STRESS AND THE IMMUNE NETWORK

by

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PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
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Finally, thank you to my family. My wife Linda gave me her total support and picked up the extra chores in my absences from the family. My daughter Naomi, who understood the work, provided useful feedback on my writing. My daughter Janneke, who sensibly took no interest in all of this, kept me anchored in the real world. My parents Carmelo and Vittoria, who migrated to Australia almost 50 years ago, provided me with a future. You all gave me exactly what I needed - thank you again.
The work presented in this dissertation is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for another degree at this or any other institution.
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<th>Definition</th>
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<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Az</td>
<td>Azide (sodium azide)</td>
</tr>
<tr>
<td>B7</td>
<td>APC surface co-stimulatory molecule</td>
</tr>
<tr>
<td>BAI</td>
<td>Beck Anxiety Inventory</td>
</tr>
<tr>
<td>BDI</td>
<td>Beck Depression Inventory</td>
</tr>
<tr>
<td>C1-C9</td>
<td>Proteins of the complement system</td>
</tr>
<tr>
<td>C gene</td>
<td>Constant region gene segment</td>
</tr>
<tr>
<td>C region</td>
<td>Constant region of an immunoglobulin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster differentiation</td>
</tr>
<tr>
<td>CD40</td>
<td>A B cell membrane molecule</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand, a T cell membrane molecule</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CIS</td>
<td>Central immune system</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTX</td>
<td>Cholera toxin virus</td>
</tr>
<tr>
<td>D gene</td>
<td>Diversity gene segment</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EMAI</td>
<td>Elizabeth Macarthur Agricultural Institute</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment of an immunoglobulin</td>
</tr>
<tr>
<td>FACS-Fix</td>
<td>1% paraformaldehyde in PBS</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallisable fragment (constant region) of an immunoglobulin</td>
</tr>
<tr>
<td>FcγRII</td>
<td>Low affinity IgG receptor (one of the Fc receptors)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FcγRIIB</td>
<td>A component of the B cell co-receptor (one of the Fc receptors)</td>
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<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fos</td>
<td>A marker of neural cell activation</td>
</tr>
<tr>
<td>FR</td>
<td>Framework region of an immunoglobulin</td>
</tr>
<tr>
<td>FSC/SSC</td>
<td>Forward scatter/side scatter</td>
</tr>
<tr>
<td>G protein</td>
<td>Heterotrimeric GTP-binding protein</td>
</tr>
<tr>
<td>H chain</td>
<td>Heavy chain of an immunoglobulin</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venule</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen region</td>
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<tr>
<td>HPA</td>
<td>Hypothalamus-pituitary-adrenal</td>
</tr>
<tr>
<td>HV</td>
<td>Hypervariable region of an immunoglobulin</td>
</tr>
<tr>
<td>H-Y</td>
<td>Male-specific histocompatibility antigen</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>Immunoglobulin D</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>J chain</td>
<td>Polypeptide in IgA or IgM polymers</td>
</tr>
<tr>
<td>J gene</td>
<td>Joining gene segment</td>
</tr>
<tr>
<td>L chain</td>
<td>Light chain of an immunoglobulin</td>
</tr>
<tr>
<td>LCB</td>
<td>Locus of control of behaviour</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium chloride</td>
</tr>
<tr>
<td>LMP</td>
<td>Low molecular weight polypeptide</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>LOC</td>
<td>Locus of control</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
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<tr>
<td>MBL</td>
<td>Mannose binding lectin</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mlg</td>
<td>Membrane-bound immunoglobulin</td>
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<tr>
<td>NIM</td>
<td>Neuroimmunomodulation</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
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<tr>
<td>NREM</td>
<td>Non-rapid eye movement sleep</td>
</tr>
<tr>
<td>P(ANG)</td>
<td>A measure of anger/hostility</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>P(CON)</td>
<td>A measure of confusion/bewilderment</td>
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<tr>
<td>P(DEP)</td>
<td>A measure of depression/dejection</td>
</tr>
<tr>
<td>P(FAT)</td>
<td>A measure of fatigue/inertia</td>
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<tr>
<td>PIS</td>
<td>Peripheral immune system</td>
</tr>
<tr>
<td>PNI</td>
<td>Psychoneuroimmunology</td>
</tr>
<tr>
<td>POMS</td>
<td>Profile of Mood States</td>
</tr>
<tr>
<td>P(TEN)</td>
<td>A measure of tension/anxiety</td>
</tr>
<tr>
<td>P(TMD)</td>
<td>Total Mood Disturbance</td>
</tr>
<tr>
<td>P(VIG)</td>
<td>A measure of vigour/activity</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement sleep</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>sIg</td>
<td>Secretory immunoglobulin</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>SmcY</td>
<td>H-Y gene</td>
</tr>
<tr>
<td>SOC</td>
<td>Sense of coherence</td>
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<tr>
<td>SWAPS</td>
<td>South Western Area Pathology Service</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
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</tr>
<tr>
<td>TAP</td>
<td>Transporter for antigen processing</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TD antigen</td>
<td>Thymus-dependent antigen</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TH1</td>
<td>Inflammatory T cell</td>
</tr>
<tr>
<td>TH2</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>Thy-1</td>
<td>Murine T cell-specific surface molecule</td>
</tr>
<tr>
<td>TI antigen</td>
<td>Thymus-independent antigen</td>
</tr>
<tr>
<td>TME</td>
<td>A thymic extract</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>V gene</td>
<td>Light chain variable region gene segment</td>
</tr>
<tr>
<td>V region</td>
<td>Variable region of an immunoglobulin</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal (poly-)peptide</td>
</tr>
<tr>
<td>VRM</td>
<td>Variable region molecule</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>WCC</td>
<td>White cell count</td>
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ABSTRACT

The clonal selection/defence paradigm appears unable to reconcile immune function with homeostatic activity whereas organismic homeostasis is central to immune function in the network/autopoiesis paradigm. The aim of this investigation, therefore, was to test the proposition that immune function that is not clonally driven (central immune system activity) contributes to organismic homeostasis in collaboration with psychoneural responses.

In one experiment sheep were confined, either in groups or individually, and the time course of changes in cortisol levels, behaviour and T lymphocyte numbers were monitored. Results included a drop in cortisol levels over time, resumption of normal behaviour as measured in an approach-avoidance test, and a rise in CD4 cell numbers together with a drop in the number of CD8 cells. The results seemed indicative of both psychoneural and immune recovery from the adverse effects of stress on the central immune system.

In another study, soldiers were monitored during the stressful experience of recruit training. In a situation of sustained, high levels of cortisol: their psychological status improved markedly; their T and B lymphocyte numbers fluctuated about the baseline, indicating central immune system activity and the absence of immune suppression; and their NK cell numbers fell significantly, indicating both an absence of immune challenge and a low level of innate immune activity. Both the psychoneural and the immune responses ran counter to expectations based on the high observed levels of cortisol.

The combined results suggest that, at least when the immune response is not clonally driven, the psychoneural system and the central immune system may not be
operating independently of each other but rather as sub-networks of the organismic network. Consequently, homeostasis is properly characterised as a property of the whole organism. In autopoietic terms, then, homeostasis could be defined as the maintenance of network stability.
CHAPTER 1

INTRODUCTION

A diagnosis of systemic lupus erythematosus means the presence of an incurable, potentially life-threatening, autoimmune disease, the symptoms of which could involve any body system and which will flare up and go into remission in unpredictable ways. Some individuals respond by making the disease the centre of their lives, so that other elements of their lives assume lesser importance. Others respond by integrating it into their lives so that its impact on life fluctuates with the flares and remissions. Thus the same global diagnosis can appear to result in quite different outcomes for different individuals. These observations raise several questions. Are these differences characteristic of, and peculiar to, lupus? Does the individual somehow influence the course of this disease? Is this an example of a wider phenomenon?

Beginning with an examination of lupus may not necessarily permit generalisation. On the other hand, adopting a more fundamental approach could address the three questions more or less simultaneously. Such an approach has guided the present investigation. Thus, although the anecdotal evidence about lupus may have prompted this investigation, it is not a study of the disease itself. Rather, the aim has been to devise experiments to examine different responses to essentially the same situation, but in a way that might lend itself to broad generalisation. At the same time, the ways that lupus affects individuals and that individuals respond to it suggest that
there may be a psychoneural aspect to the process - is that also part of a general mechanism? Furthermore, is the underlying process antigenically-driven, as is the case in lupus?

The upshot of these deliberations has been to devise scenarios that could be extrapolated to lupus, but that are not defined by it. Thus, the subjects of this investigation will be responding to non-pathological, non-antigenic stressors. Both their immune and their psychoneural responses will be examined. In particular, evidence for possible interplay between the immune and psychoneural systems will be sought. Assuming that the subjects will be able to maintain a degree of self-regulatory equilibrium, this investigation may also contribute to an understanding of the nature of homeostasis. Although lupus (or any other autoimmune disease) will not contribute to the scenarios, the outcomes would be expected to shed some light on the original observations.
CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Homeostasis is one of the core concepts of physiology, yet its significance in immunophysiology seems less than clear. In this review both homeostasis and immune function are examined in order to clarify the issue.

Homeostasis is discussed broadly in this section, then re-integrated into immune function in section 2.6. Beginning with the historical background (section 2.2), the remainder of this review explores the immune system. Reflecting the fact that the vast majority of the work has been done within the clonal selection/defence framework, section 2.3, the largest part of the review, provides a detailed picture of the finer detail of immune function. The content of this section could be described mainly as the anatomy and physiology of the system. Therefore, section 2.4 broadens the horizon to cover other aspects of the biology of immune function. The biological perspective is continued in section 2.5 with an examination of the interactions among the immune system and other body systems. The immune network framework is introduced in section 2.6 which then re-examines some issues that arose in the context of clonal selection/defence. This section also addresses the
immune homeostasis issue in terms of an expanded view of the immune system. The various threads are drawn together (section 2.7) in order to pose a number of research questions that then form the basis of the investigations described in this dissertation. Finally (section 2.8), the experimental plan of this investigation is outlined.

