in the past month (grams)		Smoker/Non-smoker ratio
	n		Mean	SD	Mean	SD	Mean	SD	
Honours students	21	3:18	28.90	9.16	15.43	.75	.53	.63	1:20
High stress group	18	2:16	35.72	13.78	13.94	3.02	.34	.34	0:18
Low stress controls	10	2: 8	32.50	11.58	14.73	2.35	.47	.64	1:9

Table 2.2
Experiment 1: Means and standard deviations (in brackets) for the Profile of Mood States (POMS), at three separate times for the three groups

POMS VARIABLES	HONOURS STUDENTS			HIGH STRESS GROUP			LOW STRESS CONTROLS		
	TIME 1 LOW STRESS (n = 21)	TIME 2 HIGH STRESS (n = 21)	TIME 3 POST EXAM (n = 20)	TIME 1 LOW STRESS (n = 18)	TIME 2 HIGH STRESS (n = 18)	TIME 3 POST STRESS (n = 17)	TIME 1 LOW STRESS (n = 10)	TIME 2 LOW STRESS (n = 10)	TIME 3 LOW STRESS (n = 10)
Tension-Anxiety (T)	14.52 (7.73)	22.19 (7.38)	11.25 (6.73)	9.11 (6.02)	21.11 (5.05)	10.65 (6.42)	4.70 (2.83)	4.30 (1.34)	3.90 (1.73)
Depression-Dejection (D)	9.24 (7.47)	16.71 (9.67)	10.25 (8.91)	6.50 (6.83)	18.50 (11.63)	8.00 (8.36)	3.30 (4.32)	2.40 (3.44)	2.10 (2.85)
Anger-Hostility (A)	6.76 (5.22)	14.05 (8.59)	8.05 (8.36)	6.33 (7.21)	14.72 (8.31)	7.88 (6.82)	6.60 (6.82)	5.00 (3.77)	4.70 (4.76)
Vigor (V)	14.81 (6.40)	10.19 (4.93)	13.20 (6.29)	17.39 (6.13)	11.28 (5.59)	15.88 (5.34)	18.00 (4.22)	17.50 (4.03)	16.40 (3.75)
Fatigue (F)	11.43 (5.99)	17.71 (6.47)	11.00 (7.31)	9.06 (5.76)	13.83 (6.32)	9.12 (5.63)	5.80 (4.37)	5.20 (4.57)	5.40 (4.48)
Confusion - Bewilderment (C)	8.62 (4.50)	11.76 (5.31)	8.25 (4.88)	7.56 (3.45)	12.22 (3.47)	7.53 (3.02)	4.90 (2.88)	3.60 (2.01)	3.40 (1.90)
Total Mood Disturbance (TMD)	35.76 (28.23)	72.24*** (32.19)	35.60+++ (33.99)	21.17 (29.46)	69.11*** (29.51)	27.29+ (29.20)	7.30 (15.59)	3.00 (13.39)	3.10 (14.39)

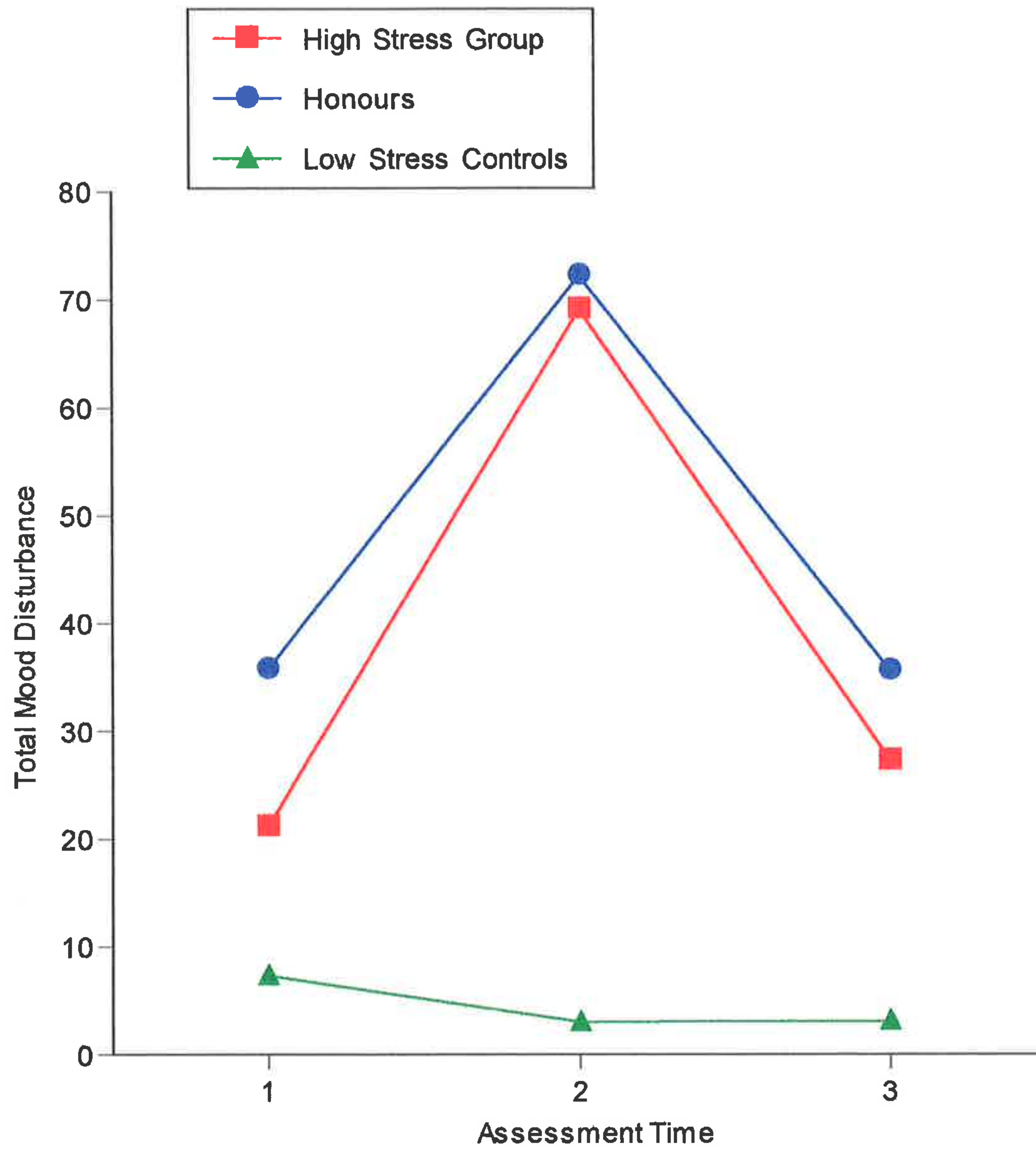
Note. Significant differences between low and high stress times assessed by k-1 planned comparisons (non-orthogonal), indicated by *** $p < .001$. Similarly, significant differences between high and post stress periods indicated by +++ $p < .001$. There were no significant differences between Time 1 and Time 3 for the TMD variable of each group. A similar pattern of results was seen on each of the subscales.

Table 2.3
Experiment 1: Means and standard deviations of the STAI-trait scores for the three groups

Group	STAI-trait		
	n	Mean	SD
Honours students	21	40.62	10.22
High stress group	18	35.22	8.17
Low stress controls	10	33.60	8.77

FIGURE 2.1

Experiment 1: The group by time interaction of the Total Mood Disturbance scores.



High Stress Group
Honours
Low Stress Controls
Total Mood Disturbance
80
70
60
50
40
30
20
10
0
1
2
3
Assessment Time

(iii) NT Results

Figure 2.2 shows the means and standard deviations of NT values for the three groups. A repeated measures analysis of variance was performed, in the same manner as for the psychological variables. A significant group by time interaction was found, with $F(4, 88) = 2.88$, $p < .05$. This interaction is shown in Figure 2.3. The honours students had a 33% lowering of their NT values during the high stress period of the study, $t(46) = 3.15$, $p < .01$. Following the examinations, their NT values rose to within 20% of their initial low stress period, and were significantly greater than at the high stress time of their study, $t(46) = 2.61$, $p < .05$. Their NT values during low stress times were not significantly different from the low stress control group data.

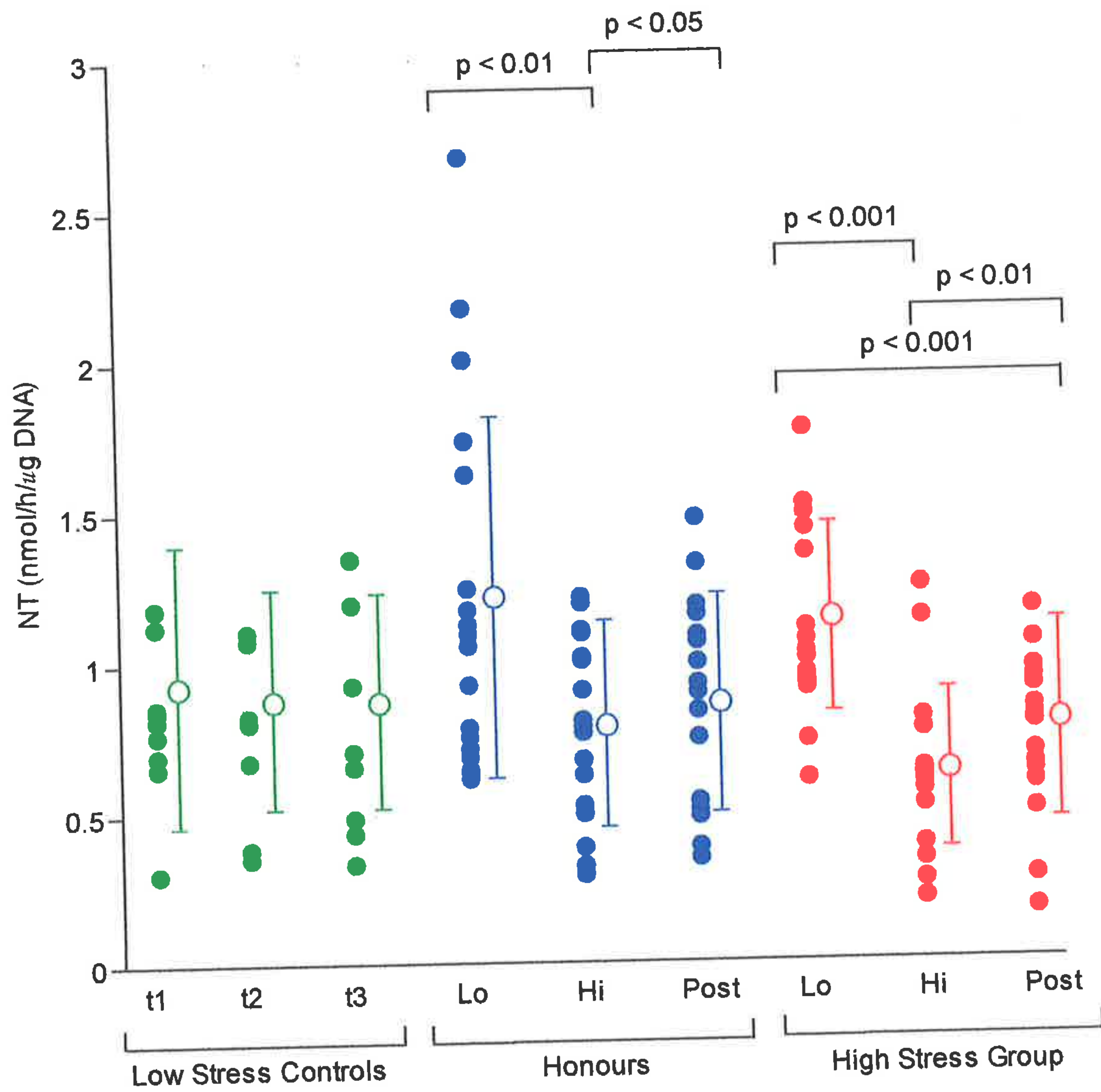
Also shown in Figure 2.2, the high stress group had a mean NT at their high stress time which was approximately half that of the initial low stress time, $t(46) = 9.56$, $p < .001$. Approximately 3 months following stress the NT activities increased significantly, $t(46) = 3.35$, $p < .01$, but were still lower than the initial low stress time, $t(46) = 4.60$, $p < .001$. In contrast, the low stress control group which was relatively unstressed throughout the 12 month period of this study had mean NT values which were not significantly altered.

There was a significant main effect for time, $F(2, 88) = 14.69$, $p < .001$, but no overall main effect for group, $F(2, 44) < 1.00$. It may be concluded that (1) NT decreased significantly in Time 2 (mean = .75) compared to Time 1 (mean = 1.08) and Time 3 (mean = .89). This was due to the lowered NT values in the honours students and the high stress group. (2) There was no overall effect of group. Means for the honours students (.98), high stress group (.84) and the low stress controls (.89) did not differ significantly.

The substantial effect sizes in this study exceeded one standard deviation for both the honours students, and the high stress group. Cohen proposes 0.8 of a standard deviation as a criterion for a large effect size (J. Cohen, 1992).

FIGURE 2.2

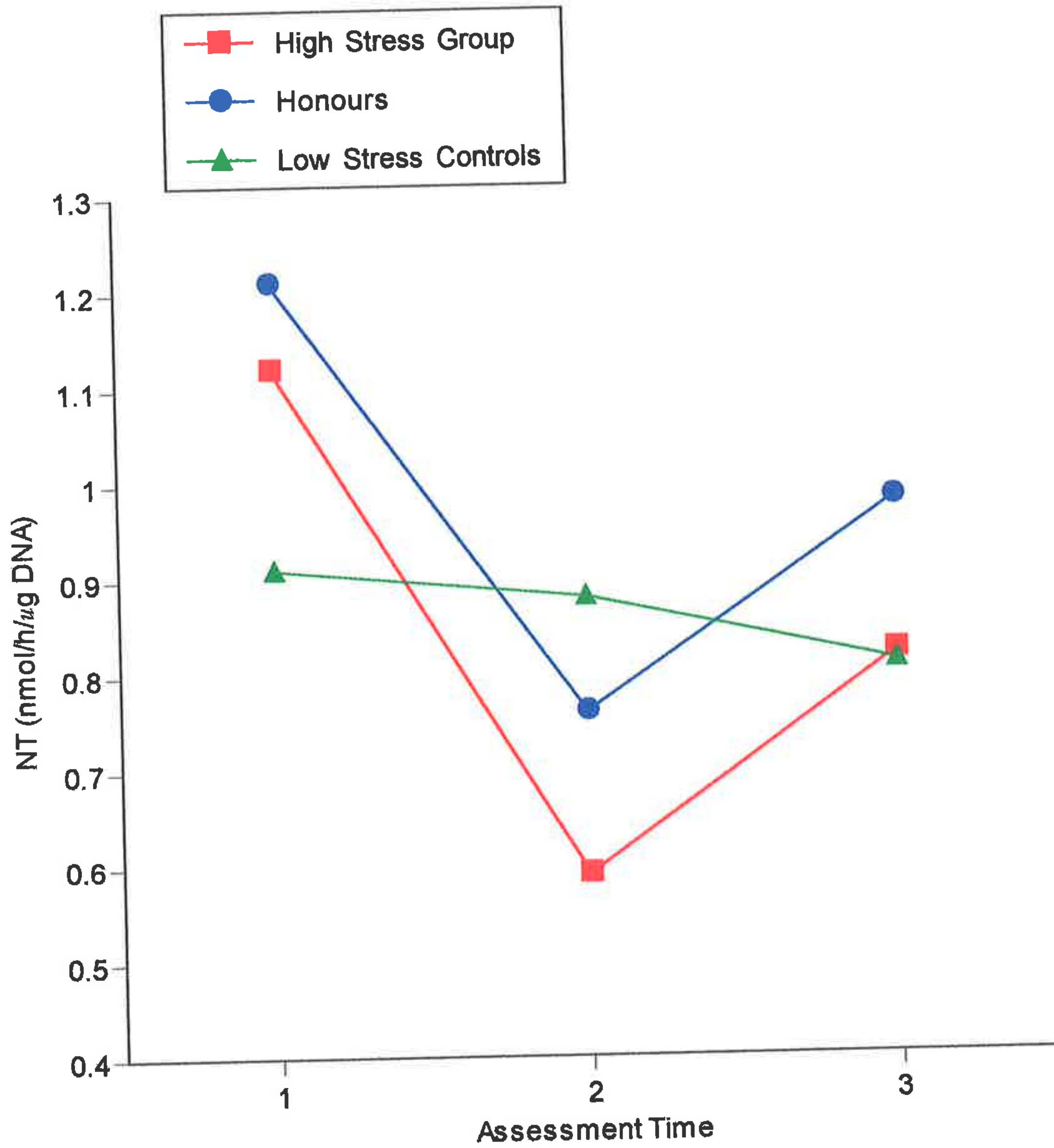
Experiment 1: NT activities in low stress controls, honours students and the high stress group at three separate times during the year. Results for the honours students and the high stress group are presented at their low, high and post stress periods. Low stress controls did not experience increased stress during the study, and results are presented during their low stress times. Data are shown as individual values (coloured circles •), means (o) and standard deviations (vertical lines). Significance of the differences between groups are indicated by horizontal bars. The absence of bars indicates non-significance.



p < 0.01
p < 0.05
p < 0.001
p < 0.01
p < 0.001
NT (nmol/h/µg DNA)
3
2.5
2
1.5
1
0.5
0
t1
t2
t3
Lo
Hi
Post
Lo
Hi
Post
Low Stress Controls
Honours
High Stress Group

FIGURE 2.3

Experiment 1: The group by time interaction of NT activities.


High Stress Group
Honours
Low Stress Controls
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
NT (nmol/h/ug DNA)
1
2
3
Assessment Time

(iv) Relationships between NT and psychological variables

It is clear from the foregoing that at times of high stress, variables indicating negative mood disturbance tended to increase, whilst assayed NT levels decreased. The relationship between these two types of variables was investigated directly using correlational analyses as shown in Table 2.4. NT values were correlated with each psychological variable, using all available scores for the two groups that were expected to vary in their stress levels (i.e., honours students and the high stress group). Correlations of NT with psychological variables were significant and negative for five of the mood variables. Figure 2.4 shows the correlation between NT and TMD. Trait anxiety did not correlate with NT. Also, NT was found not to correlate with red blood cell, neutrophil and platelet counts.

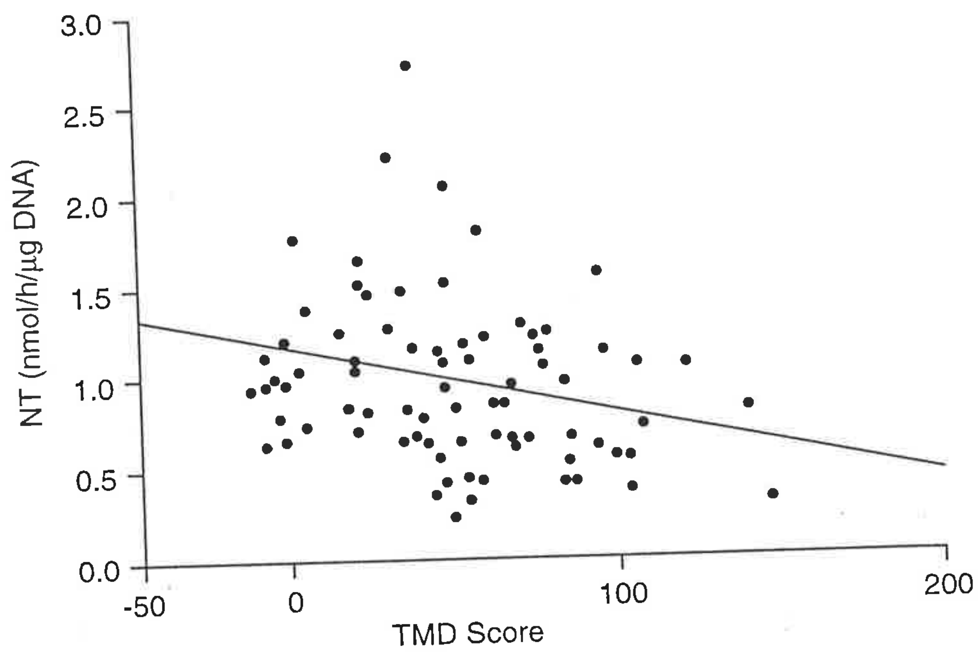
Table 2.4
Experiment 1: Correlations of NT Values with POMS variables, for honours students and the high stress group

Psychological Variables	Pearson r (df=75)
Tension-Anxiety (T) (POMS)	-.31**
Depression-Dejection (D) (POMS)	-.26**
Anger-Hostility (A) (POMS)	-.22*
Vigor (V) (POMS)	.02
Fatigue (F) (POMS)	-.12
Confusion-Bewilderment (C) (POMS)	-.30**
Total Mood Disturbance (TMD) (POMS)	-.26*

Note. Significant correlations are indicated by * $p < .05$; ** $p < .01$

FIGURE 2.4

Experiment 1: The correlation between NT and Total Mood Disturbance scores.



3.0
2.5
2.0
1.5
1.0
0.5
0.0
NT (nmol/h/µg DNA)
-50
0
100
200
TMD Score

2.3 Experiment 2

Experiment 1 showed that stress was associated with decreased NT levels in a non-clinical population. In Experiment 2 it was hypothesised that a similar reduction of NT levels would be found in a clinical population that was seriously depressed. Some of the depressed patients were on high levels of antioxidants which included ascorbate 1000 mg, d-alpha tocepherol (vitamin E) 335mg, vitamin A 3 mg, zinc gluconate 50mg, ubidecarenone (co-enzyme Q-10) 10 mg, supplements daily. Even though lowered antioxidant status may lead to immune suppression (Meydani, Wu, Santos, & Hayek, 1995) no predictions were made in relation to NT activities and a major depression group taking antioxidants. Severity of depressive symptoms was measured using the Beck Depression Inventory (BDI, Beck & Steer, 1993). Trait anxiety was assessed as in Experiment 1. It was expected that the major depression groups would have higher trait anxiety and depressive symptoms than the general population.

2.3.1 Method

(i) Subjects

This study included 60 subjects. Depressed patients were drawn from private psychiatric clinics in Adelaide, and divided into two groups according to whether or not they were on antioxidant medication. As a control group, all subjects originally allocated to the control group at their initial assessment in Experiment 1 (n = 28) were used as the controls in Experiment 2. The groups were thus specified as follows.