In its original formulation (Cannon, 1932), homeostasis was described as the self-regulatory mechanism that allows an organism to maintain itself in a state of dynamic equilibrium by restricting the fluctuation of variables within tolerance limits. Since then, feedback has been recognised as the essential mechanism of homeostasis (Capra, 1996), and the concept has been applied to individual body systems. As a result, organismic homeostasis is commonly seen as the effect of the interplay among the homeostatic functions of the individual systems. However, it is seen as something of a paradox with respect to the immune system. “All biological systems are to some extent self-regulating......However, unlike most biological systems, the immune system must also make specific responses to extrinsic substances whose nature cannot be anticipated, and these responses must be prompt enough and sufficiently strong to control a rapidly growing pathogen. Thus, the system must allow large deviations from homeostasis when called upon to do so” (Janeway and Travers, 1996).

This view of homeostasis and feedback is consistent with the view of an organism in constant input/output contact with its environment. However, a different understanding emerges when the organism is described as being autopoietic (Maturana and Varela, 1987). The core concept of autopoiesis is that an organism maintains itself as an operational whole, not by its parts alone but in the network of relationships among its parts; the distinction between organisation and structure becomes crucial here. Organisation refers to the group identity of the living system, the pattern of network relationships among components that characterises an
individual. Structure refers to the particular physical properties within the system
and the roles of the components. Although autopoietic organisation can be achieved
with different components and structures, a particular pattern of organisation will
uniquely define an organism or a related group of organisms (a species).
Furthermore, organisation and structure complement each other, so that organisation
exists only as relationships among structures and structure exists only as filling the
roles that those network relationships establish (Maturana, 1981). Indeed, given that
different components are capable of assuming different roles within a particular
organisational pattern, organisation can remain constant within a certain range of
structural changes. However, beyond that range organisational roles are breached,
the organisational pattern is altered and the system (organism) loses its identity. The
system can then either assume a new identity, based on a new organisation, or
disintegrate (Fleischaker, 1988). A correlate of these characteristics of autopoiesis is
organisational (operational) closure, whereby the autopoietic network of operations
lies entirely within its own boundary, despite requiring energy and materials from its
environment. Hence a living system (organism) is said to be organisationally closed
but energetically open (Maturana and Varela, 1987). Under these circumstances the
organism is engaged in a permanent monologue of self-stimulation and feedback.
Any influence that brings about a perturbation of the monologue is then interpreted
as a sign that conveys the meaning of the environment (von Uexküll et al., 1993).
Such a perturbation is possible because of so-called structural coupling between the
organism and the environment, whereby structural changes occur to the extent that
organisation is maintained. In other words, the organism is continually undergoing
structural changes triggered by interactions with the environment. Consequently,
homeostasis can now be defined as the capacity of an organism to maintain
organisational stability while tolerating allowable deviations in the levels of
individual components (the structure). Hence, one reason for the paradox may be
that it is couched in the pre-autopoietic view of homeostasis. In addition, it rests on
an examination of only part of the immune system, the part that does, indeed, engage
in apparently non-homeostatic responses to pathogens.

2.2 Historical background

One of the first authors in immunology was Eli Metchnikoff who presented his novel theory of immunity in 1883. According to Metchnikoff, immunity arose as an active defence mechanism in the host, mediated largely by the action of phagocytes. As well as being crucial for normal host defence against a variety of pathogens, phagocytes are also the principal effector cells of chronic inflammatory diseases, where host tissue itself becomes the pathological target. In this view, the immune process is a specialised case of inflammation, a specific expression of the body’s effort to maintain its integrity. This theory clearly identifies Metchnikoff as a cellularist within the domain of evolutionary biology. He was concerned with the development and definition of organismic identity, at both the evolutionary and life history levels (Tauber, 1994).

Metchnikoff’s microbiological contemporaries developed what has been termed the humoralist position which regarded phagocytes as no more than scavengers of killed pathogens. In this view, true defensive factors are noncellular and are found as antitoxins in the fluid phase of blood and other bodily fluids. Humoralist research questions quickly became issues of specificity and chemical identification so that Metchnikoff’s more theoretical concerns with identity were largely ignored. Thus, the humoralist approach was concerned with mechanisms and processes by which the organism maintains integrity, defending an already well-demarcated host (Tauber, 1994). Immunology began to move in the direction of a biochemically oriented science of the organism reduced to its simplest physicochemical elements (Tauber and Chernyak, 1991).
By 1908, when Metchnikoff and Ehrlich shared a Nobel Prize, the humoral and cell-mediated aspects of immune function had been integrated. Furthermore, the existence of the innate and adaptive responses was evident (Tauber, 1994). For many of the following years, an understanding of antibody formation and diversity was the dominant research issue. In historical terms, the existence of lymphocyte receptor diversity that matched the surface molecules of particular pathogens was elucidated before the generation of lymphocyte antigen receptor diversity had been understood (Ada and Nossal, 1987).

Theories on antibody formation have revolved around either instruction or selection (Talmage, 1986). Instructive theories proposed that antigen directed (instructed) the production of antibody; selection theories suggested that antigen selected the appropriate antibody from an existing repertoire.

The earliest selective theory (Ehrlich, 1900) proposed that a lymphocyte carried a variety of side chains on its surface. The particular side chain which matched an antigen was subsequently produced in excess in order to mop up the remaining antigen. Inconsistencies between the side chain theory and subsequent observations (Landsteiner, 1933) led to the development of a number of instructive theories, notably the template theory of Pauling (1940). Instructive theories, however, could not accommodate observations on the dynamics of antibody formation (Burnet and Fenner, 1949), leading to the development, over a relatively short period of time, of the clonal selection theory. Jerne (1955) proposed that an individual carries small numbers of antibodies against all antigens. In an immune response, antigen binds to antibody and the bound complex stimulates leukocytes to produce and release more of that specific antibody. Talmage (1957) extended the concept to include proliferating leukocytes in the response. Burnet (1957) took the idea further. He suggested that the antigen binds to a cell surface receptor-bound antibody,
stimulating cell division. In addition, the cell and its clones can only produce one type of receptor/antibody. The question of self-recognition and attack was addressed by revisiting earlier work that had shown that exposure to foreign tissues during embryonic development rendered the individual immunologically tolerant to those tissues (Billingham, Brent and Medawar, 1953). Burnet proposed that potentially self-destructive lymphocytes would be deleted early in life before they mature.

In its current form the clonal selection theory is considered to be the most important principle in adaptive immunity (Janeway and Travers, 1996); it rests on four basic postulates:

(i) each lymphocyte bears a single type of receptor of a unique specificity;
(ii) interaction between a foreign molecule and a lymphocyte receptor capable of binding that molecule with high affinity leads to lymphocyte activation;
(iii) the differentiated effector cells derived from an activated lymphocyte will bear receptors of identical specificity to those of the parental cell from which that lymphocyte was derived;
(iv) lymphocytes bearing receptors specific for self molecules are deleted at an early stage in lymphoid cell development and are therefore absent from the repertoire of mature lymphocytes.

Jerne, whose proposal (1955) led to Burnet's (1957) clonal selection theory, also subsequently recognised the network of relationships to be found in the immune system (Jerne, 1974). Since antibodies are glycoproteins, it is not surprising that antibody molecules themselves display antigenic determinants. This results in the generation of antibodies against antibodies and has led to the notion of a network (Cohen, 1988; Jerne, 1984), for example, among IgM (Lundkvist et al., 1989) and IgG (Berneman, Ternynck and Avrameas, 1992) antibodies.
At perhaps the finest level, immunological homeostasis is maintained by the network of interactions among T cells (section 2.3.5), B cells (section 2.3.3) and antibodies (section 2.3.1) (Jerne, 1974, 1984). Idiotopes are antigenic determinants, unique to an antibody or group of antibodies and defined by the reaction between anti-idiotopic antibodies and the antibodies bearing the idiotopes. The set of idiotopes of an antibody is termed its idiotype (Rajewsky and Takemori, 1983). External antigens and internal, anti-idiotypic, antibodies can competitively bind the combining site of specific antibodies. Thus, some anti-idiotypic antibodies can resemble the external antigen, carrying its "internal image" (Fields et al., 1995). The antibody network, therefore, consists of a repertoire of antibodies and their anti-antibodies (Berneman, Ternynck and Avrameas, 1992; Lundkvist et al., 1989) which carry the internal image of antigens. The broader immune network, which includes lymphocytes and their receptors, helps to establish and maintain a distributed repertoire of receptors at all times (Stewart and Varela, 1989).

By the time that Jerne’s idiotypic network theory emerged “the immunological community had entirely adopted Burnet’s theory of cellular clones at rest (waiting for the outside antigens)...some of the dominant views derived meanwhile from the clonal selection theory are formally incompatible with the network theory, and it took Jerne’s stature to get it off the ground” (Coutinho, 1995a). Indeed, the usual textbook view today (Coleman, Lombard and Sicard, 1992; Janeway and Travers, 1996) is that the idiotypic network is a cellular/molecular arrangement that regulates (or modulates) the activity of the major element of immune function, the clonal immune response. This is quite different from Jerne’s view that the network of immune relations is at the centre of the immune system (Vaz and de Faria, 1990).

In a historical sense, the idiotypic network theory does not appear to have made much of an impact on immunological thinking for the first 15 or so years following
its publication. In its original form (Jerne, 1974), and in an earlier paper that foreshadowed some aspects (Jerne, 1973), the proposed network seemed to be a bundle of parallel sequences of idiotypic interactions. The trigger for activity may have been an external antigen and/or its “internal image” idioype. It was presented as a theory to supplant clonal selection, yet it shared some features and appeared to be merely a regulator of clonal selection processes. Ten years later Jerne (1984, 1985) admitted that the idea had not yet been satisfactorily formulated, yet he repeated that the network is a major regulatory feature of the immune system. Nevertheless, he had further developed his ideas about antibody multispecificity and the self/nonself question.

Others had also begun addressing Jerne’s ideas. The self/nonself question became the self/nonsense concept (Vaz and Varela, 1978) whereby self is what can interact with the immunological self and nonsense is what cannot. The immune system began to be seen as a self-determined network that exhibited connectivity, degenerate (overlapping) specificity, regulation-by-suppression, and a cognitive domain of possible interactions (Coutinho et al., 1984). The concept of the repertoire of antibody specificities was refined and defined further. Thus, an individual is said to have a potential repertoire (of antibodies) consisting of what can be synthesised, limited only genetically; the available repertoire is said to consist of what has been synthesised and can be used, derived ontogenetically by the generation of diversity; the actual repertoire refers to what is actually being used, the circulating antibody repertoire that is ephemeral and determined by present experiences. The actual repertoire defines the cognitive domain of immune interactions, made up of contacts that are relevant to the immunological self. Since it is constantly being modified by experiences it can tolerate the “foreign-familiar” (diet, normal microbiota) and mount a response against the “foreign-foreign” (the new) (Vaz, Martínez-A. and Coutinho, 1984).
This development of the idiompiric network theory was happening in the context of, and was influenced by, the autopoiesis perspective. A living system is said to have autopoietic organisation, that is, it is "self-producing". It consists of processes of production that generate its components. These components themselves then participate in the processes of production in a continual recursive re-creation of self. Thus, autopoietic systems produce themselves and only themselves (Maturana and Varela, 1980, 1987). At the same time, the idiompiric network provided a new complex biological system for theoreticians to model (Farmer, Packard and Perelson, 1986; Perelson, 1989; Perelson and Oster, 1979).