1. Major depression group not on antioxidants (-A/O): 23 patients were diagnosed with major depression according to the DSM-III-R criteria (American Psychiatric Association, 1987) using the Composite International Diagnostic Interview (CIDI, Morris-Yates, Teesson, Peters, & Andrews, 1994). Four patients were classified into major depression, single episode and melancholia (code 296.23), and 19 patients were classified into major depression, recurrent episode and melancholia (code 296.33). Sixteen of the patients were hospitalised and seven were outpatients at the time of assessment.

2. Major depression group with antioxidants (+A/O): Nine patients were diagnosed as specified above into two patients with major depression, single episode and melancholia (code 296.23), and seven patients with major depression, recurrent episode and melancholia (code 296.33). They were all hospitalised inpatients at the time of assessment. These patients had been placed on a range of antioxidants as already indicated. The high antioxidant therapy of these patients was not directed by this study. The clinician attending these patients elected to use high dose antioxidants as part of his routine therapeutic management. When the clinician was questioned about why he put his patients on antioxidants he replied that he himself was taking antioxidants, and that it was his personal belief that antioxidants were beneficial to ones health.

3. Controls: 28 non-clinical subjects with a mean score of less than 35 on STAI-trait and less than 10 on the Beck Depression Inventory (BDI), as assessed at Time 1 in Experiment 1.

(ii) Materials

Apparatus for the NT assay was the same as in Experiment 1.

The Composite International Diagnostic Interview (CIDI, Morris-Yates, Teesson, Peters, & Andrews, 1994) was used in order to make a DSM-III-R diagnosis of depression in the major depression groups . In addition to this the following psychological tests were administered to all subjects: STAI-trait (Spielberger et al., 1983) and the BDI (Beck & Steer, 1993). The BDI was administered to provide a quantitative measure of the severity of depressive symptoms and as a device for screening control subjects. Subjects whose BDI scores were greater than 10 were not assigned to the control group.

(iii) Procedures

All subjects were assessed according to age, sex, number of years of education completed, smoking, alcohol consumption, drug intake and by the psychological tests specified above for stress evaluations. Assessments were done at the time of blood collection for all groups. All of the depressed subjects were assessed with the CIDI, and classified according to the DSM-III-R criteria (Morris-Yates et al., 1994). As in Experiment 1 all subjects were free of acute infections

for at least two weeks prior to assessment, and their CBP's were normal. Both major depression groups were assessed on only one occasion, and the control group data were used from Time 1 only.

The laboratory procedures were as described in Experiment 1.

2.3.2 Results

(i) Demographic data

Table 2.5 shows the demographic data for the sixty subjects in this clinical study. Both depression groups were compared to the control group in relation to male/female ratio using the Fisher Exact test. There were no significant differences between the groups in the male/female ratio. Nor did groups differ significantly in age, $F(2, 57) = 2.64$, $p > .05$. The major depression group not taking antioxidants was significantly less educated than the other groups, $F(2, 57) = 4.26$, $p < .05$. No significant association was found between antioxidant status and diagnostic classification on a Fisher Exact test. Two out of nine patients taking antioxidants were diagnosed with major depression, single episode and melancholia compared to four out of twenty three with this diagnosis who were not taking antioxidants. In addition, the proportion of depressed patients on antidepressants and major tranquillizers did not differ between high and low dose antioxidant groups. The other group in this study was drug free. There was a higher proportion of smokers in the depressed groups ($p < .05$), but the groups did not differ in the amount of alcohol consumed, $F(2, 57) < 1.00$.

Table 2.5

Experiment 2: Demographic data for the three groups

Group	n	Age Mean	Age SD	Male/Female ratio	Education (years) Mean	Education (years) SD	Alcohol consumption in past month (grams) Mean	Alcohol consumption in past month (grams) SD	Smoker/ Non-smoker ratio	Drug State: No. on anti-depressants	Drug State: No. on major tranquilizers	Drug State: No. drug free
Major depression group (-A/O)	23	41.16	11.96	6:17	12.08	2.29	1.30	.27	7:16	20	9	3
Major depression group (+A/O)	9	34.67	13.91	2:7	12.88	2.14	.20	.41	5:4	8	5	1
Controls	28	34.57	12.92	8:20	14.21	2.96	.39	.46	1:27	0	0	28

(ii) Psychological variables

Table 2.6 shows the means and standard deviations for psychological variables, for the three groups in Experiment 2. There were no significant differences between the two depression groups in terms of CIDI diagnoses as assessed by the Fisher Exact test. A one-way analysis of variance showed a significant difference between groups on BDI scores, $F(2,59)=101.99$, $p<.001$. The major depression group not on antioxidants rated themselves as significantly more depressed than the controls, $p<.05$. Moreover, the major depression group taking antioxidants was significantly more depressed (as indicated by their higher BDI scores) than the major depression group not on antioxidants ($p<.05$). As expected, there was a significant difference between groups in terms of trait anxiety, $F(2,59)=110.40$, $p<.001$. Both major depression groups scored significantly higher on STAI-trait scores than the controls, $p<.05$.

Table 2.6
Experiment 2: Means and standard deviations for psychological variables, for the three groups

Group	n	Beck Depression Inventory		STAI-trait	
		Mean	SD	Mean	SD
Major depression group not on antioxidants	23	29.29	11.23	61.92	8.37
Major depression group with antioxidants	9	39.56	9.98	62.78	3.23
Controls	28	3.43	3.32	35.00	8.27

All BDI scores were significantly different from each other using the Scheffé test, $p < .05$.
Both depressed groups scored higher on STAI-trait than the controls, using the Scheffé test, $p < .05$

(iii) NT results

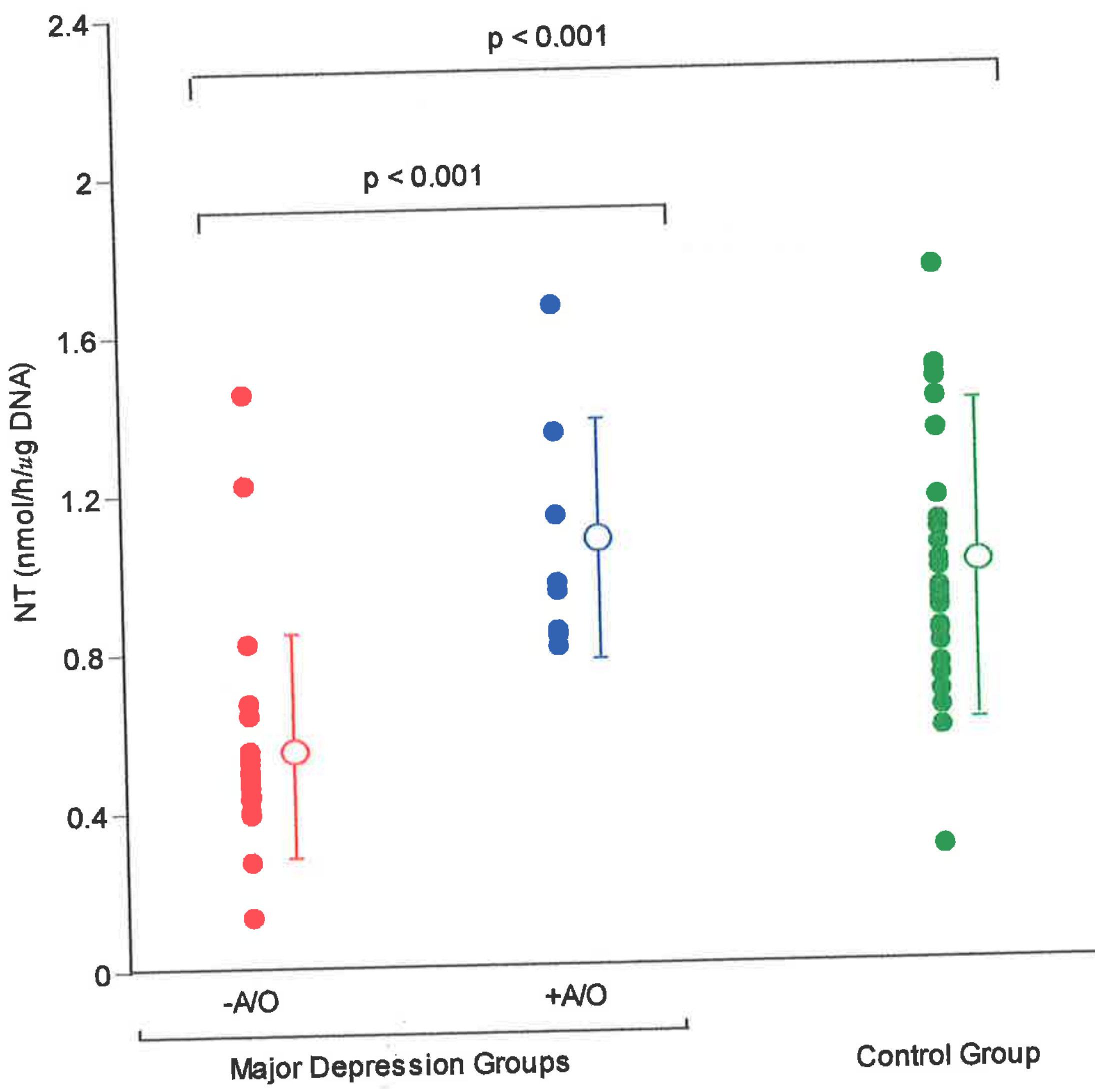
Figure 2.5 shows the means and standard deviations of NT activities in controls and major depression groups on high and low antioxidant intakes. A one-way analysis of variance between the controls, the major depression group not on antioxidants and the major depression group on antioxidants was significant, $F(2, 57) = 16.90$, $p < .001$. Planned comparisons (non-orthogonal) revealed significant differences between 1) controls and the major depression group not taking antioxidants, $t(58) = 16.20$, $p < .001$; and 2) the major depression group not on antioxidants versus the major depression group on antioxidants, $t(58) = 16.72$, $p < .001$.

A large effect size (exceeding one standard deviation) was evident when the major depression group not taking antioxidants was compared with the controls. A similar effect size was evident when the major depression group not on antioxidants was compared with the major depression group on high antioxidants. Depressed patients on antioxidants had NT values two-fold higher than the depressed group not receiving this treatment and indeed had values of NT similar to the control group. This normalization of NT activities was not due to a direct effect of ascorbate per se on NT since ascorbate added at concentrations of up to 100 umol/L above endogenous ascorbate levels to the lymphocyte homogenate of participants on low ascorbate intakes, did not affect NT activities. We calculated that the endogenous ascorbate concentrations for our lymphocyte homogenates would vary from 20-60 umol/L rising to 80 umol/L in ascorbate supplemented patients (Evans, Currie, & Campbell, 1982).

As shown in Figures 2.6 and 2.7, respectively, NT correlated negatively with the Beck Depression Inventory, $r(49) = -.39$, $p < .01$, and with STAI-trait scores, $r(49) = -.38$, $p < .01$. These analyses included the major depression group not on antioxidants and the control group. The major depression group on antioxidants was omitted because antioxidants appeared to have a protective effect on NT activities. As in Experiment 1, NT was found not to correlate with red blood cell, neutrophil and platelet counts.

FIGURE 2.5

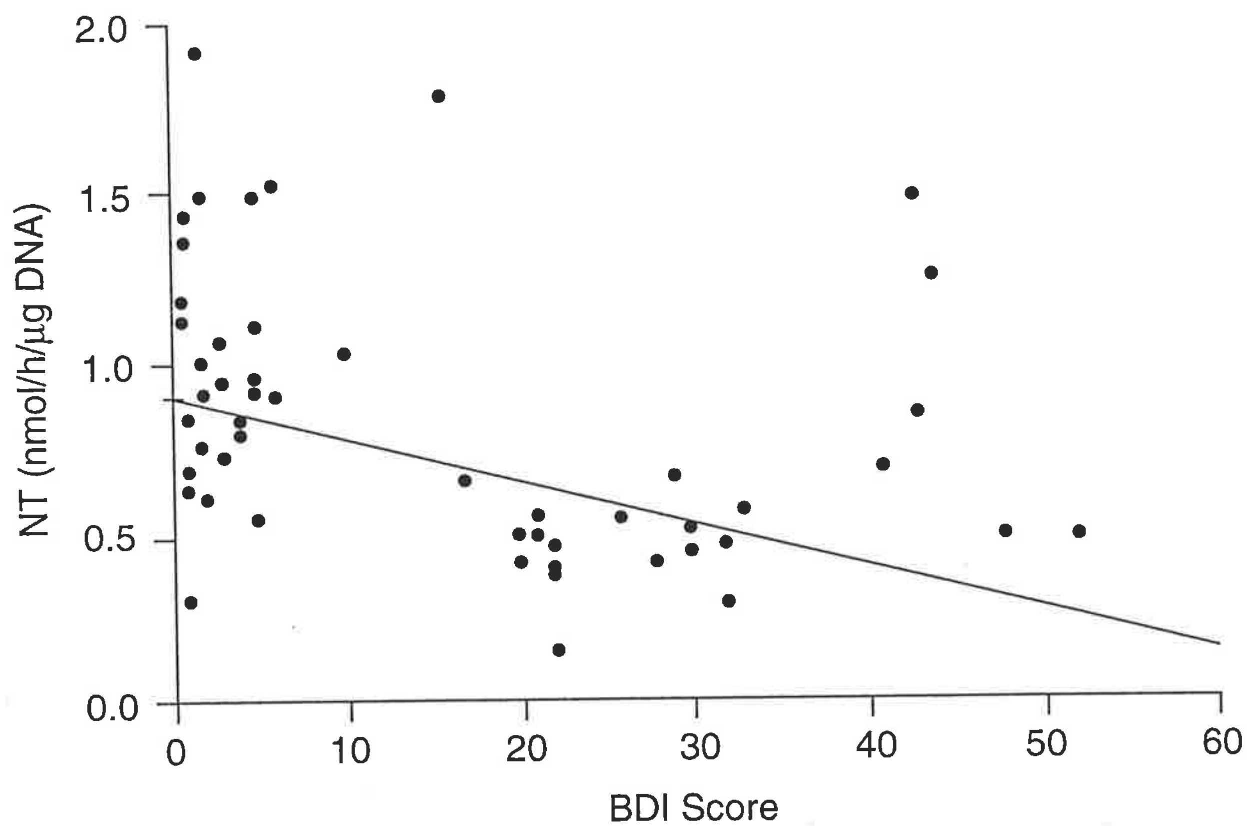
Experiment 2: NT activities in controls and major depression groups on high (+ A/O) and low (- A/O) antioxidant intakes. Data are shown as individual values (coloured •), means (○) and standard deviations (vertical lines). Significance of the differences between groups are indicated by horizontal bars. The absence of bars indicates non-significance.



p < 0.001
p < 0.001
2.4
2
1.6
1.2
0.8
0.4
0
NT (nmol/h/µg DNA)
-A/O
+A/O
Major Depression Groups
Control Group

FIGURE 2.6

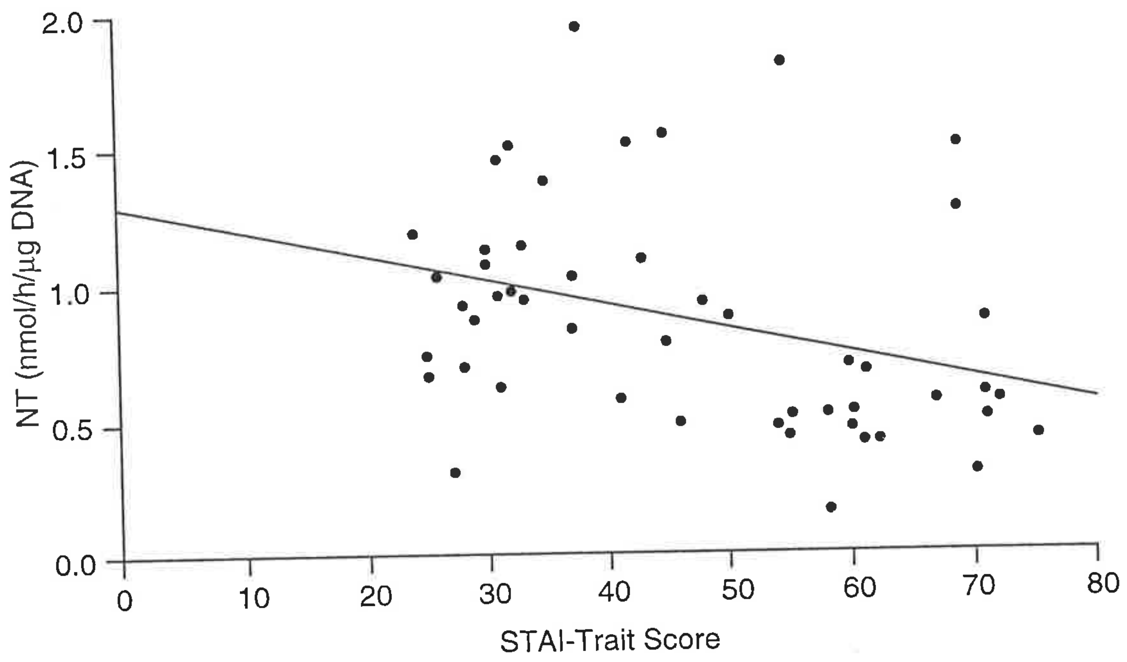
Experiment 2: The correlation between NT and the Beck Depression Inventory scores.



2.0
1.5
1.0
0.5
0.0
NT (nmol/h/μg DNA)
0
10
20
30
40
50
60
BDI Score

FIGURE 2.7

Experiment 2: The correlation between NT and the State Trait Anxiety Inventory scores.



2.0
1.5
1.0
0.5
0.0
NT (nmol/h/μg DNA)
0
10
20
30
40
50
60
70
80
STAI-Trait Score

2.3.3 Discussion

NT was found to correlate well with psychological stress in the groups examined in both Experiment 1 and Experiment 2, with up to 50 % reduction of enzyme activity in stressed individuals. The degree of reduction of NT in stressed groups was similar to that found in a previous study for persons who were well but antibody positive to HIV and, in retrospect, suggested that psychological stress in these HIV-positive patients may have been a contributing factor to their low NT activities (Chalmers et al., 1990). NT appears to be a reliable marker for depressed patients, and as shown by the control group (Figure 2.2) is not subject to the seasonal variations seen with some other enzyme markers (Maes et al., 1994d). It is difficult to put a time course on this enzyme in relation to the lag time from depression to a lowering of NT, and this probably varies from one person to another. However, the lowering of NT in the honours student group occurred over a period of 3 months from the low to high stress periods. Two weeks following examination the NT activity levels though still low were beginning to normalise. This would suggest that the changes in NT occurred on average over a 1-3 month period.

The effects seen with NT are not a generalized effect of other ectoproteins on the cell and appear to be specific to NT. For example, the activity of dipeptidyl peptidase IV (DPPIV), another lymphocyte ectoenzyme also related to immunity, was unaffected in both HIV-positive and depressed patients (Chalmers et al., 1990). This is not to imply, however, that other ectoproteins important to lymphocyte function and immunity are not affected by psychological stress, and contribute to the immune suppression seen in depressed patients. This is an aspect that perhaps should be investigated in future studies.

Other purine metabolizing enzymes besides NT, examined as indicators of immune suppression have been mainly cytoplasmic. These enzymes have included adenosine-deaminase, adenosine-kinase, purine nucleoside phosphorylase (PNP) and hypoxanthine guanine phosphoribosyltransferase (HGPRTase) (Renouf, Wood, Frazer, Thong, & Chalmers, 1989). Lymphocytic adenosine deaminase, like NT, was lowered in HIV-positive patients compared to healthy controls, whereas adenosine kinase was raised. The fact that PNP and HGPRTase were unaffected by HIV-antibody status suggests that the effect on NT in this study was not due to malnutrition. This was confirmed by the normal blood pictures seen for the subjects in this study. Moreover, it is unlikely that the lowering of NT is due to depletion of lymphocyte sub-sets since the reported changes with these are marginal and not sufficient to explain the two-fold drop in NT seen with depression (Weisse, 1992).

The mechanism of the reduction of NT appeared to be mediated by oxygen radicals with the finding of normal NT levels in melancholic patients on high antioxidant intake in this study. Ethical reasons precluded taking patients who were seriously depressed off their antidepressant medication. With the depressed groups it is possible that their drug intake may have lowered NT activities, either by direct NT-drug interaction, or through increased oxidative stress, since drug metabolism increases oxidative stress. However, if this was so one would have expected their stress levels plus drug intake to further reduce NT below the stress group not on drugs (Experiment 1). Since this did not happen it is unlikely that their drug intake is affecting NT values in these patients.

Previous studies would tend to suggest an increased oxygen radical generation with stress. These include (1) an increased inflammatory-like process reported for people suffering from major depression (Joyce et al., 1992; Maes et al., 1993); 2) Low serum levels of zinc, an antioxidant metal in depressed persons (Maes et al., 1994c); and also 3) The finding that HIV-positive patients have a low anti-oxidant status could explain their low NT activities (Sappey et al., 1994). However, the increased inflammatory process with HIV-positive homosexuals is probably not only

related to stress, but also due to reported increased rates of infections arising from sexually transmitted diseases in this group when compared to heterosexuals (Darrow, Barrett, Jay, & Young, 1981).

In summary, NT is lowered in situations of psychological stress and this appears to be related to inflammatory responses which have been shown to occur in depressed patients. In other words, NT is both a good indicator of an inflammatory process and a marker of oxygen radical damage to the cell. It is proposed that suppression of NT because of its role in cell maturation will decrease immunity in people who are depressed or stressed because of its role in cell maturation and activation.

In conclusion, despite the large effects observed in both studies, it must be acknowledged that a limitation of this research design was that it involved comparing groups whose members were not randomly assigned. Although it seems unlikely that the observed effects could have been due to subject selection, or selection related factors (Cook & Campbell, 1979) rather than to the treatment (differences in stress levels) this possibility can not be ruled out. It seems improbable, however, not only because of the lack of marked differences between the groups on demographic characteristics, but also because the within-subjects effects were just as marked as the between subject effects.