Within its first 15 years, the idiompiric network theory had established the existence of anti-idiompiric antibodies, had started the modelers modelling, yet, in the absence of investigations into its actual dynamics, had proposed nothing that clonal selection couldn't explain (Coutinho, 1995a). Thus the clonal selection theory continued to hold sway for over 30 years and these circumstances produced several outcomes that were less than satisfactory (section 2.3.10). Clonal selection and affinity maturation have been modeled as if they were immune network properties (Joshi and Krishnanand, 1996). Two of the recent models of AIDS pathogenesis refer to a destabilised immune network, but are consistent with clonal selection (Hoffmann, 1994; Süssal et al., 1993). The therapeutic use of anti-idiompiric antibodies, as a form of surrogate immunisation, has been proposed for viral infection (Rewald and Gonzalez, 1996), for the clearance of immune complexes (Carrero, Mallender and Voss, 1996), for autoimmune disease (Araga, Leboeuf and Blalock, 1993, 1994; Lenert, Lenert and Senecal, 1996), for cancer (Fagerberg et al., 1993, 1994; Masaki et al., 1996; Schmitt et al., 1994), and for a variety of conditions (Dwyer, 1992).

Despite some ambiguities in Jerne's original proposition, and its misuse in some of the early applications, the idiompiric network theory survived because of the development of so-called second generation immune networks (Varela and
Coutinho, 1991) that formed the basis of subsequent developments. These issues are explored in section 2.6, beginning with the notion of second generation networks.

Clearly, the clonal selection theory dominates immunology and has proved to be extremely useful. It has provided the theoretical basis for the immunological defence metaphor, the major organiser of thinking about immune function. In fact, most of the research questions have been asked, and most of the explanations have been developed, in the context of clonal selection and/or immunological defence. Therefore, the following section (2.3) provides a review of immunology consistent with the clonal selection framework and the defence metaphor.

2.3 The clonal selection theory and immunology

The bone marrow is the site of production of all the cells of the blood, including the white blood cells of the immune system; the process is termed haematopoiesis (Crocker and Milon, 1992). The cells all originate from the same precursor cells in the bone marrow, the pluripotent haematopoietic stem cells (Golde, 1991; Kennedy et al., 1997; Weissman and Cooper, 1993). They, in turn, give rise to stem cells of more restricted potential. The two stem cells of interest in this review are the myeloid progenitor and the common lymphoid progenitor. The myeloid progenitor gives rise to monocytes, which develop into macrophages after migrating into tissues, and granulocytes, of which there are three types, neutrophils, basophils and eosinophils (Wiltrout and Varesio, 1990). The common lymphoid progenitor gives rise to three types of lymphocytes, the effector cells of the immune system. T lymphocytes, or T cells, are significantly involved in both humoral and cell-mediated immune responses. B lymphocytes, or B cells, are responsible for humoral immunity. A small population of lymphocytes, termed null cells, do not display markers identifying them as either T cells or B cells (Anderson, 1990).
The lymphoid organs are organised tissues where lymphocytes interact with non-lymphoid cells, resulting in either lymphocyte maturation or the initiation of an adaptive immune response. The primary or central lymphoid organs, where lymphocytes are generated and mature, consist of bone marrow and the thymus, a lymphoepithelial organ located superior to the heart and posterior to the sternum. The secondary or peripheral lymphoid organs, where the adaptive immune response starts, include lymph nodes, the spleen, and lymphoid tissue associated with various mucosal surfaces (Anderson, 1990).

Innate immunity provides generalised defence against infectious agents, typically bacteria, and other foreign substances. This system uses proteins encoded in the germ line to identify such potentially noxious substances. These proteins, whether they are cell surface receptors or soluble, seem usually to recognise carbohydrate structures (Fearon and Locksley, 1996). The cells that contribute to the innate immune response include natural killer cells, macrophages and monocytes, granulocytes, and platelets. Reactions of these cellular effectors are mediated by recognition systems made up of complement and Fc receptors, cell adhesion molecules, and cytokine receptors (sections 2.3.6 and 2.3.7).

The effectiveness of the innate immune response is based on the predictable nature of the carbohydrate chemistry of the bacterial capsule. It should not be surprising, however, that many bacteria have evolved capsules that conceal the molecules that phagocytes would otherwise recognise, thus enabling the bacteria to avoid the innate immune system. The adaptive immune system, then, has developed receptors on lymphocytes which can target individual antigens. Thus the adaptive immune system is able to generate potentially a large number of specific lymphocyte receptors, yet a relatively small number of different lymphocytes is generated during each response (Ada and Nossal, 1987).
2.3.1 The antibody molecule

Antibodies are protein molecules called immunoglobulins (Potter and Smith-Gill, 1990). They are synthesised by B cells as membrane-bound antibodies and by plasma cells as secreted antibodies (Burnet, 1954) and are found primarily in plasma or serum. Pure immunoglobulins have been produced in vitro as monoclonal antibodies (Winter and Milstein, 1991).

"The Y-shaped antibody is a quintessentially bifunctional protein: unique binding sites of the arms latch on to intruders, while 'effector' sites in the stem ensure delivery of such undesirables to the destructive forces of complement and phagocytes" (Parham, 1995).

Immunoglobulin (Ig) molecules make up a family of glycoproteins with the same basic molecular architecture but with a diverse range of different functional properties. Thus, within the Ig family, five classes of antibodies (and several subclasses) can be distinguished - IgA, IgD, IgE, IgG, IgM. Classes and subclasses differ biochemically, physically and functionally. The following description of the basic features of antibody molecules is based on IgG as the example (Figure 2.1) and is derived from several sources (Edelman et al., 1969; Janeway, 1993; Mansour and Cooper, 1993; Owen and Lamb, 1988; Potter and Smith-Gill, 1990; Winter and Milstein, 1991).
Figure 2.1 Schematic representation of polypeptide chains in the basic Ig structure. The light (L) chains are divided into two homology regions: $V_L$ and $C_L$. The heavy (H) chains are divided into four homology regions: $V_H$, $C_H1$, $C_H2$ and $C_H3$. $C_H1$ and $C_H2$ are joined by a "hinge" region, indicated by the solid area. Fab and Fc refer to fragments produced by enzymatic cleavage. Glycosylation sites (cho), as well as interchain and intrachain disulfide bonds (S-S), are indicated (Edelman et al., 1969).
IgG is a large molecule with a molecular weight of approximately 150 kD. It consists of two light (L) chains and two heavy (H) chains (25 and 50 kD molecular weights, respectively) linked together by disulfide bonds. There are two types of light chain, termed kappa (κ) and lambda (λ) chains, distinguishable by their amino acid sequences in the constant regions. There appears to be no functional difference between antibodies having κ or λ light chains. The κ:λ ratio varies from species to species; in humans it is 2:1. In addition, there are five main classes of heavy chain (isotypes). They determine the functional activity of the antibody molecule and give rise to the five classes of antibodies already mentioned. IgA contains α heavy chains, IgD has δ, IgE has ε, IgG has γ, and IgM has μ heavy chains.

Each chain of an immunoglobulin molecule contains distinct amino acid sequence domains, each domain being about 110 amino acids long. Light chains have two domains and the heavy chain of IgG has four. The amino-terminal (first) domain of both chains shows significant variation in amino acid sequence between different antibodies; these variable (V) domains make up the variable region. The carboxy-terminal domains of both chains, either light (one domain) or heavy (three domains), have constant amino acid sequences within the same isotype; these constant (C) domains make up the constant region.

In three-dimensional terms, the immunoglobulin molecule consists of three roughly equal globular portions joined by a flexible polypeptide sequence termed the hinge region (Figure 2.1). Each arm of the Y shape is termed the Fab fragment (Fragment antigen binding) and contains the antigen binding activity. The stem of the Y is termed the Fc fragment (Fragment crystallisable) and carries out other effector functions (Hulett et al., 1995).
Within the variable regions of both light and heavy chains, the sequence of variability is not distributed evenly. There are three regions of particular variability, termed hypervariable regions (HV1, HV2, HV3). The rest of the V domain consists of four relatively invariant framework regions (FR1, FR2, FR3, FR4). The framework regions determine the structural framework of the domain (the β sheet structure). The hypervariable regions constitute the antigen-binding site by forming a surface that is complementary to the antigen. Thus they are also termed complementarity determining regions (CDR1, CDR2, CDR3). Because final antigen specificity is determined by CDRs from both VH and VL domains, different combinations of heavy and light chains result in combinatorial diversity in antigen specificities.

The complete antibody repertoire available within the individual mounting the response is enormous. The question is: how can antigen receptors with an apparently infinite range of specificities be encoded by a finite number of genes? The explanation was provided by Tonegawa and colleagues (Hozumi and Tonegawa, 1976; Tonegawa, 1983). They found that the genes for immunoglobulin variable regions are inherited as sets of gene segments, consisting of three unlinked families of genes located separately on chromosomes 2, 14 and 22. As B cells develop in the bone marrow, different gene segments recombine in different cells. Each cell then generates a unique gene for the variable region of each chain of the immunoglobulin. Consequently, a relatively small number of gene segments generates many B cell receptor proteins; each B cell expresses a unique receptor specificity; all the progeny of any cell (the clones) will inherit genes encoding the same receptor specificity. T cell receptor diversity is generated in a similar way. The small amount of genetic material involved in this process is sufficient to generate between $10^8$ and $10^{12}$ different immunoglobulin specificities and up to $10^{18}$ T cell receptor specificities (Fearon and Locksley, 1996; Jerne, 1985; Kuby, 1994; Pereira et al., 1989).
That repertoire is generated from a small group of variable region genetic sequences at each locus by DNA rearrangement (somatic recombination) (Janeway, 1993; Rolink and Melchers, 1991). The potential to generate diversity is further enhanced by the imperfect way in which somatic recombination occurs (Flajnik, 1994; Grawunder et al., 1997; Nussenzweig et al., 1996; Oettinger et al., 1990; Rajewsky, 1996; Ramsden, Paull and Gellert, 1997; Schatz and Baltimore, 1988; Schwarz et al., 1996; Tarlinton et al., 1995; Weaver and Alt, 1997; Wilson, Grawunder and Lieber, 1997). At the same time that immunoglobulin gene diversity is being generated both among and within B cells, only one rearranged heavy chain gene and only one rearranged light chain gene are finally expressed in each individual developing B cell. This leads to B cells exhibiting a single type of antigen receptor, the restriction being termed allelic exclusion (Nussenzweig et al., 1987; Papavasiliou et al., 1995; Rajewsky, 1996). Once functional antibody genes have been assembled, diversity can be further generated throughout the variable region by the process of somatic hypermutation (Cascalho et al., 1996; Williams, 1996b; Yélamos et al., 1995).