CHAPTER 3

EVIDENCE FOR FREE RADICAL-MEDIATED REDUCTION OF LYMPHOCYTIC 5'-ECTONUCLEOTIDASE DURING STRESS[5]

3.1 Introduction

There is persuasive evidence suggesting a positive correlation between psychological stress / depression and disease in humans (reviewed by Cohen & Williamson, 1991; Glaser & Kiecolt, 1987; Kiecolt-Glaser & Glaser, 1995). In the previous chapter it was shown that that stress was associated with depleted 5'-ectonucleotidase activity (NT) in humans in that honours students prior to thesis submission and examinations, people experiencing stressful life events, and patients with major depression and melancholia had a significant twofold reduction of their lymphocytic 5'-ectonucleotidase activities (NT). In contrast, NT levels in a major depression group with melancholia on high doses of antioxidants were normal. The main hypothesis of this thesis is that psychological stress causes an attenuated inflammatory response, with consequent sustained release of oxygen radicals which damage NT. The second hypothesis is that antioxidants provide protection against damage to NT. In this chapter these hypotheses are tested by firstly assessing if humans undergoing stressful life events have a depleted antioxidant (ascorbate) status indicative of sustained oxygen radical damage (Experiment 3). The other aspect is tested by generating the superoxide anion in in vitro systems and testing its effect on 1) Nitroblue tetrazolium (NBT) reduction in the presence of various antioxidants (Experiment 4), and 2) lymphocytic NT (Experiment 5). These related experiments are described separately, and their implications are then outlined in a joint discussion.

3.2 Experiment 3

Experiment 3 is based on the assumption that ascorbate, and possibly other antioxidants shield NT from oxygen radical damage. It was conducted in order to test whether

[5] The experimental work cited in this Chapter has been accepted for publication in the International Journal of Immunopharmacology.

humans undergoing stress / depression have depleted stores of ascorbate. It is assumed that ascorbate protects against the reduction of 5'-ectonucleotidase activity during stress, and therefore humans who are stressed / depressed may have depleted ascorbate stores resulting from excess oxygen radical-mediated damage to ascorbate during stress.

3.2.1 Method

(i) Subjects

This study included 72 subjects from non-clinical and clinical populations in the Adelaide metropolitan area. Stress groups and non-clinical controls were derived from the original study described in Chapter 2. Due to technical difficulties some of the patient specimens collected in the original study were not available. See Chapter 2 for further details regarding the original sample. The five groups were as follows.

1. Honours students: 21 students undertaking an honours psychology course were recruited from The University of Adelaide as described in the previous chapter.

2. Major depression group not on antioxidants: Eighteen of the original sample of 23 patients diagnosed with major depression according to the DSM-III-R criteria were available for analysis (American Psychiatric Association, 1987). Four of the patients were classified into major depression, single episode and melancholia (code 296.23), and fourteen patients were classified into major depression, recurrent episode and melancholia (code 296.33). Fourteen of the patients were hospitalised and four were outpatients at the time of assessment.

3. Major depression group taking antioxidants: Nine patients were diagnosed with major depression as described in the previous chapter.

4. Controls not on antioxidants: Fifteen of the original sample of 28 control subjects were available for analyses. This group had a mean score of 37.60 (SD= 8.81) on STAI-trait and 3.93 (SD=3.77) on the Beck Depression Inventory (BDI).

5. Controls on antioxidants: This group was not in the original studies described in Chapter 2, and was added to define the saturation level of the ascorbate stores in people not

suffering from depression. This control group would indicate whether ascorbate stores in the major depression group (+ ascorbate) are lower because oxygen radical species may have destroyed some of the ascorbate, and thus their antioxidant status may have been insufficiently high to afford protection of ascorbate stores. This group consisted of nine subjects with a mean score of 36.56 (SD=8.69) on the STAI-trait, and a mean score of 2.11 (SD=3.14) on the BDI.

Both control groups consisted of academic staff, general staff members and post-graduate students recruited from the University of Adelaide. Mean scores for the BDI and STAI-trait scores for both of these groups were approximately half a standard deviation below those of the standardisation samples (Beck & Steer, 1983; Spielberger et al.,1983).

(ii) Materials for laboratory tests

Apparatus for the blood processing and NT assay is described in Chalmers and Hare (1990). Ascorbate was measured by the method of Roe (1961) using trichloracetic acid (TCA) / charcoal 2,4-dinitrophenylhydrazine colour reagent (DNPH) and ascorbate standards were prepared as described by Chalmers and Lark (1985). Ascorbate and NT results were expressed respectively as pmol/ug DNA and nmol/hr/ug DNA. DNA was measured spectrophotometrically in lymphocyte extracts by the method of Warburg-Christian (1942).

(iii) Psychological tests

Psychological tests were administered as described in Chapter 2.

(iv) General procedures

Data collection and general procedures were as described in Chapter 2. As with the study described in Chapter 2, the study was conducted blind with the analyst, Dr. A. H. Chalmers, unaware of the subject's grouping.

(v) Procedure for ascorbate assay

To 0.1 mL human or rat lymphocyte extract from NT assay add 0.25 mL TCA/charcoal mix, shake and microfuge for 30 sec. To 0.125 mL of supernatant add 0.03 mL of DNPH

solution and take the absorbance immediately after (A0) incubation for 4 hours at 37° C and take the absorbance after 4 hours (A4). Standards of 200, 100, 50 and 0 (blank) uM ascorbate were run simultaneously with theunknowns to allow ascorbate concentrations to be assessed. DNA concentration was calculated according to the method of Warburg-Christian (1942) by diluting 0.02 mL of the lymphocyte extract to 0.2 mL water during the 4 hour incubation, and read the absorbances at 260 and 280 nm against a blank of water.

Calculation

$$\text{pmoles ascorbate/ug DNA} = \frac{\text{uM (ascorbate)} \times 1{,}000}{11 \times \text{DNA (ug/mL)}}$$

3.3.2 Results

(i) Demographic data

As some of the data were not available from the original study cited in Chapter 2 results were reanalysed. Table 3.1 summarises the demographic data for the 72 subjects in this study. As previously indicated (Chapter 2) there were no significant demographic differences in the groups in relation to male/female ratio, and alcohol intake, $F(4, 67)<1.00$. However, the major depression group (-antioxidants) were significantly older than the honours students and controls (-antioxidants), $F(4, 67)=5.52$, $p<.001$, Scheffé, $p<.05$. The major depression groups tended to smoke more than the other groups studied as assessed by a Fisher Exact Test. They were generally less educated, $F(4, 67)=10.83$, $p<.001$, Scheffé, $p<.05$, and were taking psychotropic drugs.

Table 3.1
Experiment 3: Demographic data for the five groups

	n	Male/ Female Ratio	Age		Education (years)		Alcohol consumption in past month (grams)		Smokers vs Non-smokers	Drug State: No. on Anti-Depressants	Drug State: No. on Major Tranquilizers	Drug State: No. Drug Free
			Mean	SD	Mean	SD	Mean	SD				
Honours	21	3:18	28.90	9.16	15.43	.75	.54	.63	1:20	0	0	21
Major depression group (+A/O)	18	4:14	43.50	10.18	12.11	2.56	.37	1.47	4:14	17	7	3
Major depression group (-A/O)	9	2:7	34.67	13.91	12.89	2.15	.20	.41	5:4	8	5	1
Controls (- ascorbate)	15	2:13	29.40	11.89	15.53	2.00	.44	.52	1:14	0	0	15
Controls (+ ascorbate)	9	2:7	41.11	14.97	15.89	2.67	1.05	2.02	1:8	0	0	9

(ii) Psychological variables

A similar pattern of results to that described in Chapter 2 was obtained for psychological parameters. Results of BDI and STAI-trait variables are summarised in Table 3.2. Similarly, the means and standard deviations of the POMS variables for the honours students at their low (Time 1) and high stress (Time 2) are shown in Table 2.2 of the previous chapter. The Time 3 measurement occasion was not available for analyses. A related samples t-test for the TMD scores showed that during the time of high stress in the honours group, TMD scores increased significantly, $t(20) = 4.37$, $p < .05$. Groups differed significantly in their BDI scores, $F(3,47)=55.69$. Both depressed groups had significantly higher BDI scores than the control groups, Scheffé test, $p<.05$. The major depression group (+antioxidants) had significantly higher BDI scores than the depressed group (-antioxidants) as assessed by a Scheffé test, $p<.05$. Both depressed groups had significantly higher scores on the STAI-Trait than the control groups, but did not differ from each other, $F(4, 71)=35.50$, $p<.001$, Scheffé test, $p<.05$.

Table 3.2
Experiment 3 : Means and standard deviations for psychological variables, for the five groups

Group	n	Beck Depression Inventory		STAI-trait	
		Mean	SD	Mean	SD
Honours	21	NA		40.62	10.22
Major depression group not on antioxidants	18	31.11	12.10	62.17	8.91
Major depression group with antioxidants	9	39.56	9.98	62.78	3.23
Controls (-ascorbate)	15	3.2	1.82	36.07	7.46
Controls (+ascorbate)	9	3.33	1.11	36.44	7.30

Note. NA=not assessed.

(iii) NT activities

Figure 3.1 shows the means and standard deviations of NT activities for the six groups in Experiment 3. As expected, a similar pattern of results to that described in Chapter 2 were obtained for NT activities. A one-way analysis of variance on NT activities of the five groups was significant, $F(4, 67) = 5.89$, $p < .001$. Planned comparisons (non-orthogonal) revealed that the NT activities of the major depression group not taking antioxidants was significantly lower than the other groups, $t(70) = 4.05$, $p < .05$. A related samples t-test on NT scores for the honours group showed that NT was reduced significantly during the high stress time when compared to the low stress time, $t(20) = 3.42$, $p < .05$.

(iv) Ascorbate results

Figure 3.2 shows the means and standard deviations of ascorbate stores in the control groups, major depression groups on high and low antioxidant intakes, and honours students at low and high stress times. A one-way analysis of variance between groups on their ascorbate stores showed a significant difference between groups, $F(4,67) = 6.34$, $p < .05$. Planned comparisons (non-orthogonal) revealed a significant reduction of ascorbate stores in the major depression group not taking antioxidants compared to the other groups, $t(70) = 4.65$, $p < .05$. A repeated measures t-test for ascorbate stores showed a significant reduction in ascorbate stores for honours students at their high stress time compared to their low stress time, $t(20) = 1.80$, $p < .05$.

FIGURE 3.1

Experiment 3: Means and standard deviations of the NT values in the control groups, major depression groups on high and low antioxidant (+ and – A/O respectively) intakes, and honours students at low and high stress times.

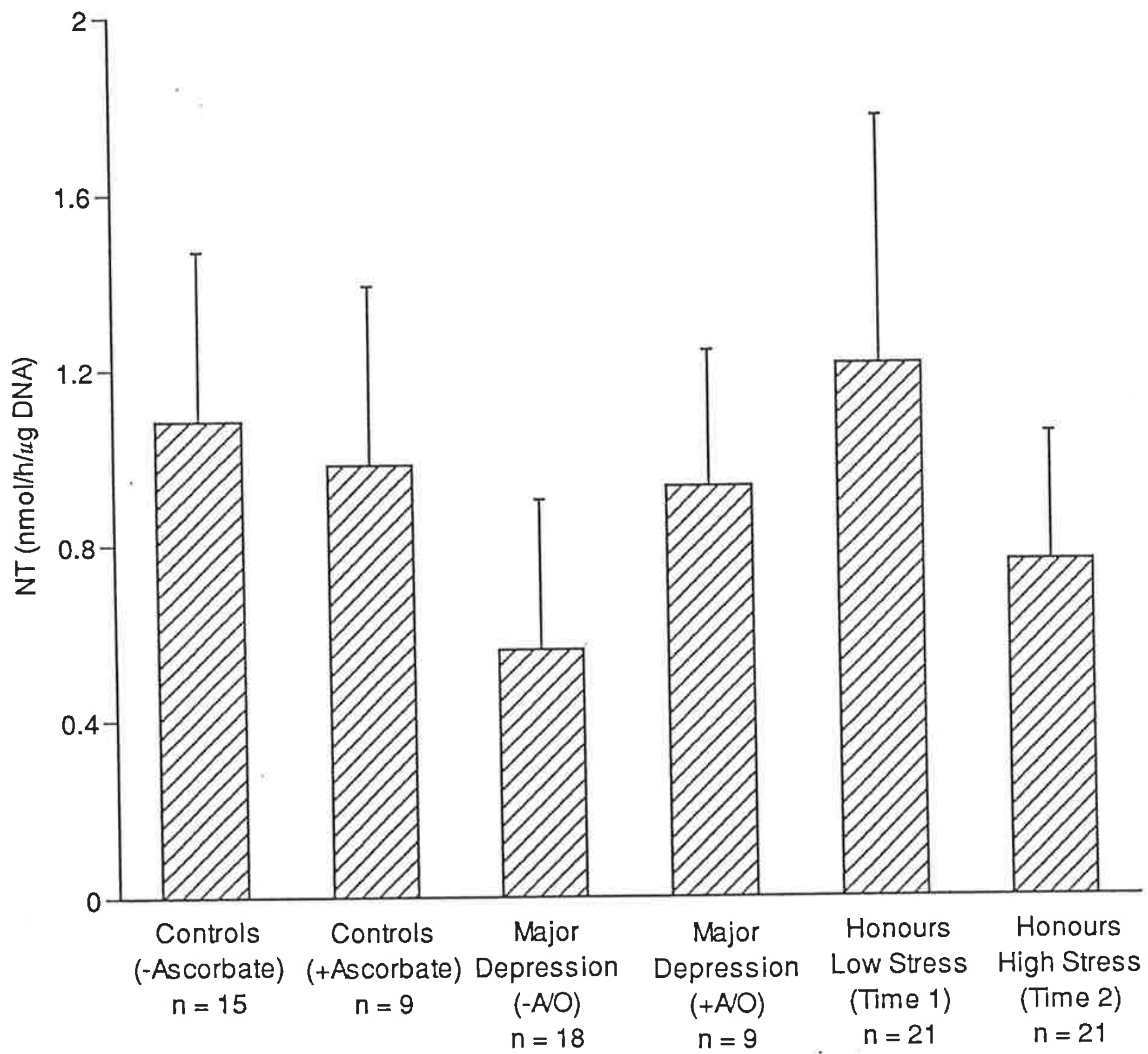
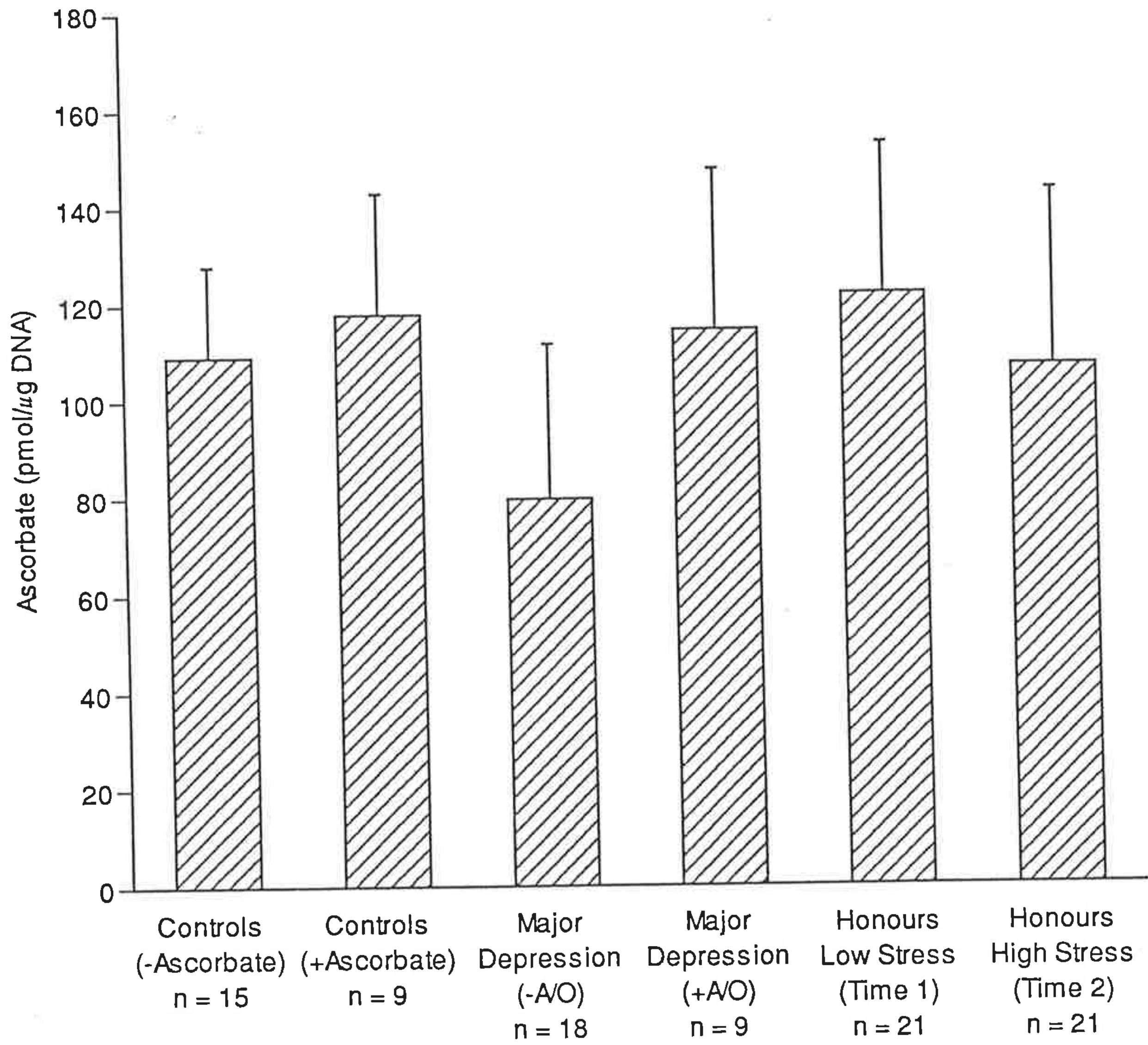

2
1.6
1.2
0.8
0.4
0
NT (nmol/h/ug DNA)
Controls (-Ascorbate) n = 15
Controls (+Ascorbate) n = 9
Major Depression (-A/O) n = 18
Major Depression (+A/O) n = 9
Honours Low Stress (Time 1) n = 21
Honours High Stress (Time 2) n = 21

FIGURE 3.2

Experiment 3: Means and standard deviations of ascorbate stores in the control groups, major depression groups on high and low antioxidant intakes (+ and – AO respectively), and honours students at low and high stress times.



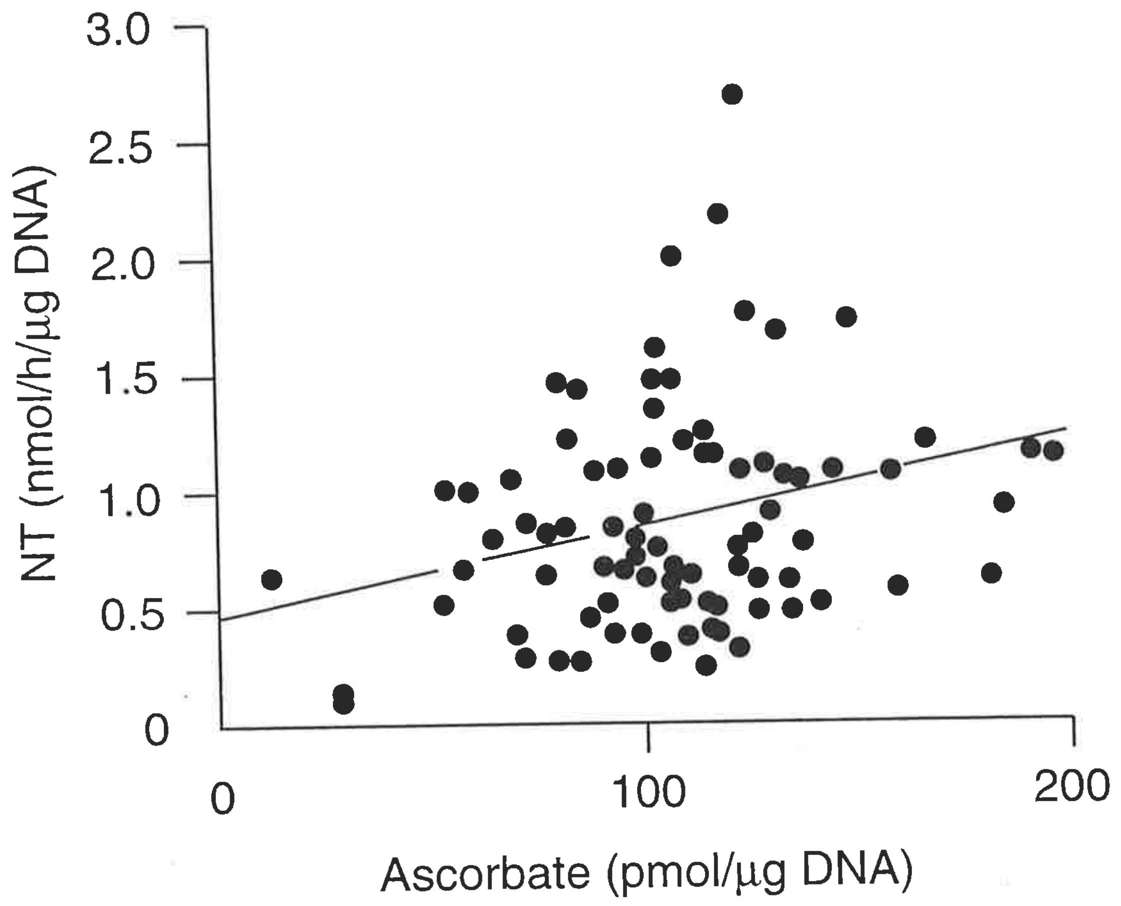
180
160
140
120
100
80
60
40
20
0
Ascorbate (pmol/μg DNA)
Controls (-Ascorbate) n = 15
Controls (+Ascorbate) n = 9
Major Depression (-A/O) n = 18
Major Depression (+A/O) n = 9
Honours Low Stress (Time 1) n = 21
Honours High Stress (Time 2) n = 21

(v) Relationships between NT, psychological variables, ascorbate and blood cell counts

As previously described in Chapter 2, NT correlated negatively with both BDI and the STAI-trait scores, $r(31) = -.39$, $p<.05$; $r(31)= -.48$, $p<.01$, respectively. In contrast to the findings in Chapter 2, NT did not significantly correlate inversely with TMD scores, $r(41)=-.22$, $p>.05$. This negative result was probably due to the smaller sample size. The correlation in Chapter 2 also included the high stress group at their low and high stress times. Ascorbate correlated positively with NT, $r\ (31)=0.39$, $p <.05$. These analyses included only the groups that were not taking antioxidants because it was assumed that antioxidants would have a protective effect on NT activities. NT was found not to correlate with red blood cell, neutrophil and platelet counts.

FIGURE 3.3

Experiment 3: The correlation between NT and ascorbate values.



3.0
2.5
2.0
1.5
1.0
0.5
0
NT (nmol/h/µg DNA)
0
100
200
Ascorbate (pmol/µg DNA)

3.3 Experiment 4: In vitro study on NBT

Experiment 4 was conducted to assess the in vitro effect of the superoxide anion reductions of NBT and to establish if antioxidants were protective against this damage. Thus the NBT assay allowed a convenient screening of the antioxidants that were protective of NBT reduction by the superoxide anion. The antioxidants used in this study are shown in Table 3.3.

3.2.1 Materials and Method

(i) Reagents

1. Xanthine (400 uM) solution in 10 mM tris pH 7.4 / isotonic saline (tris saline) buffer.

2. Nitroblue tetrazolium (NBT;Sigma Cat No. N-6876) 600 uM solution in tris saline buffer. Store at 4°C.

3. Xanthine oxidase (XO; 20 Units/mL from cow milk; Boehringer Mannheim Cat No 110434).

4. Superoxide dismutase (SOD; 75,000 units lyophilized powder, Sigma Cat No S-2515) was reconstituted into 1 mL of water and stored at 4°C.

In vitro studies on NT and NBT described below used the following compounds as potential modulators of the superoxide anion: (+)-Tocopherol acetate (Sigma Cat No T-3001), L-ascorbic acid (Ajax Chemicals, Australia Cat No 308709), selenium dioxide (Sigma Cat No S-9379), retinol acetate (Sigma Cat No R4632), zinc sulphate (Sigma Cat No Z4750), glutathione reduced (Boehringer Mannheim Cat No 127736), dehydroascorbic acid (DHA; Aldrich Chemical Co Milw, WI, USA. Cat No 26,155-6), 1,4-dithiothreitol (DTT, Boehringer Mannheim Cat No 197777) and uric acid (Sigma Cat No U-0881. All solutions were made prior to testing at 100 times the physiological concentration. All were made in tris saline except ascorbic and dehydroascorbic acids which because of their instability at pH 7.4 (Chalmers, Cowley, & McWhiney, 1985) were made in water and used within 10 minutes. Retinol acetate and tocopherol acetate were dissolved in ethanol. The volumes of alcohol used ($\leq 20\ \mu$L), added as blanks to the assay, did not affect the rate of the assay.

(ii) Procedure

Studies on Nitroblue tetrazolium (NBT). In this assay a direct monitor of superoxide anion reduction of NBT to its blue formazan dye was measured spectrophotometrically at 560 nm (Robak & Gryglewski, 1988). Mix 0.5 mL of xanthine solution with 0.5 mL of NBT and start the reaction with 5 uL XO. The initial rate, maximal in the first minute of the reaction, was used to study the effect of various compounds on this reaction. The inhibitors used were made up at about 100 times the physiological concentration and 10 uL were added to this assay. Those substances which affected NBT reduction were further tested directly on xanthine oxidase to determine whether the effects seen were due to oxygen radical scavenging or to direct effects on xanthine oxidase. The test was done as follows. To 0.5 mL xanthine solution (400 umol/L) add 0.5 mL tris/saline buffer and 10-20 uL of inhibitor/activator. Start the reaction by the addition of xanthine oxidase (see above) and measure uric acid formation at 295 nm. The reaction was only linear in the first minute and absorbances were measured every 15 sec.

(iii) Inhibitor concentrations used in oxyradical tests.

Reference ranges for blood levels were obtained from Combs (1992) and Underwood (1977).

(iv) In vitro study on NBT: Effects on NBT and XO.

The rate of reduction of NBT to formazan by the superoxide anion is shown in Fig. 3.4. The reduction was complete at about 10 minutes and ascorbate at 100 umol/L inhibited the reaction by about 50 %.

The results of this study shown in Table 3.3 indicate that ascorbate, zinc and glutathione at physiological concentrations inhibit the reduction of NBT by the superoxide anion. The inhibition shown by superoxide dismutase confirms that the reaction is mediated by the superoxide anion. The inhibitions shown by urate and oxidized glutathione were due mainly to a direct inhibition of xanthine oxidase rather than a scavenging of the superoxide ion exclusively.

Figure 3.5 shows a dose response curve which indicates the effect of zinc (O), ascorbate (●) and the combined effects of zinc and ascorbate (Δ) on inhibiting the reduction of NBT by superoxide anion. These data indicate that the inhibition by ascorbate and zinc is additive.

FIGURE 3.4

Experiment 4: The rate of reduction of NBT to formazan by the superoxide anion. The reduction was complete at about 10 minutes and ascorbate at 100 umol/L inhibited the reaction by about 50 %.

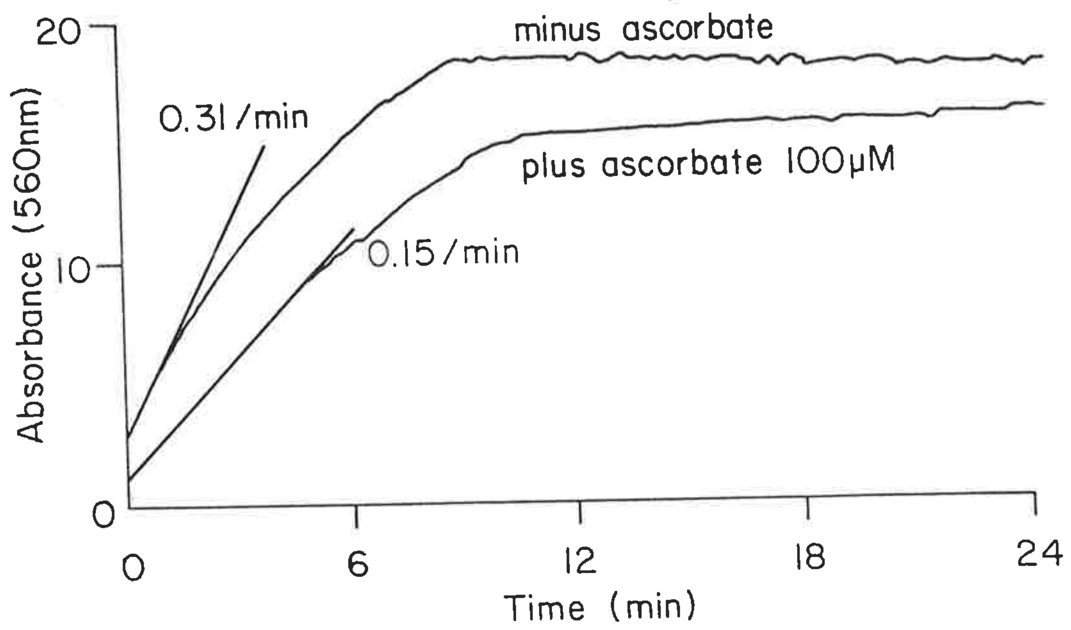

minus ascorbate
plus ascorbate 100μM
0.31/min
0.15/min
Absorbance (560nm)
20
10
0
0
6
12
18
24
Time (min)

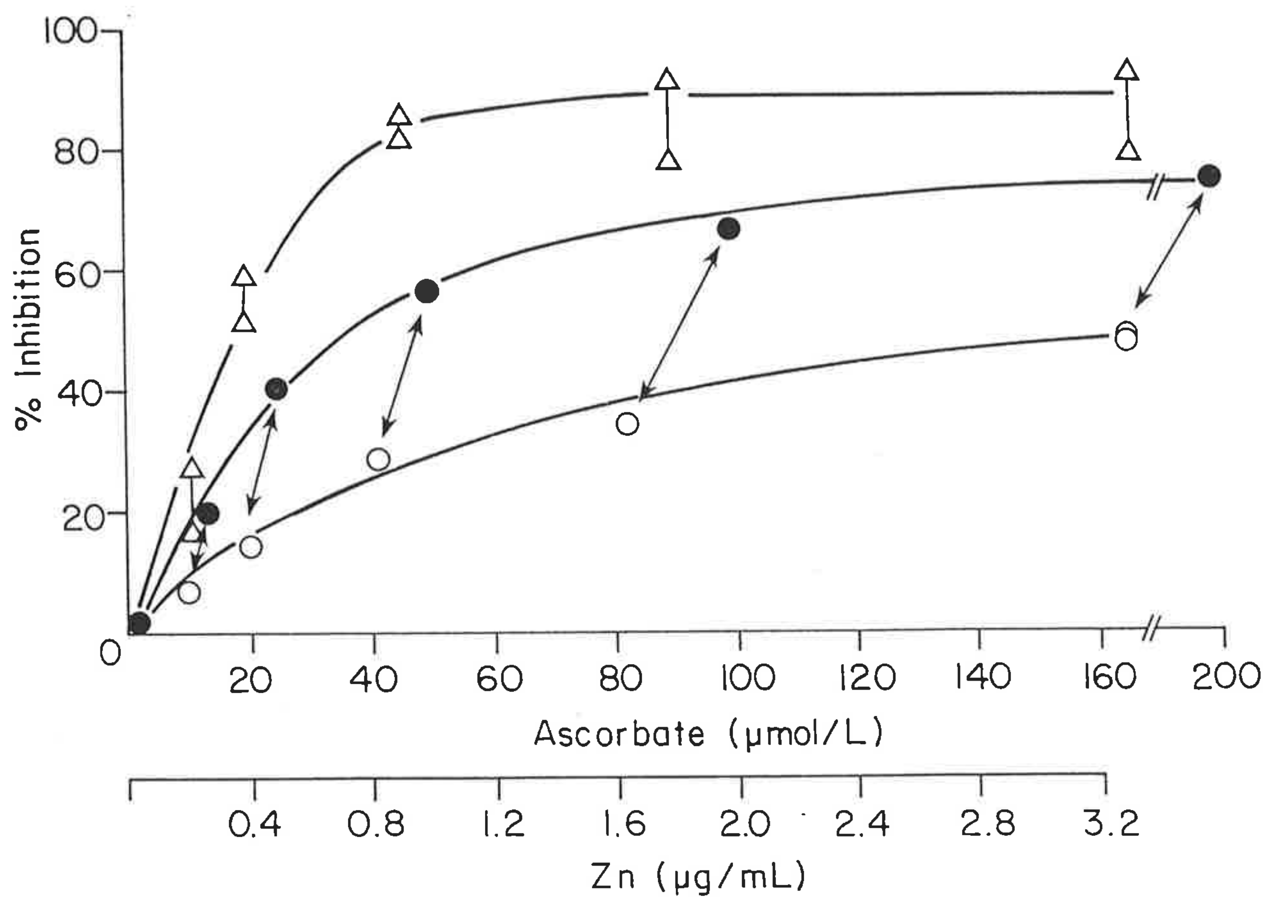
Table 3.3
Experiment 4: Inhibitor studies on NBT reduction by superoxide anion.

Compound	**Assay Concentrations**	**Physiological Concentrations**	**% Inhibition**[1]
SOD	750 U/mL		83.4
GSH	50 uM		73.3
	100		79.5
	200		98.0
GSSG	50uM		61.7
DTT	50uM		-279.3
	100uM		-217.2
DHA	114uM		3.9
	228		0.0
	456		0.0
Ascorbate	25uM	50-90	32.6
	50		56.3
	100		65.9
	200		73.6
Zinc	0.41ug/mL	0.6 - 1.4	14.9
			27.5
			33.0
			47.6
Urate	0.1mM	0.17-0.48	11.2
	0.5		36.3
	0.83		50.2
Selenium	0.45 ug/mL	0.14-0.56	0
	0.91		0
	1.36		0
	1.82		0
Retinol Acetate	2.5 ug/mL	0.37-1.20	0
	5.0		0
	10.0		0
Tocopherol Acetate	10.0 ug/mL	2.85-14.1	0
	20		0

[1] Ascorbate, zinc, DTT and GSH at 100 umol/L did not affect xanthine oxidase directly. This would indicate that ascorbate, zinc and glutathione inhibit by scavenging free oxygen radicals. DTT must enhance electron transfer reduction of NBT to formazan since it did not affect xanthine oxidase directly. GSSG at 100 umol/L inhibited xanthine oxidase 48 %. Since the inhibition at 50 umol/L of GSSG is 61.7 %, it must inhibit both xanthine oxidase directly and also by scavenging free oxygen radicals. Uric acid at 1 mmol/L inhibited xanthine oxidase by 66 % similar to the effects seen on NBT reduction. Uric acid has been shown to inhibit xanthine oxidase with a K_I of 0.1-0.16 mM (Chalmers, Knight, & Atkinson, 1969) and so its main effect is likely to be inhibition of xanthine oxidase. Negative values indicate stimulation of NBT reduction by superoxide anions.

FIGURE 3.5

Experiment 4: A dose response curve which indicates the effect of zinc (O), ascorbate (●) and the combined effects of zinc and ascorbate (Δ) on inhibiting the reduction of NBT by superoxide anion. The combined zinc and ascorbate concentrations used are indicated by the interconnecting arrows (↔).



100
80
60
40
20
0
% Inhibition
20
40
60
80
100
120
140
160
200
Ascorbate (μmol/L)
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
Zn (μg/mL)

3.4 Experiment 5: in vitro study on NT

Experiment 5 was conducted to assess the in vitro effect of superoxide anion directly on lymphocytic NT and to assess whether ascorbate is protective of this process.

3.4.1 Method

(i) Subjects

Subject A was a 32 year old female PhD student from the Psychology Department at the University of Adelaide. She was on a high antioxidant diet which consisted of taking the following supplements daily: d-alpha tocepherol (vitamin E) 335 mg, zinc gluconate 50 mg, ubidecarenone 10mg (co-enzyme Q10), ascorbate 1000 mg, supplements daily. In contrast, subject B was a 57 year widower from the Adelaide metropolitan area with 18 years of high school and tertiary education. The subjects initial scores on the POMS, BDI, and STAI are shown on Table 3.4.

(ii) Materials

Materials were as described in Experiment 4.

(iii) Procedure

On the recommendation of a naturopath Subject A had been on a high antioxidant diet for 6 weeks prior to her psychological, immunological and antioxidant assessment. At the beginning of the experiment, Subject B was taking no antioxidant supplements prior to his first psychological, immunological and antioxidant assessment (Condition 1). Subsequently, subject B was placed on ascorbate 0.5 grams for the duration of six weeks prior to his second psychological, immunological and antioxidant assessment (Condition 2). Finally, he was placed on the same range of antioxidant supplements as subject A for the duration of six weeks prior to his third psychological, immunological and antioxidant assessment (Condition 3).

16x5ml (80mls total) of EDTA anti-coagulated blood was taken from each subject by routine venepuncture for this study, and lymphocytes were isolated as described previously

(Chalmers & Hare, 1990). The lymphocytes were then suspended in 0.5 mL of tris saline buffer, and were exposed to superoxide anions by mixing them with 0.5 mL xanthine (400 umol/L) and adding 5 uL xanthine oxidase (100 milliunits; Sigma Chemical Co., Mo, USA). At defined time points, the cells were diluted into 10 mL of tris saline, separated by centrifugation (1200 rpm for 10 min) and washed twice in 10 mL tris saline before being suspended into 0.3 mL of tris/saline and frozen at -20°C. The thawed lysate was ultrasonicated at 4°C and NT and ascorbate measured on the lysate as previously described in this thesis.

In the protection assay, 10 uL of ascorbate (10 mmol/L in water) was added to the 1 mL reaction volume of xanthine and lymphocytes to give a final concentration of 100 umol/L of ascorbate. Xanthine oxidase was added as above and at defined time points, the reaction stopped by dilution into 10ml tris saline followed by immediate centrifugation and washing steps.

3.4.2 Results of in vitro study on NT

(i) Psychological data.

Table 3.4 shows psychological variables and antioxidant regime for the two subjects in this experiment.

Table 3.4
Experiment 5: The scores for psychological variables for subject A and B

				POMS Variables						
	Antioxidant Therapy (A/O)	BDI	STAI -trait	T	D	A	V	F	C	TMD
Subject A	High A/O	2	40	5	2	4	16	5	3	3
Subject B	No A/O	21	55	17	27	3	13	20	10	64
Subject B	Ascorbate only	22	60	20	25	2	12	20	12	67
Subject B	High A/O	20	45	7	6	3	15	12	10	49

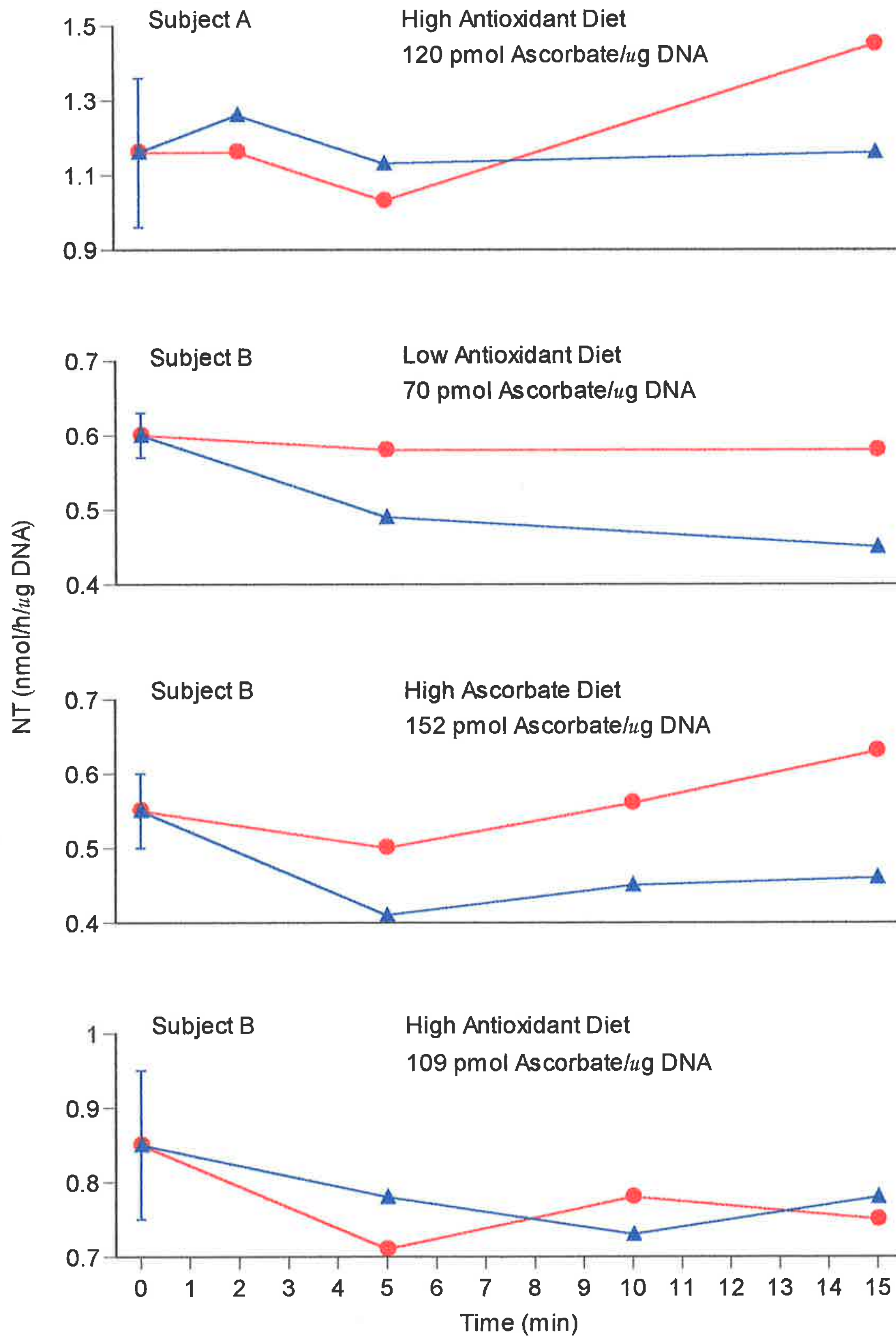
(ii) NT and antioxidant data

Figure 3.6 summarises the effect of the superoxide anion on lymphocytic NT in subject A during a high antioxidant intake, and in subject B on low antioxidant intake (Condition 1), high ascorbate intake (Condition 2), and high antioxidant intake (Condition 3). In contrast to subject B, ascorbate was not required in subject A to protect NT from superoxide radical damage. When subject B had his ascorbate stores increased from 70 to 152 pmol/ug DNA by a daily oral intake of 0.5 g ascorbate/day for 6 weeks, this increased ascorbate did not protect NT from damage by oxygen radicals. The NT activity of subject B was about half that of subject A on both high and low ascorbate intakes. When subject B was taking the same antioxidant supplements as subject A for a period of six weeks, his NT value increased from 0.55 to 0.85. In addition, subject B's high antioxidant diet protected NT from superoxide radical damage.

FIGURE 3.6

Experiment 5: The effect of the superoxide anion on lymphocytic NT in subject A during a high antioxidant intake, and in subject B on low antioxidant intake (Condition 1), high ascorbate intake (Condition 2), and high antioxidant intake (Condition 3).

Lymphocytic NT measured in the presence of xanthine/ xanthine oxidase/ ascorbate (●) and xanthine/ xanthine oxidase (▲) are indicated. Time points shown as the means (±SD) represent 6-8 measurements.