The major function of IgG is activation of the complement cascade by binding to C1q, the initial component (Brekke et al., 1993, 1996). The most efficient activator of complement, however, is IgM (Colten, 1994). Both IgG and IgM exhibit significant levels of natural autoantibody reactivity, mainly to enzymes, intracellular structural elements, MHC class I and II molecules, and TCRs. However, they mutually inhibit each other's autoreactivity in whole serum (Berneman, Ternynck and Avrameas, 1992). IgG is the most abundant mammalian antibody.

IgA is the antibody characteristic of mucosal surfaces. Secretory IgA is found in exocrine secretions such as milk (Newman, 1995), tears, saliva and perspiration, urogenital secretions, and seromucous secretions of the lung and intestine (Coleman, Lombard and Sicard, 1992). Its major biological function is to trigger the alternate
pathway of complement activation, under the influence of interleukin-6 (Husband et al., 1996; Ramsay et al., 1994). IgA and IgM are able to form polymers, aided by the presence in a polymer of an additional polypeptide, termed the J chain. IgM mainly forms pentamers, useful in binding to repetitive epitopes and to C1q of the complement cascade (C1q has six Ig binding sites); IgA forms dimers which enable it to be transported through epithelia (Potter and Smith-Gill, 1990).

The primary biological function of IgD is unknown (Gleeson et al., 1987) but its location on the surface of B cells suggests that it is likely to be an antigen receptor (Chen et al., 1993).

IgE exhibits high-affinity binding to mast cells and basophils, the cells of the allergic response, and lower-affinity binding to macrophages and other phagocytes (Sutton and Gould, 1993). IgE production is dependent on interleukin-4 (Yoshimoto et al., 1995). IgE has been implicated in allergic disease (MacDonald et al., 1995), bronchial hyperreactivity (van Herwerden et al., 1995), environmental stress (Parker, 1991) and parasitic infections, especially helminthic infestations (Janeway et al., 1967). IgE is usually thought to be the prime trigger for systemic anaphylaxis, yet a non-IgE pathway for the anaphylactic response exists in mice, perhaps mediated by IgG (Oettgen et al., 1994), linked to the low affinity IgG receptor FcyRII (CD28) (Hulett et al., 1995; Takai et al., 1996).

The heavy chain is encoded by only one set of variable region gene segments (which determine antigen specificity) and a cluster of constant region genes (which determine isotype and effector function). Thus, in responding to an antigen, a B cell begins by producing IgM (of the appropriate specificity). It can then exchange CH gene segments, altering the class and effector functions of the antibodies being produced. The process, termed isotype switching or class switching, occurs between highly repetitive DNA sequences, termed the switch (s) regions, and is directed to
particular Ig classes by cytokines (Lorenz, Jung and Radbruch, 1995), vasoactive intestinal peptide (VIP) (Kimata, 1996) and nerve growth factor (Kimata et al., 1991). The expression of IgD, however, differs from the expression of the other isotypes in that, when/if it is produced, it is co-expressed with IgM on the surface of mature naive B cells (Cheung et al., 1982).

2.3.2 Antigen recognition

Antigens make up a diverse collection including foreign cells and a wide variety of molecules such as proteins (either in native form or substituted with groups such as dinitrophenyl), polysaccharides and polypeptides. Any antigen consists of an array of antigenic determinants (epitopes), each of which is a chemical grouping capable of recognition by antigen receptors, antibodies and/or T cell receptors (Klaus, 1990). The diversity seen in presenting antigens is matched by the diversity and variety exhibited by antibodies. Antibodies bind to molecular structures such as protein macromolecules, nucleic acid macromolecules, and carbohydrate macromolecules. However, antibodies recognise neither lipids found inside membranes nor hydrocarbons polymerised as plastics (Kaplan, 1987).

The amino acid pattern of an epitope can arise either through folding of the protein, giving rise to a conformational or discontinuous epitope, or through the amino acid sequence of a single segment of polypeptide, yielding a linear or continuous epitope (Sheriff et al., 1987). The number of different epitopes displayed by one antigenic molecule can be considerable, with the differences not necessarily being absolute (Read et al., 1994); some degree of cross-reactivity is also possible (Riley et al., 1995). Also, proteins showing interspecific homology can display common epitopes, giving rise to antibody cross-reactivity (Kumar, Folgar and Lubega, 1992).
The interaction between antigen and antibody is non-covalent, most commonly electrostatic in nature. It can also be hydrophobic, especially for larger antigens. These two forces, together with overall surface complementarity, appear to be the determining features of antibody specificity (Novotny and Sharp, 1992). In fact, antibodies are capable of binding to viral surfaces, irrespective of the location and size of the cell recognition site (whether the exposed site of poliovirus or the buried site of human rhinovirus 14), largely because of electrostatic forces (Smith et al., 1996a).

2.3.3 The B cell

2.3.3.1 Generation and selection of B cells

B cell differentiation in mice and humans begins in fetal para-aortic tissue, spleen and liver and, after birth, takes place in bone marrow. Four steps have been identified in the differentiation pathway leading to an immature B cell. In the first step, a haematopoietic stem cell (Golde, 1991) develops into an early pro-B cell, in which immunoglobulin gene rearrangement has not yet begun while characteristic cell surface proteins are evident. The following steps are defined by stages in the rearrangement of immunoglobulin genes, changes in cell surface molecules, dependence on growth factors, and location in the bone marrow. In the second step, D-JH joining within the immunoglobulin heavy chain gene has occurred, resulting in the late pro-B cell. In the third step, a VH gene segment in the late pro-B cell becomes joined to the rearranged DJH, resulting in a pre-B cell that is expressing both low levels of surface and high levels of cytoplasmic μ heavy chain. In the fourth step, the light chain genes (VJL) are rearranged and the cell, now termed an immature B cell, expresses surface IgM (Janeway, 1993; Klaus, 1990; Rolink and Melchers, 1993; Sprent and Tough, 1994; Viale, Freitas and Coutinho, 1994;
Weissman and Cooper, 1993).

B cell development depends on the productive, sequential rearrangement of a heavy- and a light-chain gene (Janeway, 1993). The chances of generating an unsuccessful gene rearrangement are high and cells that do so will be lost (Tarlinton et al., 1995).

Immature B cells express only sIgM. The completion of B cell development involves emigration from the bone marrow and expression of both sIgM and sIgD. Self antigens in bone marrow, however, can lead to deletion or inactivation of immature B cells, the process taking place during the few days before the cells develop into mature B cells. When developing B cells express either antinuclear antibodies or receptors that recognise ubiquitous, multivalent self cell surface antigens, such as those of the MHC, they are eliminated from the repertoire by a process termed clonal deletion (Chen et al., 1995a; Namazee and Bürki, 1989; Nossal and Pike, 1975). These B cells are believed to undergo apoptosis (programmed cell death) (Boise and Thompson, 1996; Duke, Ojcius and Young, 1996; Steller, 1995; Vaux and Strasser, 1996). Immature B cells that bind soluble self antigens, on the other hand, are rendered unresponsive, or anergic, to the antigen and lose sIgM (Fulcher et al., 1996; Nossal, 1989, 1996). They migrate to the periphery where they express sIgD but remain anergic (Nossal and Pike, 1980). Only immature B cells that do not encounter antigen in the bone marrow at this early stage of development mature normally and migrate to peripheral lymphoid tissues as naive B cells expressing both sIgM and sIgD (Klaus, 1990).

Depending on their affinity for antigen, a small proportion of autoreactive B cells may survive in bone marrow long enough to be rescued from clonal deletion if their receptor is replaced by a non-autoreactive one, in a process termed receptor editing (Gay et al., 1993; Radic et al., 1993; Rajewsky, 1996; Tiegs, Russell and Nemazee, 1993).
The central position of clonal deletion in regulating B cell auto-antibody responses has been questioned (Diener, Fotedar and Sinha, 1993) in view of the existence of potentially auto-reactive B cells in normally functioning immune systems (Yung et al., 1973) which can become activated under appropriate conditions (Unlig, Rutter and Derrick, 1985).

B cells are produced continuously but only some contribute to a relatively stable peripheral pool. The total number of sIgM+ B cells generated in the bone marrow is quite large, about $2 \times 10^7$ per day in young mice. Few die, so most leave the bone marrow bound for the secondary lymphoid tissues. The majority of these mature B cells die within a few days and only a small proportion enter the pool of mature recirculating B cells (Sprent and Tough, 1994).

### 2.3.3.2 B cell heterogeneity

B cells are not a single homogeneous population. In fact, two major subpopulations arise from distinct progenitor cells and have distinctive properties. CD5 B cells (B-1 cells) first arise early in ontogeny and, in adults, form a self-renewing population of B cells; they can be further subdivided into B-1a and B-1b populations, produced by separate progenitors (Tornberg and Holmberg, 1995). Conventional B cells (B-2 cells) appear later in ontogeny and are replaced by new cells from bone marrow throughout life (Herzenberg and Kantor, 1993).

B-1 cells, which predominate in the peritoneal (Marcos et al., 1994) and pleural cavities, account for most of the immunoglobulin in normal serum, especially IgM. They are distinguishable from conventional B cells in surface phenotype (CD5+; Bikah et al., 1996), antigen specificity (Paglieroni, Ward and Holland, 1995),
signalling, and growth properties (Rickert, Rajewsky and Roes, 1995).