Subject A
High Antioxidant Diet
120 pmol Ascorbate/ug DNA
1.5
1.3
1.1
0.9
Subject B
Low Antioxidant Diet
70 pmol Ascorbate/ug DNA
0.7
0.6
0.5
0.4
NT (nmol/h/ug DNA)
Subject B
High Ascorbate Diet
152 pmol Ascorbate/ug DNA
0.7
0.6
0.5
0.4
Subject B
High Antioxidant Diet
109 pmol Ascorbate/ug DNA
1
0.9
0.8
0.7
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (min)

3.5 Discussion

The reported studies indicate that the superoxide anion can directly affect NT and that antioxidants protect against this damage. The finding of low tissue ascorbate stores in the human models is similar to a report by Maes and co-workers who found reduced serum levels of zinc, a metal antioxidant, in depressed patients (Maes et al., 1994c). Though the mechanism for reduced ascorbate stores is not known, it may be due to oxygen radical-mediated damage of this vitamin. Similar findings have been found in other clinical scenarios where increased oxygen radical levels have been shown to occur. For example, patients with diabetes mellitus have a 30% reduction of reduced erthrocytic glutathione compared to healthy controls, and the demand for vitamin E is increased in this condition (Jain & McVie, 1994; Sundaram et al., 1996). Similarly, HIV-seropositive patients have a reported severe b-carotene deficiency attributed to overproduction of oxidative radicals (Sappey et al., 1994). In fact, several antioxidants are destroyed by oxidative stress both in vivo and in vitro (Murphy, Kolvenbach, Aleksio, Hansen, & Sies, 1992; Palozza & Krinsky, 1991). Thus low levels of both zinc and ascorbate stores in stressed subjects may partly explain the depleted levels of NT seen in these groups. The finding of a significant positive correlation between NT and tissue ascorbate would suggest that ascorbate is a factor contributing to NT protection from free radical damage.

Subject B, described in this study is a widower of four years standing, who neither ate healthily, nor took any vitamin supplements. His BDI scores suggested that he was moderately depressed during the study. In contrast, subject A was not depressed, and was conscious of her diet, supplementing it with antioxidants as described above. The finding that a high ascorbate intake of 0.5 g/day for one month in subject B failed to protect NT against oxygen radical damage despite a two-fold increase in tissue ascorbate stores would suggest that ascorbate given as a sole antioxidant was unable to protect NT (Figure 3.6). It is likely that a combination of antioxidants is required to protect NT fully and this was confirmed in

Condition 3 (Figure 3.6). The assay used in the in vitro study on NT measures both dehydro and ascorbic acid. Therefore, the ascorbate in subject B may have been in the dehydro form since NT was not protected from oxyradical attack in this subject. Assays using high pressure liquid chromatography can differentiate ascorbate from its dehydro form and would have, in retrospect, been more appropriate for this study. Also antioxidants, like ascorbate, given alone can be prooxidant and therefore a more physiological mixture of antioxidants may be required to give antioxidant protection against free radicals (Herbert, 1994). The results on subject B shown in Figure 3.6 confirmed this hypothesis.

The NBT study suggested that glutathione in addition to ascorbate and zinc may have a protective role to play against oxygen radical-mediated damage to NT. This is consistent with findings by Liu & Mori (1994) that the administration of reduced glutathione showed a protective effect on the stomach bleeding in stressed rats. They also hypothesised that stress induces the formation of oxygen radicals and leads to oxidative damage. The previous finding in Chapter 2 that both people diagnosed with major depression and highly stressed subjects have a low NT would indicate that the levels of these antioxidants in these people may be too low to give cell protection. Therefore, it may be judicious to increase a patient's intake of antioxidants during stressful periods. In fact recent studies have shown suppression of HIV expression in infected monocytic cells by glutathione (Kalebic, Knites, Poli, Anderson, & Meister, 1991), and various antioxidants including ascorbate have been shown to protect against infection (Meydani, Wu, Santos, & Hayek, 1995). The lack of inhibition seen with Vitamin A, and Vitamin E is perhaps not surprising since these substances are active in a lipid milieu, or, like selenium, must first be incorporated into an enzyme in order to show anti-oxidant activity (Combs, 1992).

It must be acknowledged that a limitation of this research is that numbers were small in the in vitro study due to the large volume of blood required and the complexity of the experiment. Though it is not possible to generalise from a sample of two the results were consistent and

indicated that the reduction of NT in stressed and depressed persons can be mediated by reactive oxygen species, and that ascorbate and other antioxidants may be protective of this process.

CHAPTER 4: THE EFFECT OF DEPRESSION IN AN ANIMAL MODEL ON 5'-ECTONUCLEOTIDASE, ANTIBODY PRODUCTION AND TISSUE ASCORBATE STORES[6]

4.1 Introduction

The previous experiments in humans in this thesis have shown that NT (Experiment 1 and 2), and ascorbate stores (Experiment 3) were significantly depleted in individuals who were seriously depressed, or experiencing stress associated with thesis submission / examinations for an honours Psychology course. Moreover the in vitro studies (Experiments 4 and 5) support the hypothesis that this process is mediated by oxygen radical damage.

There is also persuasive evidence suggesting a relationship between psychological stress/depression and disease in animals (reviewed by Sheridan et., al., 1994; Sternberg et al., 1992). The aim of the experiment reported in this Chapter was to see if the results obtained in humans would generalise to an animal model of depression. The animal model used is Seligman's model of learned helplessness is described in the introduction (Seligman, 1975) (Chapter 1). The following experiment is modelled on the principles and apparatus originally described by Seligman and his colleagues to test their theory of learned helplessness (Seligman, 1975; Peterson, Maier, & Seligman, 1993).

4.2 Experiment 6

This experiment examined three hypotheses in relation to a learned helplessness model of depression in albino Sprague Dawley rats. First, NT is a sensitive measure of immune suppression induced by learned helplessness. According to the learned helplessness model it was hypothesised that NT would be significantly reduced in rats experiencing uncontrollable events. Second, ascorbate levels are depleted in rats that are exposed to uncontrollable events. It was hypothesised that rats exposed to uncontrollable events would have depleted ascorbate levels relative to a control group, and rats which could control aversive events. Third, the

[6] The experimental work cited in this Chapter has been submitted for publication to the International Journal of Immunopharmacology.

stress induced by learned helplessness reduces antibody responses to sheep red blood cells (SRBC). It was hypothesised that rats exposed to uncontrollable events would have a reduced humoral immune response relative to a control group and rats which could control events.

4.2.1 Method

(i) Subjects

Subjects consisted of 47 Albino Sprague Dawley rats aged between 140 and 180 days, and weighing 400-600g at the commencement of the experiment. They were housed individually, maintained on a 12-hr light/dark cycle, and had free access to food and water. Animals were randomly assigned to one of seven groups as follows:

1) A no shock group which was not immunised with sheep red cells, NS (-SRBC) (n=5)

2) A no shock group which was immunised with SRBC, NS (+SRBC) (n=5)

3) A control group, C (n=7)

4) An acute escapable shock group, ES (n=7)

5) An acute inescapable shock group, IE (n=7)

6) A chronic escapable shock group, ES (n=8)

7) A chronic inescapable shock group, IE (n=8)

In this experiment there are three treatments rats may undergo. 1) The pretreatment which is the shock treatment rats undergo to generate learned helplessness in these animals. 2) The test task is the test rats undergo to demonstrate their learned helplessness. 3) Immunisation with antigen (SRBC).

The no shock groups were not involved in pretreatment or test task, and were included to control for the possibility that exposure to SRBC or electric shocks may affect NT, ascorbate and antibody measures. The E and IE rats in the acute and chronic conditions received the pretreatment and test task as described below. The C rats received the test task, but no pretreatment.

(ii) Pretreatment apparatus

As illustrated in Figure 4.1 pretreatment was conducted in clear plexiglas boxes, measuring 24 cm long, 21.5 cm high and 13.5 cm wide. The floor consisted of 15 stainless steel bars, 2 mm in diameter and spaced 10mm apart. A plexiglas wheel, measuring 9.5 cm diameter and 7 cm wide extended 3 cm into the box through an opening in the front wall, 8 cm from the floor. Unscrambled shock was delivered by a Grason-Stadler generator (Series 700) to the bars of the grid floor at an amplitude of 1 mA. Twelve aluminium rods 7 cm long and 0.5 cm in diameter were spaced evenly around the circumference of the wheel. The wheel turned smoothly when downward pressure was applied to the rods (see Figure 4.1).

A photoelectric sensor and light emitting diode (LED) was attached to the frame of the wheel. As the wheel turned the infra-red beam generated by a LED was transmitted to the photoelectric sensor. The pulses generated by the sensor were read by the computer (1 pulse represented 1/12 of a turn of the wheel). The computer was programmed to turn off the shock after a specified criterion was reached which is discussed in the procedure section.

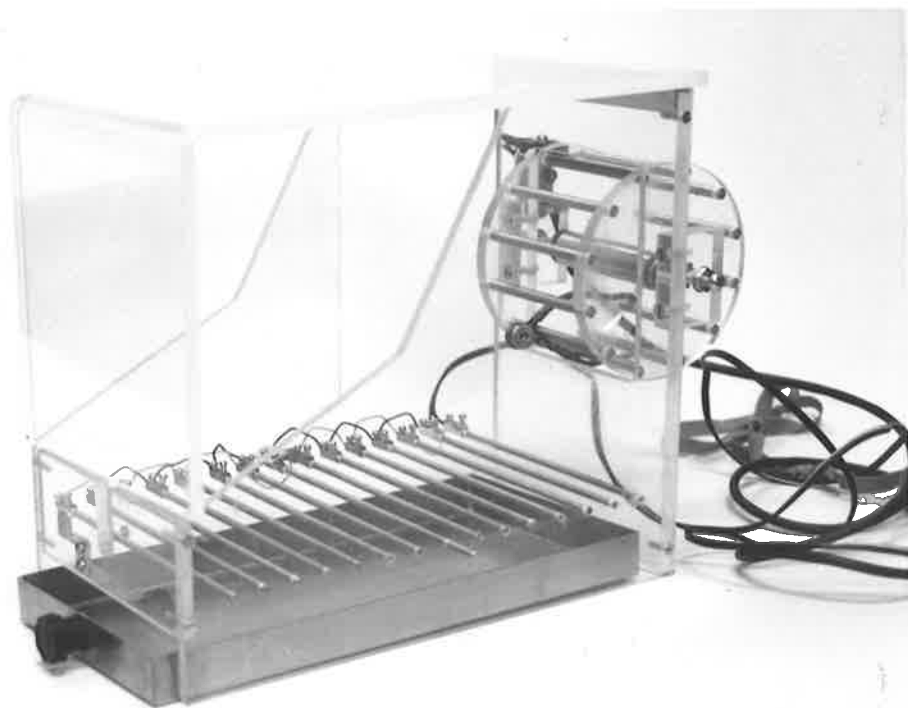


FIGURE 4.1

Experiment 6: The apparatus used in the pretreatment task.

(iii) Test task apparatus

Figure 4.2 illustrates one of the shuttleboxes used as the test task apparatus. Briefly, the test task apparatus consisted of two of these shuttleboxes measuring 490 cm long, 220 cm high and 220 cm wide. The front and back of the shuttleboxes were made of plexiglas, with aluminium side walls. The shuttleboxes were divided into two equal compartments by a central barrier measuring 21cm long, 5cm high, and 1.5mm wide. Constant lighting was provided by two 3-W incandescent lights located in the centre of each side wall of the shuttlebox, 185 cm from the floor. The floor consisted of 15 bars, 2 mm in diameter and spaced 10mm apart. Scrambled shock was delivered to the grid floor by a Grason-Stadler generator (Series 700). Each shuttlebox was housed in a sound attenuating chamber, measuring 550 cm long, 330cm high, and 320 cm wide. The sound attenuating chamber contained an exhaust fan which masked extraneous noise (See Figure 4.2). The experimental program and data recordings were controlled by IBM 286 PC computers. Two photoelectric sensors were attached to the grid floor on either side of the shuttle. Both sensors were positioned 1 cm from the centre of the shuttle. The photoelectric sensors were triggered by the movement of the rat from one side of the shuttle to the other. The computer terminated the shock after an FR-1 (a single crossing) or FR-2 (a two-way crossing) response, as specified by the program.

FIGURE 4.2

Experiment 6: The apparatus used in the test task.

(iv) Pretreatment procedure

Pretreatment differed in the acute and chronic conditions. In the acute condition escapable rats were given 100 shocks on one day. During the chronic condition escapable rats were given 20 shocks daily for 5 days on a variable 60-s schedule (range = 30-90 s). Apart from this difference between chronic and acute conditions the procedure was as follows. A yoked triadic design was used. Rats in the escapable (E) and inescapable (IE) conditions were yoked. The E rat could turn a wheel to stop the electric shocks, whereas the yoked IE rat had no control over the shocks. Each E rat was yoked to its IE counterpart so that it received identical shocks, but the IE rat was unable to switch off the shock by turning a wheel. Rats in the C group remained in their home cage for the duration of the task, and received no pretreatment shock. Shock was 1.0 mA in intensity. As previously described, (Maier, 1990), an automated shaping program was employed which ensured that the animal's response on any trial was contingent on their current performance. A response was defined as one quarter of a turn of the wheel. Responses during the first 0.8 s of shock had no effect (Maiėr & Jackson, 1977). One response (a quarter of a turn of the wheel) was needed to turn off the shock on the first five trials. The response requirement was then altered depending on the rat's performance. If the rat's escape latencies were under 5 s for the previous three consecutive trials, the response requirement was increased by 1, by 2 after four consecutive trials, by 3 after 5 consecutive trials, and continued in this manner until a maximum requirement of 16 responses (four turns of the wheel). If however, the escape latency on the previous trial exceeded 5s, the requirement remained the same. A failure on any trial to turn off the shock within 30s caused the requirement to revert to 1 response. This procedure was designed to eliminate "reflexive" and "superstitious" responding (Maier & Jackson, 1977).

(v) Test task procedure

To demonstrate the learned helplessness effect in the IE group a test task involving a shuttle test was applied. The procedure for this test was as follows. Rats in the E, and IE groups in both conditions, and the C group underwent this test. In the acute condition, the test task was conducted on Day 2, 24 hours after exposure to pretreatment. In the chronic condition, the test task was given on Day 6, 24 hours after pretreatment. The test task involved five trials in which a single crossing of the shuttle was required to turn off the shock, (FR-1 trials), followed by a three minute interval, and then 25 trials in which rats had to cross to the other side of the shuttlebox and back to stop the shock (FR-2 trials).

All of the rats except for the NS (-SRBC) group were immunised intraperitonally (i.p.) with 5×10^8 SRBCs in 1 mL isotonic saline 24 hours after the test task was completed. In the acute condition immunisation occurred on Day 3 and in the chronic condition it occurred on Day 7. Five days after immunisation, rats were anaesthetised with an i.p. injection of Nembutal (1 mg/250 g rat), and blood was removed by cardiac puncture and serum separated from the clotted blood. The spleen was also removed and was gently teased open and filtered into 10 mL of Hanks BSS. In the acute and chronic conditions this occurred on Day 8, and Day 13 respectively, or Day 5 post immunisation. Antibody studies were done on the serum and spleen cells were used for measuring NT and ascorbate. Rats in the NS (+ SRBC) group were treated in a corresponding manner, but did not receive any shocks.

(vi) Materials for immunological, antioxidant and antibody assays

1. Guinea pig complement (lyophilized; Cat No S-1639) as a lyophilizate purchased from Sigma Chemical Company, St Louis Mo. USA, was dissolved in 5 mL of veronal gelatin buffer and stored frozen in 0.2 mL aliquots at -20°C.

2. Sheep Red Blood Cells (SRBC) were collected fresh into EDTA by the Animal House at Flinders Medical Centre (FMC) and washed once in isotonic saline, twice in 0.1 M EDTA (K^+) / isotonic saline and twice in veronal buffer with 0.1 % gelatin. The washed cells were then stored at 5 % (v/v) in veronal / gelatin buffer at 4°C.

3. Buffers. All buffers were prepared as described in Mayer (1971) and Weir (1978).

(vii) Procedure for ascorbate assay

Ascorbate in the rats was measured as described in the human research in Experiment 3.

(viii) Procedure for rat antibody assay

The basic principle of this assay is that antibody (in rat serum) bind antigen (SRBC) which then lyses the SRBC in the presence of complement. The antibody in rat serum is diluted by serial two-fold dilution. The dilution where 50% lyses occurs is used as an index of antibody concentration. Antibody titres were expressed as the two-fold serial dilution of serum giving 50 % lysis in the complement assay. The method was adapted from Mayer (1971). On the day of the assay dilute the (1) 5 % SRBC 5-fold in veronal/gelatin buffer to give 1 % SRBC and check the concentration spectrometrically at 540nm (1 mL of the 1 % SRBC solution plus 2 mL of water should give an absorbance at 540 nm of 0.7); (2) rat serum 10, 20, 40, 80 and 160 fold in veronal/gelatin buffer. To 0.4 mL veronal/gelatin buffer add 0.05 mL serum (diluted as described), 0.2 mL SRBC (1%) and 0.02 mL complement. Incubate at 37°C for 1 hour, centrifuge and read the absorbance of the supernatant at 540 nm and 650nm. The 650 nm absorbance allows for different thicknesses of cuvettes, and other extraneous background absorbances. With the assay run a blank of 0.45 mL veronal/gelatin, 0.2 mL

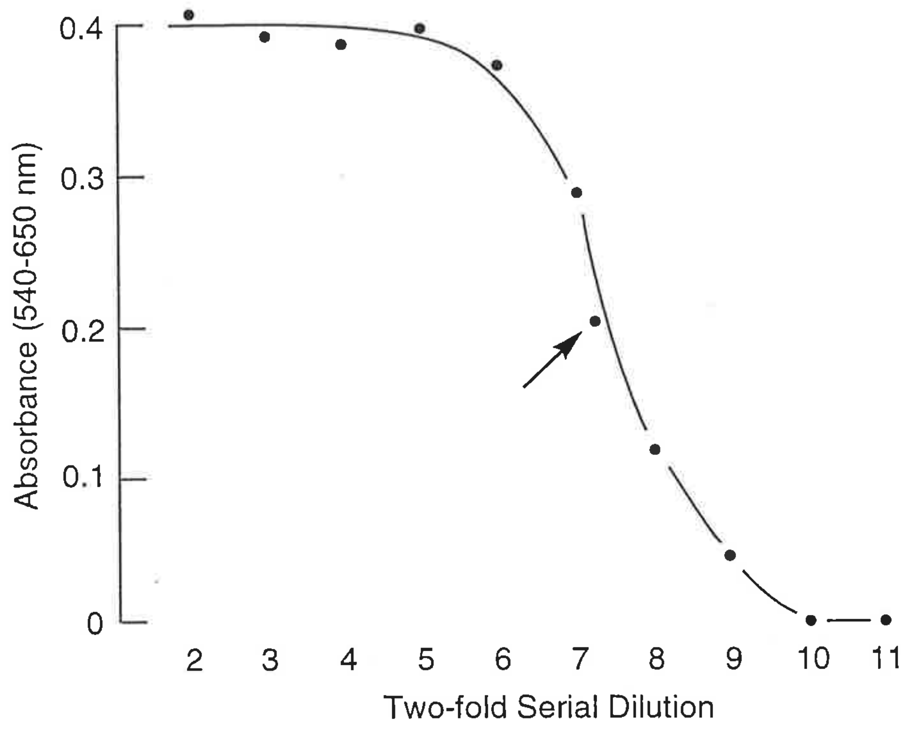
SRBC and 0.02 mL of complement and a test of 0.45 mL water, 0.2 mL SRBC and 0.02 mL complement. Read the absorbances against the blank and the test represents 100 % lysis. Plot the net absorbance at 540 nm (OD540nm-OD650nm) against the serial two-fold dilution of serum on the x-axis. The dilution which gives 50 % lysis is an indicator of antibody response against SRBC. Figure 4.3 shows an example of how antibodies were measured. 50% inhibition is indicated by the arrow, and is the value that was used for quantifying antibodies in serum.

(ix) NT assay in rats

Spleens were teased and filtered into 10 mL Hanks balanced salt solution (BSS). The large cellular components were allowed to settle for about 5-10 min. 3 mL of the supernatant was diluted with 5 mL tris (10mM, pH7.4) / isotonic saline buffer (tris saline), floated gently onto 3 mL of Lymphoprep (Nycomed Pharma, Oslo, Norway), and centrifuged at 1800 rpm for 20 min. The lymphocyte band was gently removed and washed three times with 10 mL tris saline by cell pelleting at 1200 rpm for 10 min. The final cell pellet was made to 0.5 mL with tris saline and frozen at -20°C. The thawed homogenate was then treated as the cell isolates from humans for DNA estimations as previously described (Chalmers & Hare, 1990) by sonicating on ice with 15 pulses of microwave energy. The DNA content of the homogenates was calculated by diluting 0.02 mL of homogenate into 0.5 mL of water, reading absorbances at 260 and 280 nm and using the Warburg-Christian (1942) formula. DNA concentrations of the rat extracts were adjusted to between 4-8 ug/mL (after dilution) with tris/saline. Higher concentrations of DNA gave NT values beyond the range of the assay. Concentrations lower than 4 ug/mL allowed measurement of NT. NT was assayed by the method of Chalmers and Hare (1990).

FIGURE 4.3

Experiment 6: Complement-mediated lysis of SRBC in the presence of serum containing antibodies against SRBC. The serial two-fold dilution giving 50% lysis (indicated by the arrow) is used as an index of the antibody concentration in serum.



Absorbance (540-650 nm)
0.4
0.3
0.2
0.1
0
2
3
4
5
6
7
8
9
10
11
Two-fold Serial Dilution

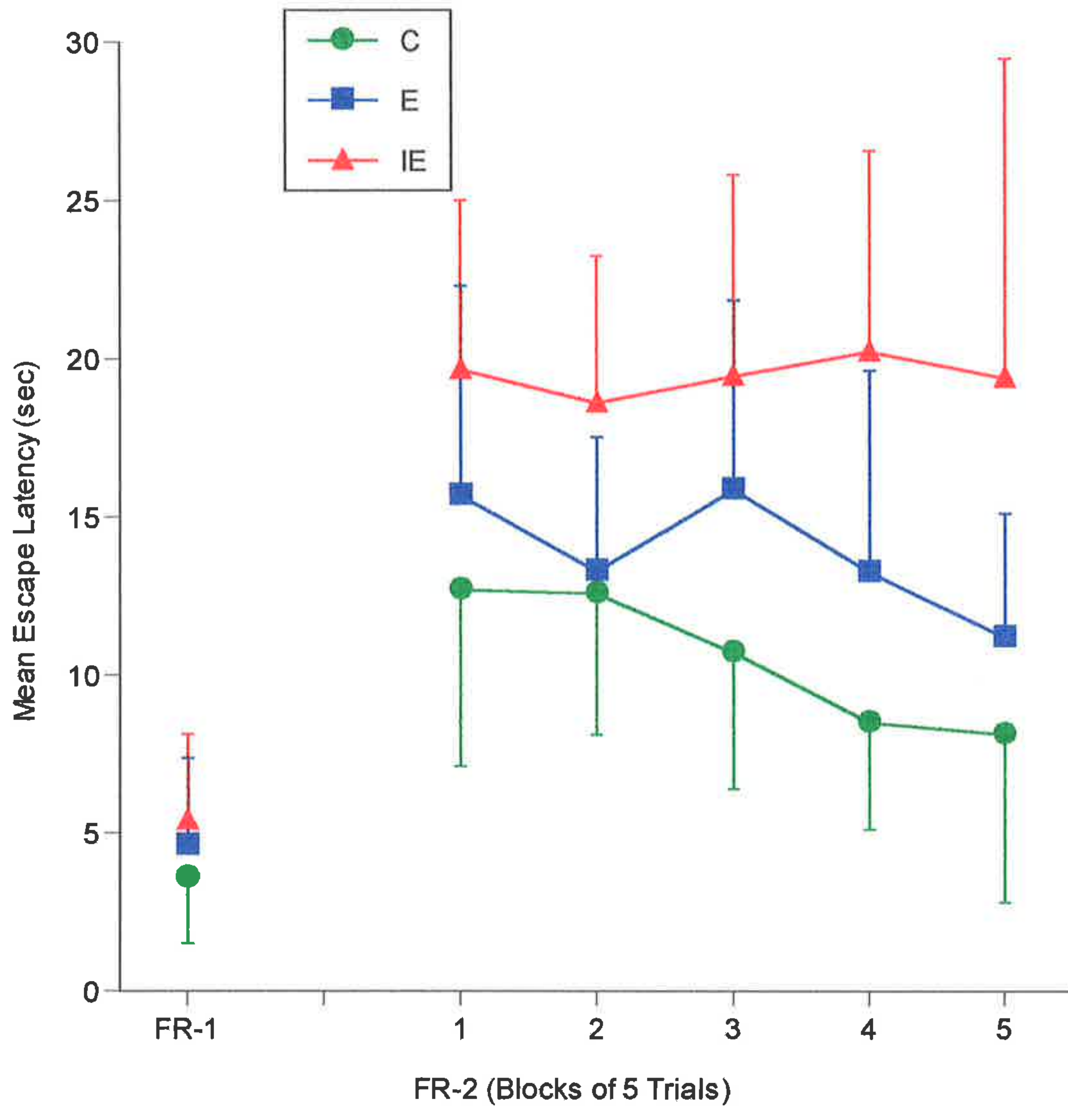
4.2.2 Results

(i) Behavioural variables

In both acute and chronic conditions all of the rats in the E group learnt to escape the shock by turning the wheel, and by the end of training reached the criterion of 16 responses. Figure 4.4 shows mean shuttle escape latencies for FR-1 and FR-2 trials in the acute condition. In the acute condition escape latencies did not differ between groups during FR-1 trials, $F(2, 18) < 1.00$. As expected, group differences became apparent when the requirement was increased to an FR-2 response. Pre-exposure to IE shocks resulted in significant performance deficits in the IE group compared to E and C groups. Group differences were analysed by a two-way analysis of variance (Groups x Trials) on FR-2 latencies. This resulted in a significant main effect of group, $F(2, 18) = 6.10$, $p<.01$. Post hoc Fisher Protected t-tests on the overall means indicated that the IE group was significantly slower in their performance than both E and C groups ($p<.05$), while performance in E and C groups was not significantly different. There was also a main effect for trials, $F(4, 72) = 2.48$, $p < .05$, indicating that overall performance improved over time: Trial 1 mean (SD) = 16.02 (6.40) relative to Trial 5 mean (SD) = 12.91 (8.21). The interaction between variables was not significant $F(8, 72) = 1.26$, $p > .05$.

FIGURE 4.4

Experiment 6: Mean shuttle escape latencies and standard deviations for FR-1 and FR-2 trials in the acute condition. A circle (O) indicates the C group, a square (□) indicates the E group, and a triangle (Δ) indicates the IE group. Standard deviations are shown as vertical bar lines.



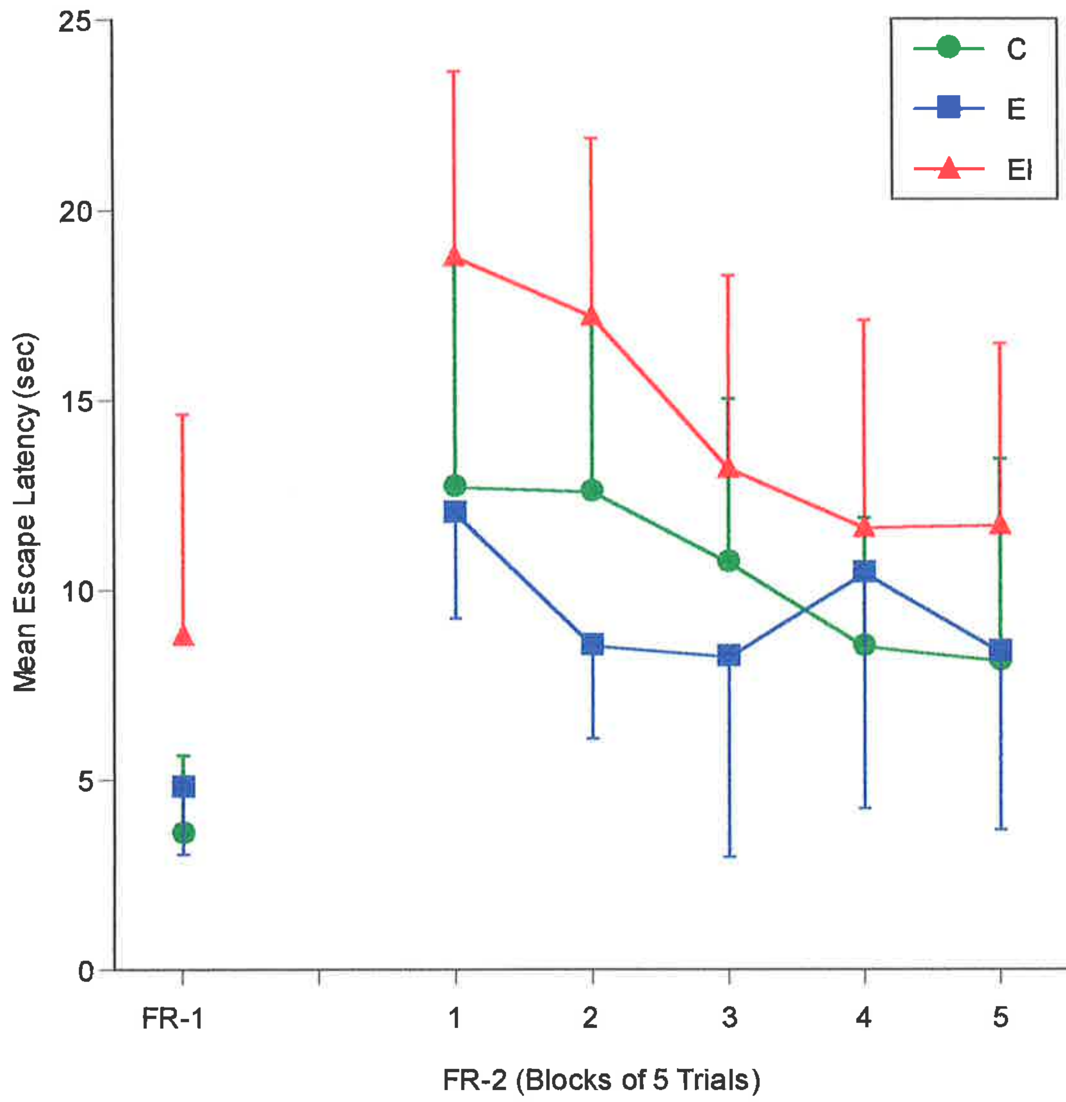
C
E
IE
Mean Escape Latency (sec)
30
25
20
15
10
5
0
FR-1
1
2
3
4
5
FR-2 (Blocks of 5 Trials)

Figure 4.5 shows mean shuttle escape latencies for FR-1 and FR-2 trials in the chronic condition.[7] Analyses were performed in the same manner as for the acute condition. In contrast to the acute condition, pre-exposure to IE shocks on the FR-1 schedule in the chronic condition resulted in significantly poorer learning in the IE group relative to the E and C groups, $F (2, 20) = 3.94$, $p < .05$. Post hoc Fisher Protected t-tests on the overall means indicated that the IE group was significantly slower in their performance than the E and C groups ($p<.05$). There was no main effect of trials, $F(4, 80) = 1.76$, $p > .05$, suggesting that overall performance for the groups did not change significantly over time. The interaction between variables was not significant $F (8, 80)<1.00$. During the FR-2 response there was a significant main effect of group, $F (2, 20) = 4.67$, $p < .05$. Post hoc Fisher Protected t-tests on the overall means indicated that the IE group was significantly slower in their performance than both E and C groups which did not differ ($p<.05$). There was also a main effect for trials, $F(4, 80) = 7.30$, $p < .001$, indicating that overall performance improved over time (Trial 1 mean (SD) = 14.57 (5.44) relative to Trial 5 mean (SD) = 9.45 (4.98). The interaction between variables was not significant $F (8, 80)=1.52$, $p>.05$.

[7] In this thesis all multifactorial ANOVA's with unequal sample sizes use the regression approach (unweighted means) recommended by Overall & Spiegel, (1969) to overcome the problem of nonorthogonality.

FIGURE 4.5

Experiment 6: Mean shuttle escape latencies and standard deviations for FR-1 and FR-2 trials in the chronic condition. A circle (O) indicates the C group, a square (□) indicates the E group, and a triangle (Δ) indicates the IE group. Standard deviations are shown as vertical bar lines.



Mean Escape Latency (sec)
25
20
15
10
5
0
FR-1
1
2
3
4
5
FR-2 (Blocks of 5 Trials)
C
E
EI

(ii) NT results

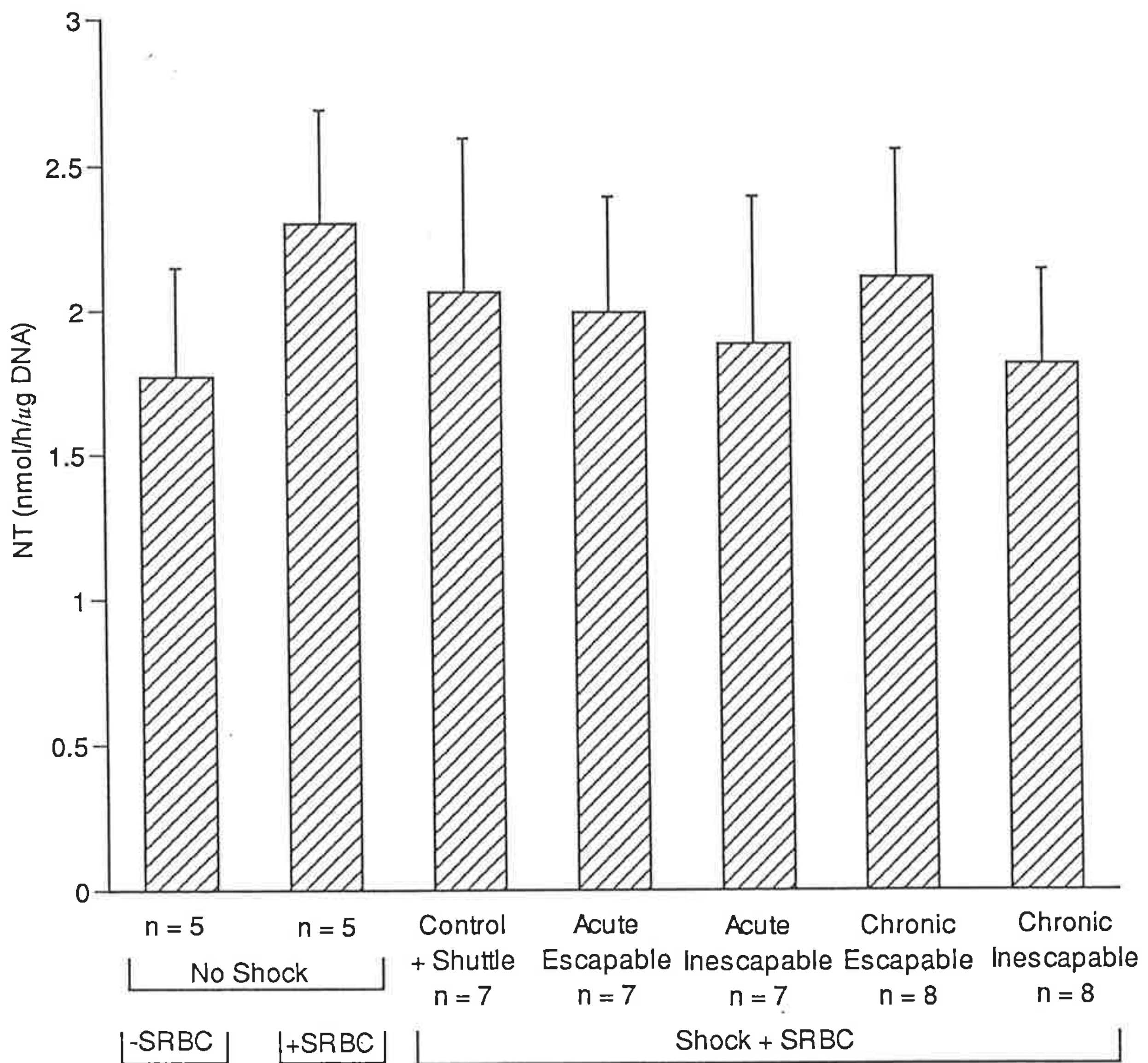
Figure 4.6 shows the means and standard deviations of NT activities for the seven groups. Although the mean scores of both the acute and chronic inescapable groups were less than their respective escapable groups they did not differ significantly. No significant differences were found among the rat groups, $F(6, 40) = 1.11$, $p > .05$.

(iii) Antibody results.

Figure 4.7 shows the means and standard deviations of antibody response to SRBC for the seven groups. As expected, all of the groups immunised with SRBC showed a significantly higher humoral response, compared with the no shock group (-SRBC) not immunised with SRBC. Contrary to learned helplessness theory there were no significant differences between the C group and other E and IE groups receiving shock. However, the groups receiving shock had significantly elevated antibody responses, compared with the no shock groups, $p < .05$. Group differences were analysed by a one-way ANOVA, $F(6,40) = 15.76$, $p < .001$. Post hoc Fisher Protected t-test comparisons revealed a significant difference between 1) the no shock group (-SRBC) compared to the other groups ($p < .05$), and 2) the no shock groups compared to the groups that experienced pretreatment and / or test-task (IE, E and C groups), $p < .05$.

FIGURE 4.6

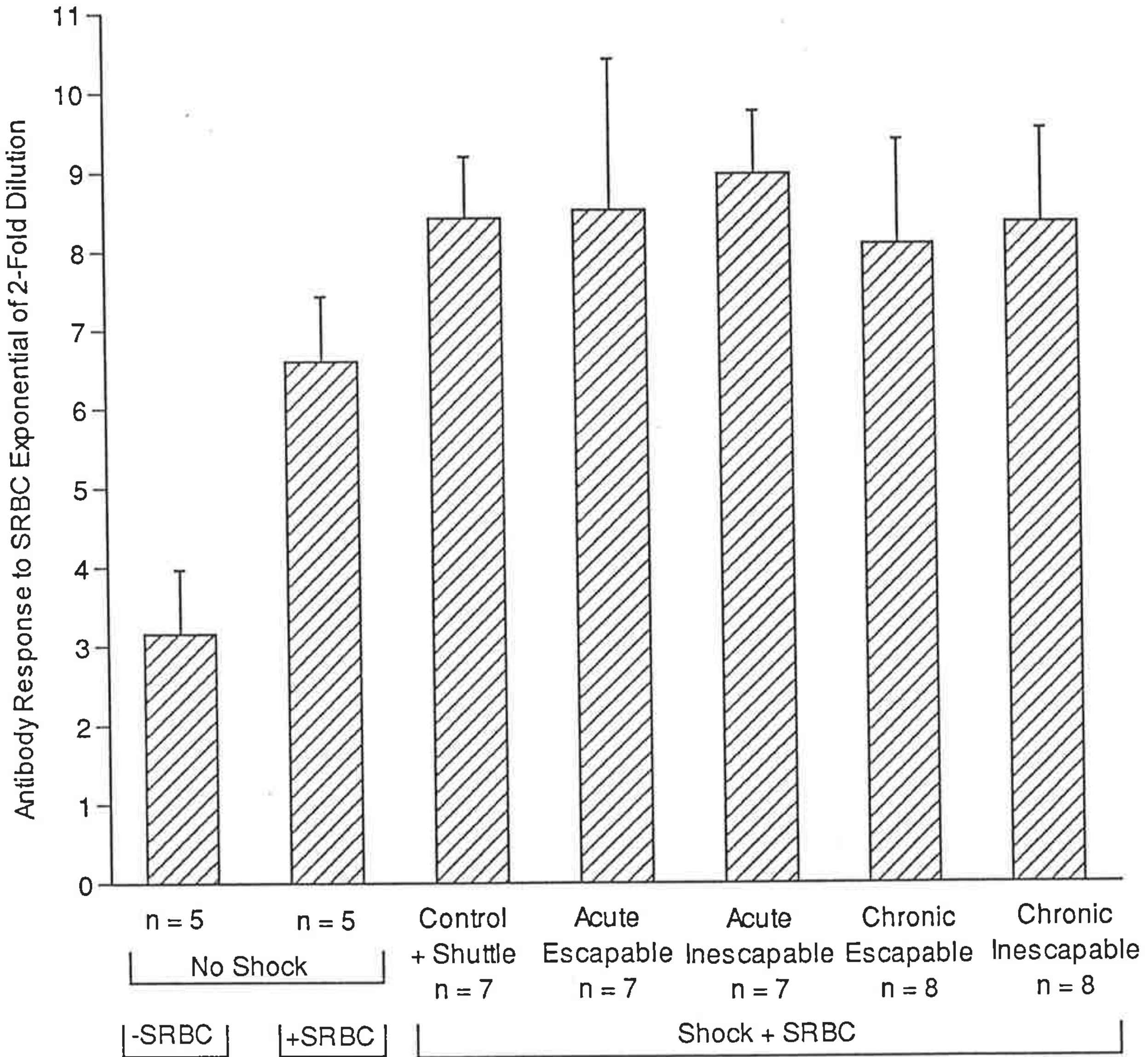
Experiment 6: Means and standard deviations of NT activities for the seven groups.



NT (nmol/h/μg DNA)
3
2.5
2
1.5
1
0.5
0
n = 5
n = 5
Control + Shuttle n = 7
Acute Escapable n = 7
Acute Inescapable n = 7
Chronic Escapable n = 8
Chronic Inescapable n = 8
No Shock
-SRBC
+SRBC
Shock + SRBC

FIGURE 4.7

Experiment 6: Means and standard deviations of antibody responses to SRBC for the seven groups.


Antibody Response to SRBC Exponential of 2-Fold Dilution
11
10
9
8
7
6
5
4
3
2
1
0
n = 5
n = 5
Control + Shuttle n = 7
Acute Escapable n = 7
Acute Inescapable n = 7
Chronic Escapable n = 8
Chronic Inescapable n = 8
No Shock
-SRBC
+SRBC
Shock + SRBC

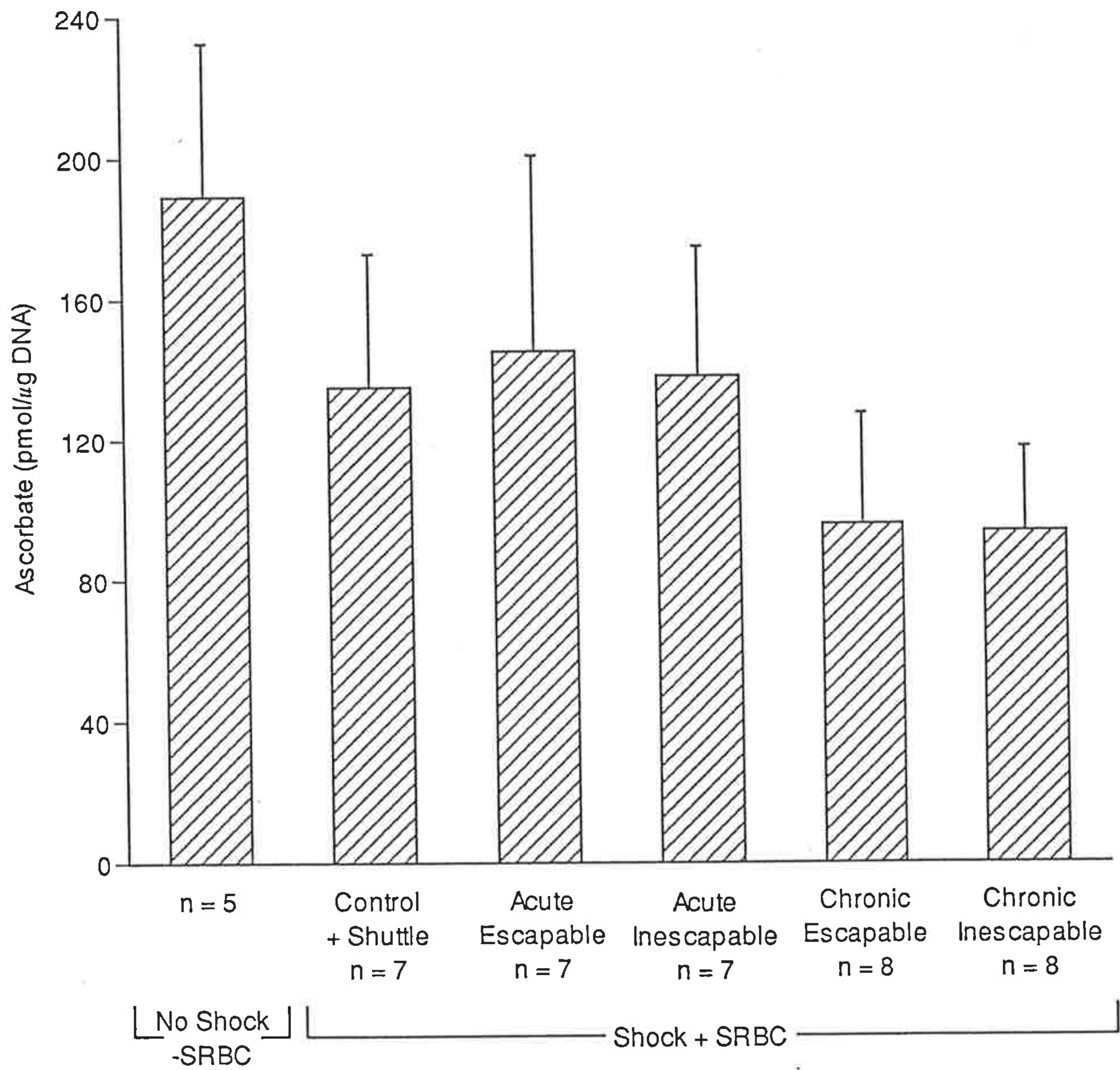
(iv) Ascorbate results

Figure 4.8 shows the means and standard deviations of the six groups. Results for the no shock group (+SRBC) were not available for technical reasons. There were significant differences between the groups in ascorbate tissue levels, $F(5, 35) = 5.43$, $p < .001$. Post hoc Fisher Protected t-tests revealed that 1) the Chronic IE group had significantly lower ascorbate stores than all groups except the chronic E group, 2) The chronic E group had significantly lower ascorbate than the no shock (-SRBC) and the acute E and IE conditions, but did not differ significantly from the C group and the chronic IE group, and 3) The no shock (-SRBC) group had significantly higher ascorbate stores than all the other groups except for the acute escapable group.