As conventional B cells mature, they leave the bone marrow and migrate to the lymphoid follicles of lymph nodes, spleen and other peripheral lymphoid tissues (Anderson, 1990). Mature B cells form part of the recirculating lymphocyte pool (Butcher and Picker, 1996), moving from blood into primary lymphoid follicles and then back into peripheral blood through specialised high endothelial venules located in lymphoid tissue (Anderson, 1990). Many B cells are found in the gut associated lymphoid tissue, where very large lymphoid follicles termed Peyer’s patches provide specialised sites where B cells mature to secrete IgA, largely under the influence of interleukin-6 (Husband et al., 1996; Ramsay et al., 1994).

Some self-reactive B cells enter the circulation but are unable to enter lymphoid follicles and so die within three days. They are denied access to the lymphoid follicles by a process of competitive exclusion whereby the passage of anti-foreign B cells is favoured (Cyster, Hartley and Goodnow, 1994; Nossal, 1994).

If B cells encounter antigen and appropriate helper T cells when they enter lymphoid tissue, and become activated, they proliferate first in the T cell areas, then some proliferating B cells establish germinal centres. A germinal centre is the site of rapid expansion of antigen-activated B cells, where both antigen-dependent secondary V(D)J rearrangement (Han et al., 1996; Hikida et al., 1996) and somatic hypermutation (Rajewsky, 1996) take place. Autoreactive B cells that develop in a germinal centre are eliminated, either by negative selection or clonal deletion (Pulendran, van Driel and Nossal, 1997). The resulting population differentiates into either memory B cells or antibody-secreting plasma cells (Arpin et al., 1995). Plasma cells are found predominantly in medullary cords of lymph nodes, in red pulp in the spleen, and in the bone marrow where they can generate a significant proportion of the circulating antibodies (Rouse et al., 1984).
2.3.3.3 B cell activation and antibody production

B cell activation is triggered when antigen binds to the B cell receptor (DeFranco, 1987). Additional signals are also provided when the B cell interacts with other cells (These interactions will be discussed later.). Antigen crosslinking of B cell receptors is often not sufficient to activate B cells. Activation is enhanced by interaction with regulatory cells such as helper T cells (TH2). The antibody response of the activated B cell is then amplified by interaction with follicular dendritic cells which retain antigen-antibody-complement complexes on their surfaces (Fearon and Locksley, 1996; Rickert, Rajewsky and Roes, 1995; Tarakhovsky et al., 1995; Zhang et al., 1995).

The intracellular pathogens that trigger T cell mediated cytotoxicity move from cell to cell through extracellular fluids; other major bacteria multiply in the extracellular space. The humoral immune response targets these extracellular pathogens, using antibodies secreted by B cells.

The binding of antigen to surface antibody signals the B cell, and the antigen is also internalised and processed into peptides that activate TH2 cells which recognise epitopes of the same molecular complex. A second signal is required for subsequent B cell activation, either by thymus-dependent (TD) or thymus-independent (TI) antigens (Rajewsky, 1996). In the case of TD antigens, such as peptides derived from bacterial protein, the second signal is delivered by the TH2 cell recognising the peptide:MHC class II complex on the B cell surface. In the case of TI antigens, such as bacterial polysaccharides, the second signal is delivered by the antigen itself (Austyn, 1989).
The specific interaction between the B cell and the TH2 cell leads to the expression of the B cell stimulatory molecule CD40 ligand (CD40L) (Grewal et al., 1996; Grewal, Xu and Flavell, 1995; van Essen, Kikutani and Gray, 1995; Yang and Wilson, 1996) on the TH2 cell surface and the secretion of the cytokines IL-4 (Mitchell et al., 1996; Yoshimoto et al., 1995), IL-5 (Dickason and Huston, 1996; Karlen, D'Ercole and Sanderson, 1996) and IL-6 (Kishimoto, Akira and Taga, 1992; Ward et al., 1996), which drive the proliferation and differentiation of the B cell into antibody-secreting plasma cells (Clemens, 1991; Hamblin, 1988; Ihle, 1990). The expression of particular antigen-specific antibody begins with sIgM and sIgD on mature naive B cells. This is followed by isotype switching so as to generate the appropriate immunoglobulin isotypes, under the direction of cytokines released by TH2 cells (Clemens, 1991; Hamblin, 1988; Ihle, 1990).

B cell proliferation and differentiation and, in particular, affinity maturation result from the interactions between activated B cells and cells in the germinal centre, even though affinity maturation and memory cell development are not absolutely dependent on the presence of a germinal centre (Matsumoto et al., 1996). This process allows selection of B cells of progressively higher affinity to contribute to the response (Graves, Doubrovsky and Beckers, 1991; Rajewsky, 1996). The two emerging types of cells (plasma and memory) appear to be derived from different precursor pools (Linton, Decker and Klinman, 1989; Linton et al., 1992; Linton, Rudie and Klinman, 1991). Plasma cells are terminally differentiated B cells - they secrete antibodies, they lack surface immunoglobulin receptors and MHC class II molecules, and they cannot switch immunoglobulin isotype or undergo somatic hypermutation. Nevertheless, they display a greater degree of affinity maturation than do memory cells (Smith et al., 1997), and are as long-lived (Manz, Thiel and Radbruch, 1997).
2.3.4 The major histocompatibility complex

The actions of T cells depend, for the most part, on their ability to recognise cells containing pathogens or their products because such cells display on their surface peptide fragments derived from the pathogen. The peptide fragments are delivered to the surface of the cell bound to specialised glycoproteins termed MHC molecules, encoded by the major histocompatibility complex (MHC) of genes (Zinkernagel and Doherty, 1974).

The MHC class I and class II molecules are cell surface glycoproteins that are closely related in overall structure and function (Madden et al., 1991). The specificity with which MHC molecules process antigens and present antigenic peptides depends on the existence of a vast array of such molecules able to match the array of antigens. The repertoire of MHC molecules must also be flexible and responsive over time, in order to overcome mutational changes which would otherwise allow pathogens to escape immune detection. MHC molecules possess these characteristics because of the mechanisms used by the MHC, the region containing the genes that encode them (Parham and Ohta, 1996). Firstly, the MHC is polygenic. The MHC occupies about $4 \times 10^6$ base pairs on chromosome 6 in the human, and consists of at least 50 genes (Human MHC is also termed HLA, the human leukocyte antigen region.). The presence of several loci introduces variety into the expression of MHC molecules (Parham and Ohta, 1996). As well as being polygenic, the MHC is polymorphic (Parham and Ohta, 1996).

Polymorphism has an impact on at least three aspects of the activity of MHC molecules: the range of peptides bound, the conformation of the peptide that is bound, and the interaction between the MHC molecule and the T cell receptor. This extensive polymorphism is extremely important in antigen recognition by T cells. A T cell will recognise antigen as a peptide bound to a particular allelic variant of an
MHC molecule, and will not recognise the same peptide either unbound or bound to other MHC molecules. This behaviour of T cells is termed MHC restriction (Zinkernagel and Doherty, 1974).

2.3.5 The T cell

2.3.5.1 T cell development and selection

The thymus plays a central role in the creation of a fully functional immune system (Miller, 1961, 1962a, b, 1994, 1996a). It provides a receptive microenvironment for bone marrow T cell precursors to enter, clonally expand, select antigen and MHC specificities, mature, and move out to the periphery (Anderson, 1990; Ritter and Crispe, 1992). As a result of positive selection for cells with appropriate MHC affinity and negative selection against self-reactive cells, the thymus releases fewer than 5% of its daily cell production, the remainder undergoing apoptosis and engulfment by macrophages (Boise and Thompson, 1996; Shortman and Scollay, 1994; Surh and Sprent, 1994) within a few days (Sprent and Tough, 1994).

Specific combinations of cell surface molecules can be used as markers of T cells at different developmental stages. When the T cell progenitor has arrived in the thymus, it interacts with the thymic stroma. The interaction triggers rapid proliferation and expression of the first T cell-specific surface molecules, CD2 and (in mice) Thy-1. At the end of this phase the immature thymocytes do not yet express the three cell surface molecules found on mature T cells, so are described as CD3-CD4-CD8-, or double negative thymocytes (negative for both CD4 and CD8). About 20% of the double negative cells belong to a separate lineage that encodes the γδ TCR; a second double negative population (about 5% of the total) expresses the αβ TCR and interacts with CD1 proteins (Bendelac et al., 1995; Porcelli, Morita and
The remaining 75% of double negative thymocytes are committed to the major αβ T cell lineage. These cells can now be further separated on the basis of expression of CD44 (an adhesion molecule) and CD25 (the α chain of the IL-2 receptor) (Zúñiga-Pflücker, Jiang and Lenardo, 1995). Initially the cells are CD44+CD25− and the TCR β chain is in germline configuration. As β chain gene rearrangement proceeds, CD25 is also expressed, so they are CD44+CD25+. Productive rearrangement of the β chain genes is found in CD44+CD25+ cells. At this stage, they are also able to act as precursors of thymic dendritic cells (Wu, Li and Shortman, 1996). The β chain then pairs with a surrogate α chain, termed pTα (Fehling et al., 1995). This pairing leads to a loss of CD25, active cell proliferation, and the expression of both CD4 and CD8. The cells are now double positive and they rearrange the α chain. These double positive cells express low levels of αβ:CD3 and become small inactive cells, most of which fail to be positively selected and die. The remaining cells down-regulate the expression of either CD4 or CD8, and express high levels of αβ:CD3. These single positive thymocytes then leave the thymus as either CD4+CD8+ or CD4+CD8− T cells, respectively (Fowlkes and Pardoll, 1989; Janeway and Travers, 1996; Kydd et al., 1995 Ritter and Crispe, 1992; Ruiz, Schwätzler and Günthert, 1995; von Boehmer, 1994; Weber et al., 1996; Weissman, 1994).

In the early stages of thymocyte maturation, T cell receptor (TCR) genes undergo a series of rearrangements resulting in large numbers of immature T cells, each expressing receptors of a single specificity. This process is very similar to that of generation of diversity in B cells, with two important differences. First, rearrangement of two different sets of receptor genes produces two T cell lineages, one expressing αβ TCRs and the other γδ TCRs. Second, there is greater scope for repeated rearrangements of TCR genes at a single locus, allowing rescue of many cells with otherwise unproductive initial gene rearrangements. In the αβ T cell lineage the outcome is double positive thymocytes expressing TCRs on which
positive and negative selection can act (Anderson, 1990; Janeway and Travers, 1996; Laufer et al., 1996; Ritter and Crispe, 1992; Shevach, 1990; Surh and Sprent, 1994).

Double positive immature thymocytes undergo positive selection for self MHC restriction and lose one of the two co-receptor molecules. They also undergo negative selection in which potentially self reactive cells are eliminated (Kersh and Allen, 1996; Marrack and Kappler, 1993) by apoptosis (Hockenbery, 1995; Hockenbery et al., 1990; Minn et al., 1997; Sentman et al., 1991; Strasser, Harris and Cory, 1991; Wyllie et al., 1990).

At the start of positive selection, a thymocyte is double positive. At the end of the selection process, mature T cells that express CD4 have receptors that recognise peptides bound to self MHC class II molecules, and those that express CD8 have receptors that recognise peptides bound to self MHC class I molecules (Bevan, Hogquist and Jameson, 1994; Sim et al., 1996; Teh et al., 1988).

Developing T cells in the thymus encounter a large selection of self peptides bound to self MHC molecules on thymic cells, and the developing cells with TCRs specific for these self peptides are deleted from the repertoire by negative selection. Negative selection in the thymus cannot eliminate T cells whose TCRs recognise proteins from other sites in the body. These proteins are protected by immunological tolerance, a mechanism that will be discussed later (Marrack and Kappler, 1993).

Whereas thymic cortical epithelial cells mediate positive selection, negative selection is mediated by several different cell types, the most important being bone marrow derived macrophages and dendritic cells. The self antigens presented by these cells are, therefore, the most important source of potential autoimmune responses, so T cells responding to such self peptides are eliminated in the thymus (Gordon, 1995; Jiang et al., 1995; Süss and Shortman, 1996).
The first T cells to appear in embryogenesis are γδ T cells. They exist in large numbers early in life and are gradually supplanted by αβ T cells. γδ T cells are found in epithelial areas of the body such as the gut, skin, lungs and uterus, and they may provide a “first line of defence” across epithelial surfaces (Brenner et al., 1986; Ferrick et al., 1995; Haas, Pereira and Tonegawa, 1993; Saito et al., 1984). γδ T cell function is considered in more detail later (section 2.3.5.2).

Not all T cells develop in the thymus. The intraepithelial lymphocytes of the gut are a large heterogeneous population of immune cells in the intestinal epithelium, predominantly CD4⁺CD8⁺, which exhibit NK- or CTL-like activity (Anderson, 1990; Shanahan, 1997; Wang and Klein, 1994; Wang, Whetsell and Klein, 1997). Human T cell development can also take place in lymph nodes, via a thymus-independent pathway (Clegg et al., 1996); this mechanism does not operate in the mouse (Modigliani et al., 1994).

2.3.5.2 T cell ligands

The truly intracellular compartment, the cytosol and adjacent nuclear spaces, is where viruses and some bacteria replicate. Cells infected in this manner are killed by cytotoxic T cells (also termed cytotoxic T lymphocytes or CTL), which are distinguished by the cell surface molecule CD8 (Garcia et al., 1996, 1997; Luescher et al., 1995). CD8 T cells are able to recognise their target cells because the peptide antigen is presented on the cell surface by MHC class I molecules which process materials from the cytosol. Thus, a CD8 T cell recognises both the antigen and the MHC class I molecule together (Bjorkman et al., 1987).
The vesicular compartment, which includes endosomes, lysosomes, endoplasmic reticulum and Golgi apparatus, is considered to be continuous with extracellular fluid. It is the location for some major pathogenic bacteria and eukaryotic parasites which are detected by T cells carrying the CD4 surface molecule. CD4 T cells generate one of two modes of action and fall into two different functional categories (Bank and Chess, 1985; Mosmann et al., 1986; Pawelec, Schneider and Wernet, 1983). TH1 cells (also termed inflammatory T cells) rid the body of intracellular pathogens by activating macrophages to kill the intravesicular bacteria they harbour; TH2 cells (helper T cells) regulate the destruction of extracellular parasites by activating B cells to make antibody (Ferrick et al., 1995; Flajnik, 1994). Because MHC class II molecules process and present materials from the vesicular compartment, CD4 T cells recognise a complex of the antigen and the MHC class II molecule together (Brown et al., 1993a).

Thus, protein antigens that are actively synthesized by the cell (viral proteins) are processed and presented (as peptides) by MHC class I molecules, while antigens obtained from outside the cell and degraded in lysosomes are processed and presented (as peptides) by MHC class II molecules (Babbitt et al., 1985; Buus et al., 1986; Hoffman, 1992; Shevach, 1990; Shimonkevitz et al., 1984).

αβ T cells predominantly recognise foreign and self-peptides presented by MHC molecules (Germain and Margulies, 1993). However, antigen recognition by γδ T cells is not so well understood (Haas, Pereira and Tonegawa, 1993), yet γδ T cells do respond to foreign microbial pathogens. This group of nonpeptide antigens recognised by γδ T cells is fundamentally distinct from peptide antigens or protein superantigens recognised by αβ T cells (Tanaka et al., 1995). The manner of their presentation to γδ T cells is equally distinct. The pathway is an extracellular, accessory cell-independent one that does not require antigen internalisation or
processing by any antigen-presenting molecule. This pathway allows for an early, rapid response to bacterial infection by γδ T cells (Morita et al., 1995). In some other instances, γδ T cells appear to bind to products of the host itself (Havran, Chien and Allison, 1991), including products of damaged cells, such as heat shock proteins (Born et al., 1990), so perhaps one of the functions of γδ T cells is to recognise the appearance of foreign material in the body by its effects on host cells themselves (Marrack and Kappler, 1994).

2.3.5.3 The T cell receptor

The T cell antigen receptor (TCR) consists of two different polypeptide chains (either α and β chains or γ and δ chains). The TCRαβ accounts for all known peptide antigen recognition (Germain and Margulies, 1993; Yewdell and Bennink, 1992); the TCRγδ has already been mentioned in another context (section 2.3.5.2). Thus, reference to the TCR in this section will be confined to the TCRαβ.

The TCR heterodimer consists of two transmembrane glycoprotein chains, α and β. The extracellular part of each chain contains two domains, one variable (V) domain and one constant (C) domain; the V domains of both chains include V and J elements while the β chain also has a D element. Both chains have carbohydrate side chains attached to each domain. Thus, this structure is homologous to a Fab fragment of an immunoglobulin. A short segment, similar to an immunoglobulin hinge region, connects the Fab-like structure to the cell membrane (Flajnik, 1994; Haskins et al., 1983; Meuer et al., 1983).

The arrangement and function of TCR genes are very similar to those of immunoglobulin genes (described in section 2.3.1). The generation of TCR diversity depends on several mechanisms: the germline multiplicity of V, D and J elements;
combinatorial joining of the V, D and J gene segments; the addition of non-germline nucleotides at gene segment junctions by terminal deoxynucleotidyl transferase (N diversity); and the combinatorial heterodimeric associations of any one of many α chains and many β chains. The DNA sequence of the human TCR β chain locus, part of the raw material for these mechanisms, has now been determined (Rowen, Koop and Hood, 1996).

There are two main differences between the immunoglobulin genes and the TCR genes. Firstly, TCR genes do not show somatic hypermutation (Flajnik, 1994). Secondly, there is no secreted form of the TCR. Thus there is only one Cα gene and two Cβ genes (with functionally indistinguishable products); the C genes only encode transmembrane polypeptide - an alternative secreted form is not coded for (Janeway and Travers, 1996).

Not all antigens that bind to MHC class II molecules are presented as peptides in the peptide-binding groove. One distinct group, termed the superantigens, do not undergo processing and presentation. They are proteins that bind directly to MHC class II molecules on the cell surface and to the Vβ region of the TCR. A superantigen is able to circumvent the normal mechanism for T cell activation by specific MHC/peptide complexes by acting as a wedge between the TCR and MHC so as to displace the antigenic peptide away from the TCR combining site (Fields et al., 1996). As there are only 20 to 50 different Vβ gene products, a superantigen can stimulate up to 20% of T cells. This nonspecific level of stimulation does not lead to an adaptive immune response, rather it produces systemic toxicity and immunosuppression, consequent on large scale production of cytokines by CD4 T cells (Herman et al., 1991; Mandelboim et al., 1996; Marrack and Kappler, 1993; Phillips et al., 1995).
During antigen recognition, CD4 and CD8 molecules associate with components of the TCR where, in broad terms, they modulate TCR-ligand interactions. Thus, they are termed co-receptors (Damle and Engleman, 1990; Henkart, 1990; Janeway and Travers, 1996; Luescher et al., 1995; Shevach, 1990). For T cell activation and signalling to occur, sufficient TCRs must engage sufficient peptide:MHC complexes. In the absence of the co-receptor (CD4 or CD8), 10,000 identical complexes are required; the involvement of the co-receptor reduces the number to 100 complexes (Corr et al., 1994; Davis, 1995; Janeway et al., 1985; Rothenberg, 1996; Valitutti et al., 1995; Viola and Lanzavecchia, 1996).

2.3.5.4 Activation of effector T cells

Adaptive immune responses are initiated in the organised peripheral lymphoid tissues, such as the lymph nodes, where a pathogen or its products are transported in the lymph. Naive T cells travel from the blood to the cortex of a lymph node by crossing the walls of high endothelial venules. The continual passage of naive T cells past antigen presenting cells (APCs) in a lymph node is crucial as only one naive T cell in $10^5$ is likely to be specific for a particular antigen. The remaining T cells return to the blood so as to recirculate through other lymphoid organs. The small number of T cells that recognise their specific antigen on the surface of an APC remain in the cortex and begin the process that generates armed effector T cells (Picker and Butcher, 1993).

The three main types of professional APCs found in peripheral lymphoid organs are macrophages, dendritic cells and B cells. Each is specialised to process and present antigens from different sources; macrophages and B cells are also, in turn, the targets of armed effector T cells. Only these three cell types express the appropriate co-
stimulatory molecules that enable them to activate naive T cells. These professional
APCs are distributed differentially in a lymph node: dendritic cells are found
throughout the cortex, in the T cell areas; macrophages are distributed throughout
the lymph node; B cells are found mainly in the follicles (Anderson, 1990; Austyn,
1989).