A two-way ANOVA (chronicity x escapability) in respect to ascorbate values indicated a main effect for chronicity, $F(1, 28)=11.41$, $p<.01$, but no main effect for escapability, $F(1, 28)<1.00$, and no interaction, $F(1,28)<1.00$. This indicated that the acute and chronic conditions differed significantly in ascorbate values as shown in Figure 4.8.

FIGURE 4.8

Experiment 6: Means and standard deviations of tissue ascorbate stores for the six groups.



240
200
160
120
80
40
0
Ascorbate (pmol/μg DNA)
n = 5
Control
+ Shuttle
n = 7
Acute
Escapable
n = 7
Acute
Inescapable
n = 7
Chronic
Escapable
n = 8
Chronic
Inescapable
n = 8
No Shock
-SRBC
Shock + SRBC

4.2.3 Discussion

The lack of effects seen on NT in the rat experiments suggests that antioxidant levels in the rat are still protective of NT under the experimental conditions used. This is perhaps not surprising since rats are able to synthesise their ascorbate from sugar precursors, unlike humans who require its intake in the diet (Levine, 1986). Nonetheless, ascorbate stores were significantly lower in the chronically stressed rat groups (Figure 4.8) indicating that for these groups more than 5 days post stress are required for these stores to normalise. Contrary to learned helplessness theory, antibody synthesis appeared to be unaffected after acute or chronic uncontrollable stressors (Figure 4.7).

In general it is difficult to correlate the rat findings with those in humans, and this probably reflects the difficulties associated with attempting to generate an animal model of classical depression as experienced by humans. With humans, unlike rats, stressors may persist for long periods of time, and recur frequently. Indeed, controversy exists over the adequacy of learned helplessness in animals as a model of depression (Weisse, 1992). The literature on animal models of depression is confusing. Stress is associated with immune suppression in some animal models (eg. Laudenslager et al., 1983; Mormede et al., 1988, Shavit et al., 1983), no changes in immune suppression in others (eg. Sandi, Borrell, & Guaza, 1992), and sometimes enhancement is reported (reviewed by Weiss & Sundar, 1992). In the current study, the stressor enhanced antibody responses in all groups that experienced electric shocks, relative to the unstressed groups (Figure 4.7). Though the mechanism of this immune enhancement is unknown it may be similar to the enhancement seen with clinical immunosuppressive drugs when given at an inappropriate time in relation to antigen administration (Chalmers, Rotstein, Rao, Marshall, & Coleman, 1985; Santos, 1967).

The interpretation of results in animal models is also made difficult mainly due to variability in the timing between antigen and stressor administration, the nature and concentration of antigen used and the quality, quantity and duration of the electric shock

administered. If the antigen is given before the stressor, immune suppression is generally reported (reviewed in Ader & Cohen, 1993). However it could be argued quite persuasively that one is really studying the effect of the stressor on immunity rather than depression on immunity. For example, if the antigen had been given before the stressor in Experiment 6 this would have been a study on the effect of electric shocks on immunity. Indeed, certain studies done this way have demonstrated lowered immunity in the shocked animals (reviewed in Ader & Cohen, 1993). Electric shocks, per se have been demonstrated to suppress lymphocyte proliferation (e.g. Batuman et al., 1990; Keller, 1981; Weiss et al., 1989); and NKCA (e.g. Cunnick et al., 1988, Keller et al., 1988, Lysle et al., 1990a; Lysle & Maslonek, 1991; Shavit et al., 1984; Weiss et al., 1989). Thus, it is difficult to assess whether the suppressed immunity reported in some studies using a learned helplessness paradigm (e.g. Laudenslager et al., 1988; Zalcman, Minkiewicz-Janda, Richter, Anisman, 1988) is due to a sense of "helplessness" and depression induced in the rat by the experience of uncontrollability or if it is due to the effects of electric shock per se. The aim of Experiment 6 was to generate depression in rats (inescapable group) and demonstrate altered immunity in IE rats when compared with control and escapable rats. Thus the antigen was given after the shocks.

Perhaps longer term administration of stressors may generate more profound depression in animals since animals chronically stressed for five days in our experiment gave some hint of falling NT, and ascorbate stores. Thus a more chronic delivery of stress may be required in order to see significant decreases in NT, ascorbate stores and antibody responses. The notion of developing a better animal model of depression and immunity in future research is discussed in the following Chapter.

CHAPTER FIVE

GENERAL CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

5.1 A potential mechanism

So what is the biological significance of lowered NT activities on lymphocytes of stressed or depressed persons? Homeostatic mechanisms operating between the immune system and the hypothalamic-pituitary adrenal axis have been reviewed (Sternberg et al., 1992), and are shown diagramatically in Figure 5.1 (circle A). After infection, the immune system releases cytokines which stimulate the hypothalamus to release corticotrophin releasing hormone (CRH), which in turn promotes the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland. The subsequent ACTH-mediated corticosteroid release from the adrenal gland then dampens the immune response (Pathway A, Figure 5.1; Sternberg et al., 1992; Barnes & Adcock, 1993).

With stress or depression there appears to be an inhibition of the corticosteroid-mediated down regulation of immunity. It has been suggested that lymphoid tissue may become resistant to the effects of corticosteroids due to the attenuated release of these hormones with stress (Barnes & Adcock, 1993). The model proposed in this thesis suggests that as a consequence of diminished Pathway A activity, Pathway B (circle B, Figure 5.1) becomes more active resulting in an increased production of oxygen radicals by the immune system which then decrease lymphocytic NT. Thus stress inhibits lymphocyte maturation through inhibition of NT and thereby lowers immunity.

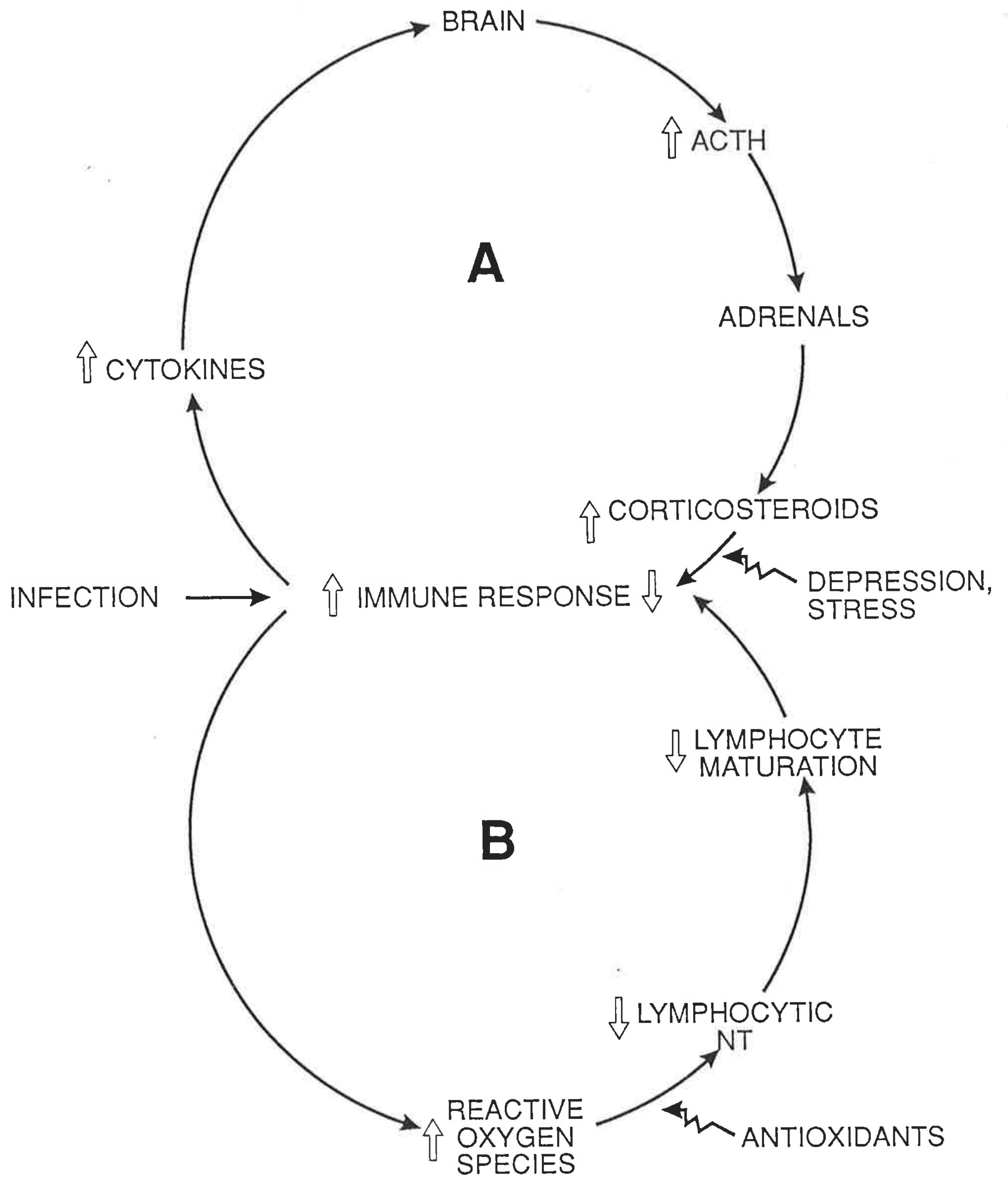
To sum up, in the normal situation corticosteroids control lymphocyte activity through the feedback mechanism shown in Pathway A (Figure 5.1). With stress the effect of this feedback loop decreases and the oxygen radical pathway (Circle B, Figure 5.1) assumes a greater role in controlling immunity. In other words, it is proposed in this model that the HPA and oxygen radicals act to control immunity by the homeostatic mechanisms outlined in circle A and circle B (Figure 5.1). With the intervention of stress, circle B becomes more

activated to control immunity. This in turn may lead to stress-induced sickness when the host is confronted by new pathogens.

Several lines of evidence converge to provide support for the model proposed in this thesis. 1) The finding of normal NT levels in melancholic patients with high antioxidant intakes (Experiment 2); 2) The finding of low tissue stores of ascorbate (Experiment 3), and zinc, an antioxidant metal, in depressed persons (Maes et al., 1994c); 3) The finding of a heightened inflammatory-like process in people with major depression (Joyce et al., 1992; Maes et al., 1993); 4) The finding that HIV positive patients have a low antioxidant status (Sappey et al., 1994). This could explain the lowered NT activities previously reported in these patients (Chalmers et al., 1990); 5) The downregulation of glucocorticoid receptors and impaired sensitivity reported to occur in depressed patients (e.g. Yehuda, Boisoneau, Mason, & Gillin, 1993; Wodarz et al., 1991). No impairment in glucocorticoid sensitivity was found in patients after clinical recovery from major depressive disorder (Wodarz et al., 1992).

FIGURE 5.1

Two homeostatic mechanisms (circle A and circle B) operating to control immunity. Open arrows indicate increased (⇧) or decreased (⇩) activities or concentrations, and wavy arrows inhibitions. Full arrows (→) show the effect of different processes on the production and subsequent interactions of various chemicals with tissues influencing immunity.


BRAIN
ACTH
A
ADRENALS
CYTOKINES
CORTICOSTEROIDS
DEPRESSION,
STRESS
INFECTION
IMMUNE RESPONSE
LYMPHOCYTE
MATURATION
B
LYMPHOCYTIC
NT
REACTIVE
OXYGEN
SPECIES
ANTIOXIDANTS

5.2 Future directions for research into this mechanism

Several directions of future research seem evident and arise from the work presented in this thesis.

(i) Future directions for human research

The research presented in this thesis suggests that lowered NT activity in lymphocytes is a marker of increased oxygen radical production in stressed/depressed humans. This in turn is consistent with the finding of lower ascorbate and zinc stores in depressed groups (Experiment, 3; Maes et al., 1994c). From this it is evident that other antioxidants stores in depressed patients should be assessed such as reduced glutathione, vitamin E, vitamin A and melatonin. Also, measuring ascorbate in its oxidized and reduced forms using HPLC may confirm the increased oxygen radical production with stress. One would expect, for example, with increased stress mediated prooxidant activity that the ratio of dehydroascorbic acid to ascorbic acid to be increased.

The proposed model of an increased production of oxygen radicals by the immune system during circle B (Figure 5.1) suggests that this process may be inhibited by administering antioxidants to humans during times of stress or depression, and this was borne out by the findings in this thesis (See Figures 2.5 and 3.6). The quantity and combination of antioxidants required to provide optimal protection against oxygen radical damage requires further investigation. As McGuigan (1994) points out on p. 248, "If administering antioxidants is beneficial, the need for research is great on how to effectively administer them". Herbert (1994) cautions against taking excessive supplements of antioxidants. He maintains that antioxidants are redox agents, they may be protective against oxygen radical damage mainly at physiological levels. However if used in an unbalanced form some can generate oxygen radicals. Herbert's work implies that assessing each individual's antioxidant status at the commencement of research is important. Vitamin C, for example, can be prooxidant if administered in the presence of high iron stores (Herbert, 1994). Research in

this area needs to proceed cautiously. Ideally one could investigate if antioxidants are beneficial by using a randomised controlled trial in a prospective study. To test which antioxidant combinations have a protective effect on NT in stressed or depressed patients would be an important study. Patients could be randomly allocated to a routine care (no antioxidant supplementation) and compared with patients who are placed on physiological levels of various antioxidant combinations taken from ascorbate, glutathione, zinc, vitamin E and vitamin A. A similar prospective design could be used with patients who have been recently diagnosed with a positive HIV status since they, like the major depression group not taking antioxidants reported in this thesis, have low NT values. In short then it would be possible to assess which combination of antioxidants most effectively re-establish or normalise NT values. The study in subject B (Figure 3.6, Chapter 3) suggests that combinations of antioxidants may prove useful in normalising NT activities, and that one could not rely on a single antioxidant such as ascorbate to protect NT.

The model of oxygen radical damage proposed in this thesis provides a possible mechanism to explain the prolongation of life found in Spiegel's (1989) study of terminal patients with breast cancer. The group of patients who received group psychotherapy were significantly less depressed than a control group and lived approximately twice as long as a similar group of breast cancer patients not receiving this psychotherapy. It is conceivable that those patients who survived longer had higher NT levels due possibly to significantly less oxygen radical damage to their cells. This hypothesis could be tested by replicating Spiegel's (1989) study and including measures of NT and antioxidant levels. Another study related to this would be to study the prevalence of disease in clinically depressed patients and relate it to NT and antioxidant status.

(ii) Future directions for animal research

Using the animal model proposed in this thesis it was difficult to correlate the rat findings with those in humans, and possibly reflects the difficulties associated with attempting to generate an animal model of classical depression as experienced by humans. In humans the stressor is either more chronic or has chronic long lasting effects on the subjects. A more chronic or long term administration of stressors to animals may generate depression in animals with concomitant decreases in NT, ascorbate stores and antibody responses. An example of a more chronic stressor would be to give rats 10 daily shocks for two weeks, since our rats which were shocked only over a 5 day period showed signs of depleted ascorbate stores. Alternatively, it may be more appropriate to use guinea pigs, instead of rats, to test the animal model of depression because like humans they are unable to synthesise ascorbate (Levine, 1986). Another approach would be to place guinea pigs on low antioxidant diets before stressing them. That is, depression of antibody responses, NT and ascorbate may only become significant in animals with low antioxidant stores who are subjected to stress. However giving attenuated shock treatment to rats or guinea pigs may not be viewed favourably by animal ethics committees.

The animal research cited in this thesis and performed by other groups (e.g. Batumen et al., 1990; Cunnick et al., 1988; Keller et al., 1988; Laudenslager et al., 1988; Lysle et al., 1990; Lysle & Maslonek, 1991; Shavit et al., 1984; Weiss et al., 1989) may also be criticised on the grounds that it lacks ecological validity. Indeed, controversy exists over the adequacy of learned helplessness as a model of depression (Weisse, 1992). In real life, stressors may persist for long periods of time, recur frequently, and stressors such as electric shock bear little relationship to that experienced in the animal's natural habitat (Dantzer & Mormede, 1995). Therefore it may be appropriate in animal studies to use models that employ social stressors that may occur in the animals natural environment. For example, peer or maternal separation studies generally show reduction in immune function during the separation (reviewed by

Weisse, 1992). An exploration of dominance-subordination relationships suggests that adoption of submissive postures correlates with a reduction in the immune response (Maier et al., 1994). Maier's study has the advantage of separating physical assault (being bitten) from the psychological state of being defeated (Maier et al.,1994). Social stressors of this kind may yield a more chronic physiological animal model of stress/depression in which to measure our parameters of NT, antibody and antioxidants.

Another possibility would be to use an animal immobilisation model such as that proposed by Liu and Mori (1994) who have shown that the administration of reduced glutathione had a protective effect on stress-induced oxidative damage.

5.3 Limitations of the experiments reported in this thesis

Although NT was found to correlate well with psychological stress/depression in the groups examined in both Experiment 1 and 2 in this thesis, with up to a 50% reduction of enzyme activity in stressed individuals, it must be acknowledged that a limitation of this research design was that it involved comparing groups who were not randomly assigned. Although it seems unlikely that the observed effects could have been due to subject selection, or selection related factors (Cook & Campbell, 1979) rather than to the treatment (differences in stress levels) this possibility can not be ruled out. It seems improbable, however, not only because of the lack of marked differences between the groups on demographic characteristics, but also because the within-subjects effects were just as marked as the between subject effects.

It is possible that malnutrition may have contributed to the lowering of NT reported in Experiments 1 and 2. However this possibility seems unlikely because of the normal blood pictures reported for the subjects in these experiments. Also, the fact that PNP, DPPIV, and HGPRTase were unaffected by HIV-antibody status (Chalmers et al., 1990) suggests that the effect on NT in this study was not due to malnutrition. Another possibility is that the drug intake of the depressed patients in Experiment 2 may have lowered NT activities, either by direct NT-

drug interaction, or through increased oxidative stress, since drug metabolism increases oxidative stress. However, if this was so one would have expected their stress levels plus drug intake to further reduce NT below the stress group not on drugs (Experiment 1). Since this did not happen it is unlikely that their drug intake is affecting NT values in these patients.

It must also be acknowledged that a limitation of the in vitro study (Experiment 5) on NT cited in Chapter 3 is that the numbers were small. This was due to the large volume of blood required and the complexity of the experiment. Though it is not possible to generalise from a sample of two the results were consistent, and confirmed that the reduction of NT in stressed and depressed persons can be mediated by reactive oxygen species and that ascorbate and possibly other antioxidants may be protective of this process.

5.4 Summary

In summary, NT is lowered in situations of psychological stress in humans, and this appears to be related to inflammatory responses which have been shown to occur in depressed patients. In other words, it is proposed that NT is both a good indicator of an inflammatory process and a marker of oxygen radical damage to lymphocytes in humans. It is also suggested that suppression of NT will decrease immunity in people who are depressed or stressed because of its role in cell maturation, and that antioxidants are able to protect against this damage. It must be acknowledged that although the experimental evidence cited in the previous chapters of this thesis converges to support the mechanism of oxygen radical damage in stressed/depressed individuals summarised in Figure 5.1, as Rosch (1994) cautions on p. 219, "association never proves causation."

Also Ader, Cohen, & Felten (1995) in their recent review of the field of psychoneuroimmunology, conclude that the "association between stressful life experiences and changes in immune function do not establish a causal link between stress, immune function, and disease. This chain of events has not been definitively established" (p. 102). The work cited in this thesis has given a biochemical link or mechanism to help either confirm

further or disprove this model. Thus, this model awaits future research to elucidate possible links between stress, lowered NT activities, lowered antioxidant status and illness.