The steps leading from a naive T cell to a clone of armed effector T cells through to
interactions with target cells involve, for the most part, cell-cell interactions that are
not antigen-specific. These interactions are controlled by a diverse collection of
adhesion molecules, mainly the selectins, the integrins, members of the
immunoglobulin superfamily, and some mucin-like molecules (Heath et al., 1997;
Horwitz, 1997; Parkos, 1997; Springer, 1994). All T cells express the β2 integrin
known as lymphocyte function-associated antigen-1 (LFA-1), thought to be the most
important adhesion molecule for lymphocyte activation (Larson and Springer, 1990).

On the rare occasion when a naive T cell recognises its specific MHC:peptide ligand,
signalling through the TCR induces a conformational change in LFA-1. The change
stabilises the association between the cells; the association can persist for days,
during which the naive T cell proliferates and its progeny differentiate into armed
effector T cells (Dustin and Springer, 1989). That association on its own, however, is
insufficient to stimulate proliferation. A second co-stimulatory signal is required.
Armed effector T cells, however, can successfully engage target cells without a
second signal (Allison and Krummel, 1995; Corr et al., 1994; Janeway, 1993;
Jenkins et al., 1991; Leach, Krummel and Allison, 1996; Marengère et al., 1996;
Pardoll, 1996; Plas et al., 1996; Rothenberg, 1996; Schwartz, 1992; Viola and
Lanzavecchia, 1996; Waterhouse et al., 1995; Zenner et al., 1995). The requirement
that the same cell presents both the specific antigen and the co-stimulatory signal is
so strict that antigen binding to the TCR in the absence of co-stimulation not only
fails to activate the T cell but leads to a state of anergy whereby the T cell becomes
refractory to activation. T cells usually encounter self antigens on cells other than APCs, so that anergy is a crucial mechanism in preventing immune responses to self antigens (Bretscher and Cohn, 1970; Fields, Gajewski and Fitch, 1996; Jenkins, Ashwell and Schwartz, 1988; Jenkins and Schwartz, 1987; Li et al., 1996; Marrack and Kappler, 1993; Quill and Schwartz, 1987; Williams, 1996a).

Macrophages are both "professional phagocytes" (Brown, 1995; Spilsbury et al., 1995) and professional APCs (Gordon, 1995). They occur in every tissue, where they have a role in surveillance against infection, in wound repair, and in tissue remodelling. When macrophages ingest bacteria, they also respond to some of the bacterial DNA by activating inflammation (Stacey, Sweet and Hume, 1996). Depending on their location in tissues, macrophages acquire a characteristic function and phenotype: in the brain they are known as microglia; capillaries contain serosal macrophages; the lungs have alveolar macrophages; liver macrophages are termed Kupffer cells; they are mesangial cells in the kidneys and peritoneal macrophages in the abdomen (Austyn, 1989). If protein antigens are taken up and presented by macrophages in the absence of bacterial components that could induce co-stimulatory activity in the macrophage, T cells specific for the antigen become anergic. Thus, self peptides derived from scavenged dead or senescent cells (that are not infected) and presented by macrophages will induce T cell anergy and tolerance, rather than an autoimmune response (Liu and Janeway, 1991).

Dendritic cells are a unique class of leukocytes which function primarily as APCs (Adema et al., 1997; Cella et al., 1997; Kronin et al., 1996; Pierre et al., 1997; Watts, 1997). They occupy discrete sites in lymphoid and nonlymphoid organs: those in lymphoid organs are termed lymphoid dendritic cells and interdigitating cells; the circulation contains afferent lymph veiled cells and blood dendritic cells; nonlymphoid organs contain Langerhans' cells and interstitial dendritic cells (Steinman, 1991). The dendritic cells in lymphoid tissue are able to process antigen
from extracellular and intracellular pathogens, prime both CD4 and CD8 naive T cells, and provide the co-stimulatory signal (Steinman, 1991). Although the ability of lymphoid dendritic cells to take up antigen is limited, they direct captured antigen from the extracellular space to intracellular antigen-processing compartments (Jiang et al., 1995). Because of their efficiency in presenting antigen, and their location in the T cell areas of lymphoid organs, dendritic cells play a significant role in clonal deletion of self-reactive T cells (Steinman, 1991). A specialised subgroup of dendritic cells, with the ability to kill CD4 T cells by apoptosis, is involved in the regulation of peripheral T cell responses (Süss and Shortman, 1996). The best known nonlymphoid tissue dendritic cells are the epidermal Langerhans' cells. They are able to ingest antigen but have no co-stimulatory activity. In an infection they take up antigen locally in the skin and migrate to lymph nodes. There they differentiate into dendritic cells that have lost the ability to ingest antigen but that can stimulate T cells (Steinman, 1991).

Most natural antigens are particulate and are taken up effectively by macrophages or dendritic cells. Some natural antigens (insect toxins and anticoagulants, snake venom) are soluble and can bind to the cell surface immunoglobulin of B cells. Surface immunoglobulin allows B cells to bind, internalise, process and present specific antigen very efficiently (Austyn, 1989). The range of antigens that B cells can present, however, is restricted to these specific types (Epstein et al., 1995).

The differentiation of CD4 T cells into inflammatory (TH1) or helper (TH2) effector cells is the key event that determines whether a cell-mediated or a humoral immune response will prevail. The presence of interferon γ (IFN-γ) favours the development of TH1 cells, while IL-4 favours the TH2 response (Bach et al., 1995; Bazan, Timans and Kastelein, 1996; Cohen, 1995; Ferrick et al., 1995; Güler et al., 1996; Hall, 1995; Hsieh et al., 1993; Marx, 1996; Noben-Trauth, Kropf and Müller, 1996; Okamura et al., 1995; Pernis et al., 1995; Walter et al., 1995; Yoshimoto et al., 1995;
The classification of T\(_{H1}\) and T\(_{H2}\) cells as inflammatory or helper T cells, respectively, is likely to be an oversimplification, especially in light of the biological activities of the cytokines they produce. The main function of T\(_{H1}\) cells is to elicit phagocyte-mediated defence against infections, via the effects of their cytokines on macrophages. In addition, T\(_{H1}\)-dominant immune responses are often associated with inflammation and tissue injury (Abbas, Murphy and Sher, 1996). T\(_{H2}\)-dominant immune responses, such as in allergies and helminthic infections, are characterised by the presence of both IgE and activated eosinophils. T\(_{H2}\) cells are also excellent helpers for B cells and stimulate the production of high levels of IgM and non-complement-fixing IgG isotypes (Abbas, Murphy and Sher, 1996).

Naive CD8 T cells can differentiate only into cytotoxic cells (CTLs), through one of two sequences. They can be activated directly by potent APCs. Some CD8 T cell responses, however, require CD4 T cells. CD8 T cells recognising antigen on weakly co-stimulating cells may become activated only in the presence of CD4 T cells bound to the same APC (Bennett et al., 1997; Khanna et al., 1997).

CTLs are essential in host defence against pathogens that live in the cytosol, the most common being viruses (Henkart, 1990). CTLs kill any cell harbouring such pathogens, by inducing apoptosis (Smyth, 1995). The response can also evoke a certain amount of collateral damage to local host cells (Smyth, 1997). Upon recognition of antigen on the surface of a target cell (Apostolopoulos et al., 1995a, b; Luescher et al., 1995), a CTL releases granules containing two characteristic classes of cytotoxins - the perforins which polymerise to create transmembrane pores in the cell membrane (Clark, 1994; Kägi et al., 1994a, b; Lowin et al., 1994; Smyth, 1994), and the granzymes (or fragmentins), proteases which enter the cell through the pores (Darmon, Nicholson and Bleackley, 1995; Smyth, 1994; Smyth and Trapani, 1995) and induce apoptosis (Darmon, Nicholson and Bleackley, 1995; Quan et al., 1996;
Activated TH1 cells are also involved in mediating cytotoxicity (Henkart, 1990), partly by inducing apoptosis directly, and partly by activating the effector functions of macrophages. The activation of TH1 cells by infected macrophages results in the release of cytokines that both activate other macrophage and co-ordinate the immune response to intracellular bacteria (Adams and Hamilton, 1992; Auger and Ross, 1992; Austyn, 1989; Gordon, 1995; Hamilton et al., 1996; Spilsbury et al., 1995).

2.3.6 Elements of the humoral response

2.3.6.1 Accessory cells

In order to dispose of neutralised pathogens and their toxins, and attack resistant extracellular pathogens, antibodies activate a range of accessory effector cells that express Fc receptors specific for the Fc portion of antibodies of a particular isotype. The accessory cells are activated when their Fc receptors bind to the Fc parts of antibody molecules aggregated on a pathogen; however, they do not bind to free immunoglobulin in the plasma. These accessory cells consist of the phagocytes (macrophages and polymorphonuclear neutrophils) which ingest and kill antibody-coated bacteria, and other cells (NK cells, eosinophils and mast cells) which secrete stored substances (Lewis, 1986; Reynolds and Ortaldo, 1990).
A major function of the Fc receptors on phagocytes is to trigger the uptake and degradation of antibody-coated (opsonised) bacteria, especially those that are otherwise resistant to phagocytosis. Fc receptor binding also signals the phagocyte to increase the rate of phagocytosis, fuse lysosomes with phagosomes, and increase its bacteriocidal activity by generating a range of toxic products. Large parasites, such as worms, cannot be ingested by phagocytes. However, when the worm is coated with antibody, especially IgE, eosinophils can attack it. Similar attacks can be mounted on other larger targets by other Fc receptor-bearing cells (Adamson et al., 1996; Auger and Ross, 1992; Brown, 1995; Eastwood, 1996; Gordon, 1995; Hausladen et al., 1996; Korytko and Boje, 1996; Lewis, 1986; Wei et al., 1995).

Virus-infected cells that display viral protein on their surface become coated with antibody. They are then recognised by NK cells which destroy them by a process termed antibody-dependent cell-mediated cytotoxicity. The cellular mechanism employed by NK cells is the same as that used by CTLs (Flajnik, 1994; Young and Cohn, 1988).