List of Appendices

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APPENDIX A

Abbreviations for Appendix A

P	Prospective
C	Cross-sectional
MD	Major depression
MDD	Major depressive disorder
DSM	Diagnostic statistical manual
E	Enumerative
LR	Lectin response
NKCA	Natural killer activity
APP	Acute phase protein
HRDS	Hamilton Rating Depression Scale
H	Hospitalised patients
O	Outpatients

APPENDIX A

Summary of characteristics of studies of immune measures in major depression

Study	Design	Subjects	Diagnosis	Age Patients	Controls	Severity HRDS	Hospital Status	Immune Measures
Albrecht, Helderman, Schlesser, & Rush (1985)	P	18 endogenous, 9 unipolar patients. 13 age/sex matched controls	MD (RDC)	35	28	19.5	H	E LR
Alshuler et al. (1989)	CS	8 unipolar and bipolar patients. 8 age/sex matched controls.	MD (DSM-III)	31	31	23	H	LR
Andreoli et al. (1993)	CS	53 depressed patients without panic disorder. 53 age/sex matched controls	MDE (DSM-III-R)	38	38	23	O	E LR NKCA
Anesi et al. (1994)	CS	20 unipolar depressed patients. 20 age/sex matched controls.	MDD (DSM-III)	44	NR	24.5	H	E LR
Calabrese et al. (1986)	CS	10 recurrent unipolar depressed patients. 11 age/sex matched controls.	MDD (RDC)	55	50	25.9	NR	E LR
Caldwell, Irwin, & Lohr (1990)	CS	10 depressed male patients, 8 schizophrenic male inpatients, and age/sex/race matched controls.	MDD (RDC) (DSM-III)	46 42	45 46	14	H	NKCA
Darko, Lucas, & Gillin (1986)	CS	5 depressed patients. 5 age/sex/race matched controls.	MDD (RDC) MD (DSM-III)	NR	NR	NR	H	E LR
Darko et al. (1988a)	CS	20 males patients. 20 age/sex/race matched controls.	MDD (RDC) MD (DSM-III)	44	44	23	H	E LR
Darko et al. (1988b)	CS	18 depressed patients.18 sex/race matched but not age matched.	MDD (RDC) MD (DSM-III)	48	35	25.1	H	E LR IL-2 Prolactin

Summary of characteristics of studies of immune measures in major depression cont'd

Study	Design	Subjects	Diagnosis	Age Patients	Controls	Severity HRDS	Hospital Status	Immune Measures
Darko et al. (1988c)	CS	11 depressed patients 11 age/sex/race matched controls.	MDD (RDC) MD (DSM-III)	48	39	26	H	E
Darko, Rose, Gillin, Golshan, & Baird (1988d)	CS	18 unipolar and 12 bipolar male patients age/sex/race matched controls.	MDD (RDC) MDD (DSM-III)	44	43	21	H	E
Darko et al. (1989)	CS	20 depressed patients and 20 age matched controls.	MDD (RDC) MD (DSM-III)	44	44	23	H	E LR GH Prolactin
Darko, Irwin, Risch, & Gillin (1992)	CS	14 bipolar and unipolar patients. 14 age matched controls.	MD (DSM-III-R)	46	46	22	H	NKCA β-endorphin
Evans, Pederson, & Folds (1988)	CS	21 patients, 7 psychiatric (non-major depression) age matched control group.	MD (DSM-III-R)	33	29	20.2	H	E
Evans et al. (1992)	CS	40 inpatients and 4 outpatients. 48 age/sex matched controls.	MD (DSM-III-R)	31	28	17.3	H	E NKCA
Irwin, Smith, & Gillin (1987)	CS	19 depressed male patients and 15 age/sex/race matched controls.	MDD (RDC)	45	41	22.2	H	E NKCA
Irwin & Gillin (1987)	CS	11 depressed male patients and 7 age matched controls.	MDD (RDC)	48.7	44.0	23.6	H	NKCA
Irwin et al. (1990)	CS	36 depressed male patients, and 36 age/sex/race matched controls.	MDD (RDC)	42	42	21.5	H	NKCA

Summary of characteristics of studies of immune measures in major depression cont'd

Study	Design	Subjects	Diagnosis	Age Patients	Controls	Severity HRDS	Hospital Status	Immune Measures
Kronfol et al. (1983)	CS	26 depressed subjects, and 20 controls.	MDD (RDC)	42	37	16.26	NR	LR
Kronfol & House (1984)	CS	13 depressed patients, and 12 controls.	MD (DSM-III)	NR	NR	NR	NR	E LR
Kronfol, House, Silva, Greden, & Carroll (1986)	CS	11 depressed patients with high urinary cortisol, 22 depressed patients with normal cortisol, and 20 age/sex matched controls.	MDD (RDC)	49	37	19.7 15.3	NR	LR
Kronfol & House (1989)	CS	40 depressed patients, and 37 controls.	MDD (DSM-III)	43	31	NA	NR	E LR Complement Antibody
Kronfol et al. (1989)	CS	12 depressed patients, and 12 age/sex matched controls.	MDD (DSM-III) (RDC)	37	37	19.5	H O	NKCA
Krueger, Levy, Cathcart, Fox, & Black (1984)	CS	6 depressed patients, and 21 controls.	MDD (DSM-III)	28	22	26.8	H	E LR
Levy et al. (1991)	CS	30 depressed psychiatric patients, and 25 staff controls.	MDE (DSM-III)	36.3	33.9	>16	H	E LR NKCA
Maes et al. (1990-1991)	CS	Study 1:10 unipolar depressed patients and 10 age/sex matched controls.	MD (DSM III)	44	45	25.6	H	E IL-2R
	CS	Study 2: 11 unipolar major depressed patients and 11 age/sex matched controls.		48	48	25	H	
Maes et al. (1992a)	CS	38 unipolar depressed patients, and 10 controls age matched.	Md MD-M MD+M (DSM-III)	36 42 50	44	14.8 21.2 26.7	H	E

Summary of characteristics of studies of immune measures in major depression cont'd

Study	Design	Subjects	Diagnosis	Age Patients	Controls	Severity HRDS	Hospital Status	Immune Measures
Maes et al. (1992b)	CS	109 unipolar depressed patients, and 22 sex but not age matched controls.	md MD-M MD+M (DSM-III)	41 42 45	53	16 22.3 27.4	H	E
Maes et al. (1992c)	CS	106 unipolar depressed patients, and 19 sex but not age matched controls.	md MD-M MD+M (DSM-III)	42 45 53	44	16 22.4 27.4	H	E
Maes et al. (1992d)	CS	37 depressed patients and 13 age/sex matched controls.	md MD-M MD+M (DSM-III)	38. 48	51	16.4 21.2 27.6	H	NKCA
Maes et al. (1993)	CS	57 unipolar depressed patients, and 10 sex/age matched controls.	md MD-M MD+M (DSM-III)	44 47 53	44	16.2 22.3 27.5	H	E APP
Maes, Meltzer, Stevens, Calabrese, & Cosyns (1994a)	CS	36 depressed patients and 13 controls not age/sex matched.	md MD-M MD+M (DSM-III)	36.3 48.2 51.3	49.4	16.7 21.2 27.6	H	E NKCA
Maes, Meltzer, Stevens, Calabrese, & Cosyns (1994b)	CS	79 unipolar depressed patients, and 17 sex but not age matched controls.	md MD-M MD+M (DSM-III)	37 43 53	46	14.8 20.7 26.1	H	E
Miller, Asnis, Lackner, Halbreich, & Norin (1991)	CS	34 patients, and age/sex matched controls.	MDD (RDC)	42	37	20.5	O	NKCA
Mohl et al. (1987)	CS	10 bipolar and unipolar depressed patients sex but not age matched.	MDD (RDC)	NR	NR	NR	H	NKCA

Summary of characteristics of studies of immune measures in major depression cont'd

Study	Design	Subjects	Diagnosis	Age Patients	Controls	Severity HRDS	Hospital Status	Immune Measures
Nerozzi et al. (1989)	CS	22 depressed patients.	MDD (RDC)	45	44	38	H	NKCA
Schleifer et al. (1984)	CS	18 depressed patients, and 18 age/sex matched controls.	MDD	54	52	28	H	E LR
Schleifer, Keller, Siris, Davis, & Stein (1985)	CS	15 ambulatory depressed patients 15 matched controls. 16 hospitalised schizophrenic patients. 16 matched controls. 10 elective surgery patients. 10 matched controls.	MDD schizophrenia (RDC)	43	40	18.9	O	E LR
Schleifer, Keller, Bond, Cohen, & Stein (1989)	CS	91 unipolar depressed patients, and 91 sex/age matched controls.	MDD- (RDC)	41	40	25.9	H	E LR NKCA
Shain et al. (1991)	CS	16 depressed patients, and 16 age / sex matched controls.	MDD (RDC)	16	16	NA	H	NKCA
Syvalahti, Eskola, Ruuskanen, & Laine (1985)	CS	18 depressed patients, 25 controls not age/sex matched.	MDD (RDC)	49	19-24	19.7	O	
Targum, Marshall, Fischman, & Martin (1989)	CS	21 depressed women, and 77 age/sex matched controls.	MDD (DSM-III)	72	72	24.2	H	E
Tondo et al. (1988)	CS	22 depressed ambulatory patients, and 35 age/sex matched controls.	MDE (DSM-III-R)	21-51	21-47	>25	NR	E
Urch, Muller, Aschauer, Resch, Zielinski (1988)		Depressed patients with age/sex matched controls.	MD (DSM-III-R)	NR	NR	22	H	NKCA
Wilson et al. (1990)	CS	10 depressed patients, and 35 age/sex matched controls.	MDE (DSM-III)	33	31	25	NR	E
Zisook et al. (1994)	P	21 Widows, aged 45-65. 21 age matched married controls. Overall mean age=56.	MDD (DSM-III-R)	NR	NR	11.7	O	E LR NKCA

APPENDIX B

Abbreviations for Appendix B

P	Prospective
C	Cross-sectional
E	Enumerative
LR	Lectin response
NKCA	Natural killer activity
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
EBV	Epstein barr virus
HSV	Herpes simplex virus
CMV	Cytomegalovirus

APPENDIX B

Summary of characteristics of studies of immune measures in chronic stressors

Study	Design	Subjects	Stressor	Psychological Measure	Immune Measures
Bartrop, Lazarus, Luckhurst, & Kiloh (1977)	P	26 bereaved spouses, ages 20-65 and 26 age/sex/race matched controls	Bereavement	Not reported	E LR
Linn, Linn, & Jensen (1984)	CS	49 men who had recent experience of family death or serious illness, aged 40-60 and 49 age/sex matched controls	Bereavement /depressed mood	Hopkins Symptom Checklist	LR IgG, IgA, IgM
Irwin et al. (1987)	CS	10 widows mean age= 57.1 years, and 9 age-matched controls, mean age=52.5	Bereavement/ depression	Social Readjustment Scale, Hamilton Rating Depression Scale	NKCA
Irwin et al. (1988)	P	9 widows mean age= 55.4 years, 11 women anticipating bereavement, mean age=57.7 years, and 9 controls, mean age=52.5	Bereavement	Not reported	NKCA
Spratt & Denney (1991)	P	9 bereaved parents aged 38-61, mean age=49, and matched with non bereaved controls	Bereavement	Beck Depression Inventory	E LR
Zisook et al. (1994)	P	21 Widows, aged 45-65 and 21 age matched married controls, mean age=56	Bereavement	Hamilton Depression Rating Scale, Beck Depression Inventory	E LR NKCA
Schleifer, Keller, Camerino, Thornton, & Stein (1983)	P	15 spouses of women with terminal illness aged 33-76, median 57years	Anticipatory bereavement	Not reported	E LR

Summary of characteristics of studies of immune measures in chronic stressors cont'd

Study	Design	Subjects	Stressor	Psychological Measure	Immune Measures
Arnetz et al. (1987)	P	9 unemployed women aged 30-44 years, mean age=38, 8 unemployed women + psychosocial program aged 28-47years, mean age=37, 8 employed women, aged 30-44 years, mean age=38	Unemployment	Not reported	E LR
Kiecolt-Glaser et al. (1987a)	CS	76 women, 38 separated/divorced, mean age= 31.5, and 38 married women, mean age=29.9	Divorced /separated vs married women	Brief Symptom Inventory, Dyadic Adjustment Scale (DAS), Kitson Attachment Scale,UCLA Loneliness Scale, Psychiatric Epidemiological Research Inventory Life Events Scale (PERI)	E LR EBV
Kiecolt-Glaser et al. (1988)	CS	64 men, 32 separated/divorced, and 32 married men	Divorced /separated vs married men	Brief Symptom Inventory, UCLA Loneliness Scale, Dyadic Adjustment Scale, Life Events Scale, Rotters Locus of Control Scale	E EBV HVS-1
Kiecolt-Glaser et al. (1987b)	CS	34 caregivers, mean age=59.3, and 34 age/sex matched controls, aged 34-82, mean age=60.3	Alzheimer caregivers	BDI, Older Americans' Resources and Services Multidimensional Functional Assessment Questionnaire, Memory and Behavior Problem Checklist	E EBV

Summary of characteristics of studies of immune measures in chronic stressors cont'd

Study	Design	Subjects	Stressor	Psychological Measure	Immune Measures
Esterling et al. (1994)	P	14 caregivers, 5 men, 9 women, mean age=68 years, 17 bereaved caregivers, 5 men, 12 women, mean age=72.3 31 controls, 9 men, 22 women, mean age=70.9	Alzheimer caregivers, bereaved (former) caregivers	Hamilton Depression Rating Scale, Perceived Stress Scale, Impact of Events Scale	NKCA
Irwin et al. (1991)	CS	48 caregivers, 18 men, 30 women mean age =71.3, and 17 controls, 6 men, 11 women, mean age=71.3	Alzheimer caregivers	Hamilton Rating Depression Scale	NKCA Neuro-peptide Y
Kiecolt-Glaser et al. (1991)	P	69 caregivers mean age=67.3, and 69 age/sex matched controls, mean age=67.8	Alzheimer caregivers	Health Review, Social Support Interview, Hamilton Depression Rating Scale	LR NKCA
Schaeffer et al. (1985)	CS	Residents living near TMI nuclear power station and hazardous toxic waste site and controls matched for age/sex/diet/smoking	Three Mile Island Nuclear Power Station	Not reported	E
McKinnon, Weisse, Reynolds, Bowles, & Baum (1989)	P	23 subjects, 12 residents living near TMI nuclear power plant, mean age=36, and 8 controls matched for age, diet and smoking	Three Mile Island Nuclear Power Station	Not reported	E HSV CMV IgG, IgM
Kiecolt-Glaser et al. (1984b)	CS	33 psychiatric inpatients, 21 women, 12 men, aged 18-52, mean age=34	Stressful life events Loneliness	UCLA Loneliness Scale Minnesota Multiphasic Personality Inventory (MMPI), Life Events Scale, Psychiatric Epidemiologic Research Interview (PERI)	LR NKCA

Summary of characteristics of studies of immune measures in chronic stressors cont'd

Study	Design	Subjects	Stressor	Psychological Measure	Immune Measures
Locke et al. (1984)	P	114 students, 79 male and 35 female, aged 17-23, mean=19.6	Stressful life events	Life Events Scale, Hopkins Symptom Checklist	NKCA
Irwin et al. (1986)	CS	39 women, 16 with ill husbands, and 11 controls	Stressful life events	General Health Questionnaire, Social Readjustment Scale, HDRS	E NKCA
Irwin et al. (1990)	CS	8 high stress controls and 28 low stress controls	Stressful life events	Psychiatric Epidemiologic Research Interview	E NKCA
Kemeny et al. (1989)	P	30 females and 6 males	Stressful life events	Daily Hassles Scale	E LR
Jamner, Schwartz, & Leigh (1989)	CS	312 outpatients	Anxiety /depression	Marlowe-Crowne Social Desirability Scale, Taylor Manifest Anxiety Scale	E LR NKCA

APPENDIX C

Summary of characteristics of studies of immune measures and acute stress

Study	Design	Subjects	Stressor	Psychological Measures	Immune Measures
Fischer (1972)	P	21 normal healthy flying crew	Space flight	Not reported	E LR
Taylor, Neale, & Dardano (1986)	P	41 astronauts	Space flight	Not reported	E LR
Schedlowski et al. (1995)	P	25 male first time parachutists aged 19-32 years, mean age=23.6	Parachuting stress	Not reported	E NKCA
Baker et al. (1984)	CS	61 students	Academic stress	Visual Analogue Scale to assess subjective anxiety	E
Dorian et al. (1982)	P	8 psychiatry trainees, 4 males, 4 females, aged 27-37, mean age=30.5 and 16 age/sex matched controls	Academic stress	Impact of Event Scale, Analogue scale, General Health Questionnaire (GHQ), MMPI, Rotter Locus of Control Scale, Jenkins Activity Scale, Hopkins Symptom Checklist	E LR NKCA
Kiecolt-Glaser et al. (1984a)	P	75 medical students, 49 males, and 26 females, mean age=23	Academic stress Stressful life events Loneliness	Brief Symptom Inventory (BSI), Social Readjustment Rating Scale, Symptom Check List-90 (SCL-90), UCLA Loneliness Scale	NKCA IgA, IgM, IgG
Kiecolt-Glaser et al. (1984b)	CS	33 psychiatric inpatients	Loneliness	UCLA Loneliness Scale, MMPI, Life Events Scale	LR NKCA
Kiecolt-Glaser et al. (1986)	P	34 students, 22 men and 12 women, mean age=23.5	Academic stress	Brief Symptom Inventory, (BSI) Shaver Loneliness Scale	E NKCA
Glaser et al. (1986a)	P	40 medical students, mean age=24.4	Academic stress	Brief Symptom Inventory (BSI)	NKCA Interferon

Summary of characteristics of studies of immune measures and acute stress Cont'd

Study	Design	Subjects	Stressor	Psychological Measures	Immune Measures
Halversen & Vassand (1987)	P	23 undergraduate students	Academic stress	State-Trait Anxiety Inventory (STAI), Analogue scale	E IL-2 LR
Baker et al. (1985)	P	61 medical students	Perceived anxiety	Visual Analogue Scale to assess subjective anxiety	E
Linn, Linn, & Jensen (1981)	CS	75 chronically ill patients	Elective hospitalisation	Hopkins Symptom Checklist, Visual Analogue Scale for anxiety	LR IgG, IgA, IgM,
Schleifer, Keller; Siris; Davis; Stein (1985)	CS	10 elective surgery patients and 10 matched controls	Elective hospitalisation	Hamilton Depression Rating Scale (HRDS)	E LR
Linn & Linn (1987)	CS	24 male inpatients	Preoperative stress: hernia repair	Not reported	LR
Tonnesen et al. (1987)	P	20 inpatients	Preoperative stress coronary artery bypass grafting	Not reported	E LR NKCA
Ironson et al. (1990)	P	46 homosexual men and 25 healthy controls	HIV-1 antibody status notification	Life Experience Survey, Impact of Events scale, State Trait Anxiety Inventory	E LR NKCA
Antoni et al. (1990)	P	46 homosexual males, ages 18-39, mean age=30	Anticipation of HIV-1 antibody status notification	STAI; Profile of Mood States (POMS) COPE Inventory Dispositional Version, Impact of Events Scale (IES)	LR ß-endorphin cortisol
Moss, Moss, & Peterson (1989)	P	10 healthy medical or graduate students, 6 males, 4 females aged 22-30, mean=24.1	Microstressors/Mood Rating	Hassles and Uplifts subscale, Profile of Mood States (POMS)	NKCA
Palmblad et al. (1979)	P	12 normal subjects	Sleep deprivation	Not reported	LR

P=prospective design, CS=cross sectional design, E=enumerative, LR=lectin response, NKCA=natural killer cell activity

APPENDIX D

Summary of characteristics of studies of immune measures in acute laboratory stressors

Study	Subjects	Stressor	Psychological Measures	Group/Condition	Immune Measures
Landmann et al. (1984)	11 men and 4 women	Stroop (8 min)	Not reported	Stress group No control group	E
Manuck et al. 1981	30 men aged 16-30	Stroop (20 min)	Not reported	High reactors Low reactors No stress controls	E LR
Bachen et al. (1992)	44 men aged 19-25	Stroop (21 min)	Not reported	Stress group vs controls	E LR
Herbert et al. (1994)	41 subjects, 22 men and 19 women aged 18-29, mean=22.3	Stroop (21 min)	Not reported	Stress group vs control	E NKCA
Naliboff et al. (1991)	12 young women mean age =31 11 older women mean age =71	Maths (12 min)	Stress Symptom Ratings (SSR)	Young vs old	E NKCA
Brosshot et al. (1992)	86 subjects aged 24-55, mean=40.5	Uncontrollable interpersonal stressor	General Health Questionaire Visual Analogue Scales for mood changes	Stress group vs controls	E LR
Weisse et al. (1990)	22 men mean age = 28	Intermittent electric shock/loud Noise (30 min)	Schedule of Recent Events, Revised Symptom Checklist, SCL-90R, Profile of Mood States (POMS), Analogue for mood	Controllable vs uncontrollable noise/ electric shock	E LR
Sieber et al. (1992)	55 men aged 18-26	Two sessions of noise (20 min). Rest (30min) between noise sessions	Attributional Style Questionnaire (ASQ), Life Orientation Test (LOT), Self Control Schedule (SCS), Desire for Control Scale (DC)	Escapable noise/ inescapable noise/ response/ escapable noise/response/ inescapable noise/ no response, no noise	E NKCA

Summary of characteristics of studies of immune measures in acute laboratory stressors cont'd

Study	Subjects	Stressor	Psychological Measures	Group/Condition	Immune Measures
Zakowski, McAllister, Deal, & Baum, (1992)	29 men, aged 18-46, mean = 31.3	30 min viewing and recall of a gruesome combat surgery film"	Modification Life Change Inventory, The Perceived Stress Scale (PSS), The Symptom Check List-90-R, Beck Depression Inventory (BDI),The Cook-Medley Hostility Scale	Stress group vs controls	LR IL-1 IL-2
Knapp et al. (1992)	10 men, and 10 women, aged 18-30	40 min recall -ve "maximally disturbing" and +ve "maximally pleasurable" emotional experiences	Draw-Two-Persons test Visual analogue for emotional feelings	Within subjects design 3 day sequence	E LR NKCA
Futterman et al. (1994)	16 male actors, aged 24-47, mean= 35, and 9 healthy nonactor controls, aged,18-43, mean=29.4	Reliving personal emotions according to method acting techniques. Baseline, 20 min mood induction, 20 min recovery time	Health Questionaire Affect Balance Scale(ABS) List life events in past week	Mood induction: negative high arousal, negative low arousal, positive high arousal, vs neutral condition 5 experimental days	E LR NKCA
Kiecolt-Glaser et al. (1993)	90 couples, wives mean age=25, 21 husbands mean age=26.1 and overall aged 20-37	Negative behaviour during 30 min marital conflict 24 hour admission	Marital Adjustment Test, POMS, Positive and Negative Affect Schedule, Social Support Questionnaire, Hamilton Depression Rating Scale (HDRS), Marlowe Crowne Social Desirability Scale	High and low negative interaction groups	E LR NKCA antibody to T3 receptor

E=enumerative, LR=lectin response, NKCA=natural killer cell activity, IL-1= Interleukin 1, IL-2=Interleukin 2

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