Mast cells are large cells found in mucosal and connective tissues that can be distinguished by secretory granules containing many inflammatory mediators, such as histamine (Lewis, 1986); they are derived from a progenitor mastocyte which is distinct from both the myeloid progenitor and the common lymphoid progenitor (Rodewald et al., 1996). Mast cells bind stably to monomeric IgE antibodies. Antigen cross-linking of the bound IgE molecules triggers rapid degranulation, releasing inflammatory mediators into the surrounding tissues (MacDonald et al., 1995). These mediators trigger local inflammation, which recruits cells and proteins required for host defence to sites of infection (Echtenacher, Männel and Hüllner, 1996; Malaviya et al., 1996). It is also the basis of the acute phase of the allergic reaction causing asthma (van Herwerden et al., 1995), hay fever and systemic
anaphylaxis (Colten, 1994; Galli and Wershil, 1996).

2.3.6.2 The complement system

The complement system is the major soluble protein effector of innate immunity. It consists of a large number of distinct plasma proteins; one is directly activated by bound antibody to trigger a cascade of reactions, each of which results in the activation of another complement component. The sequential activation of the components follows two main pathways, termed classical and alternative, which join to form a common terminal sequence (Figure 2.2). The classical pathway is initiated immunologically, by antigen-antibody complexes; the alternative pathway is initiated non-immunologically, before antibody can be synthesised, by carbohydrate-rich particles that lack sialic acid (endotoxin or bacterial cell wall polysaccharide) (Adinolfi, 1993; Borsos and Leonard, 1990; Fearon and Locksley, 1996; Law and Reid, 1988; Lewis, 1986). In a third proposed activation pathway, termed the lectin pathway, the complement cascade begins when a serum lectin, mannose binding lectin (MBL - first described as mannose binding protein), binds to mannose-containing surface molecules of bacteria or viruses (Lu, 1997; Thiel et al., 1997; Weiss, Drickamer and Hendrickson, 1992). This is probably no more than a subset of the classical pathway, however, since MBL is structurally similar to, and mimics the role of, C1q which interacts with bacteria-antibody complexes (Thompson, 1995a). More correctly, complement fixes antigen-antibody complexes, whether on bacteria or on multicellular parasites (Read et al., 1994). The subsequent uptake and destruction of complement-bound pathogens by phagocytes occurs via complement receptors on the phagocytes (Borsos and Leonard, 1990; Bozic et al., 1996; Dempsey et al., 1996; Fearon and Locksley, 1996; Law and Reid, 1988).
Figure 2.2 The complement system and its inflammatory mediators. Two routes exist by which C3 is converted into active, C3b (active components are designated by a horizontal bar) with the loss of a small fragment, C3a: these are the classic and alternative complement pathways. In the classic pathway antigen-antibody complexes activate C1, the first component of the classic sequence. C4 and C2 are then activated with the loss of small fragments, C4a and C2a. The major fragments form C4b2a which is the C3 convertase enzyme of the classic pathway. The alternative pathway is activated by bacterial cell wall polysaccharides, even in the absence of antibody; these are themselves able to convert C3 to C3b (Lewis, 1986).
Local inflammatory responses can be induced by the small complement fragments, sometimes also termed anaphylatoxins. They are differentially active - C5a is more active than C3a, which is more active than C4a. They act directly on local blood vessels and C5a also acts indirectly by activating mast cells. Like mast cell activation by IgE, these fragments stimulate local increases in blood flow, increased binding of phagocytes to local endothelial cells, and increased local vascular permeability leading to fluid, protein and cell accumulation in local tissues. The fluid increases lymphatic drainage, carrying antigen to local lymph nodes. The antibodies, complement and cells recruited in this way enhance pathogen clearance by phagocytosis. The complement fragments also directly enhance phagocyte activity (Henson, 1996; Höpken et al., 1996; Ilschner, Nolte and Kettenmann, 1996; Law and Reid, 1988; Lewis, 1986; Nolte et al., 1996).

The most significant effect of complement activation is the assembly of the terminal components to form the membrane attack complex (MAC) which creates a pore in the target cell membrane. Up to 16 C9 molecules bind to the complex and polymerise to form a pore of 100Å diameter in the cell membrane, killing the target cell (Borsos and Leonard, 1990; Law and Reid, 1988; Lewis, 1986; Young and Cohn, 1988). Host cells are protected from inadvertent damage by a series of complement regulatory proteins acting at different stages of the cascade (Christiansen et al., 1996; Law and Reid, 1988).
2.3.7 Integrated response to infection

2.3.7.1 Innate response

The first host tissues encountered by a pathogen are the epithelia of the body surfaces which constitute a mechanical, chemical and microbiological barrier that a pathogen must traverse in order to cause infection (Cavanagh et al., 1996; Kupper, 1990; McNabb and Tomasi, 1981; Schonwetter, Stolzenberg and Zasloff, 1995; Tsuchiya and Horii, 1996). When a pathogen does cross an epithelial barrier, the first line of defence is an innate response based on the alternative pathway of complement activation (Figure 2.2). This pathway proceeds in the absence of specific antibody and so is effective up to a week before antibody production can trigger the classical pathway (Borsos and Leonard, 1990; Law and Reid, 1988; Lewis, 1986).

As well as triggering the alternative pathway of complement activation, a pathogen that crosses the epithelial barrier will encounter macrophages. In addition to the Fc and complement receptors which mediate phagocytosis of opsonised particles, macrophages display an array of surface receptors, many of which facilitate microbial interactions (Auger and Ross, 1992). The interaction between macrophages and pathogens has three significant consequences. The first is the phagocytosis of the pathogen, which may be sufficient to prevent an infection from developing. The second important effect is the secretion of cytokines by the macrophages. The third revolves around the contribution by the macrophage, acting as an APC, in induction of the adaptive immune response (Gendelman and Morahan, 1992; Sadick, 1992; Speert, 1992).

The actions of complement and macrophages described here take place in the first hours following local infection. If they are not effective, the next phase of the
response occurs rapidly, as it is also innate, unlike the time the adaptive response takes to induce clonal expansion and maturation. This next phase of the innate immune response includes the recruitment of more phagocytes and effector molecules to the site of infection, in response to monokines (Alcamf and Smith, 1996; Dickinson et al., 1995; Güler et al., 1996; Hachicha, Naccache and McColl, 1995; Hall, 1995; Hsieh et al., 1993; Lewis, 1986; Lloyd et al., 1995; Marx, 1996; Parker, 1991; Rajarathnam et al., 1994; Smith et al., 1996b; Zhan and Cheers, 1995; Zheng et al., 1995). Macrophages release a variety of other molecules in response to infectious agents, while complement activation will have also generated the inflammatory mediators C5a, C3a and C4a (Bazan, 1995; Herschman, Xie and Reddy, 1995; Lewis, 1986). The combined local effect of these mediators is the inflammatory response, characterised by heat, redness, swelling and pain brought about by local vascular changes (Beeson, 1994; Bozic et al., 1996; Cook et al., 1995; Devchand et al., 1996; Serhan, 1996; Sylvestre and Ravetch, 1994). Another effect of these mediators on endothelium is to induce the expression of adhesion molecules that bind to the surface of circulating phagocytes and increase the rate at which they migrate across local small blood vessel walls into the tissues (Butcher and Picker, 1996; Diacovo et al., 1996; Felsenfeld, Choquet and Sheetz, 1996; Ferrara, 1995; Friedlander et al., 1995; Gallicchio et al., 1996; Ilic et al., 1995; Khew-Goodall et al., 1996; Koch et al., 1995; Laudanna, Campbell and Butcher, 1996; Lloyd et al., 1996; Manjunath et al., 1995; Pandey et al., 1995). A third local effect of these mediators is to trigger blood clotting, occluding local small blood vessels. Thus, fluid leaves the blood vessels and carries pathogens to lymph nodes, rather than to the rest of the body (Beutler and Cerami, 1990). As well as their important local effects, the monokines IL-1, IL-6 and TNF-α have a range of biological activities that help coordinate whole body responses to infection. They are endogenous pyrogens, raising body temperature by resetting the hypothalamic set point. They also activate hepatocytes to synthesise acute phase proteins (Sipe, 1990) which act as opsonins. This activity is augmented by enhanced recruitment of neutrophils from
the bone marrow. Finally, they help to initiate the adaptive immune response (Van Snik, 1993).

Cells infected with virus, however, produce anti-viral interferons that have several major functions. First, they inhibit viral replication. Second, they induce MHC class I expression by most cells in the body except virus-infected cells, enhancing resistance to NK cells and increasing susceptibility to CTLs. Third, they activate NK cells which then kill virus-infected cells selectively (Hamilton et al., 1996; Ihle, 1995; Kyriakis and Avruch, 1996).

NK cells are an early component of the host response to virus infection. IFN-α, IFN-β and IL-12 appear first, followed by a wave of NK cells, which together control virus replication but do not eliminate the virus entirely. Viral elimination is accomplished when specific CTLs are produced (Helander et al., 1996; Reynolds and Ortaldo, 1990; Warren et al., 1996). Cytotoxic actions of NK cells are inhibited by the presence of MHC class I molecules on the target cell and an NK cell receptor (Barinaga, 1995; Colonna and Samaridis, 1995; Fan et al., 1997; Gumperz and Parham, 1995; Held, Roland and Raulet, 1995; Karlhofer, Ribaudo and Yokoyama, 1992; Ljunngren et al., 1989; Pazmany et al., 1996; Phillips et al., 1995). The stability of the MHC class I molecules is enhanced when they are complexed with a self peptide (Kärre, 1995; Malnati et al., 1995).

Together with NK cells, CD5 B cells (B-1 cells) and γδ T cells constitute the lymphocytes with receptors of limited diversity. They appear to provide early protection from a limited range of pathogens but do not generate lasting immunity. In the absence of antigen-specific T cell help, only IgM is produced so that these responses trigger the activation of complement (Rajewsky, 1996).
2.3.7.2 Adaptive response

The cells and molecules that mediate the adaptive immune response have already been described. So too have the interactions at the level of the individual steps in the response. Consequently, sources referred to in earlier descriptions will not be cited again. This section, then, deals with the coordinated adaptive response to a pathogen, leading to a state of protective immunity.

The first step in an adaptive immune response leading to protective immunity is the activation of T cells in the draining lymphoid organs (McHeyzer-Williams and Davis, 1995). The trapping of antigen by APCs in the lymphoid tissues and the continuous recirculation of T cells through them ensures that T cells will encounter their specific antigen on a professional APC. The recirculation of naive T cells through lymphoid organs is facilitated by adhesive interactions with endothelial cells of high endothelial venules. The interactions are essentially the same as those described for the extravasation of phagocytes to sites of infection. In the cortex of the lymph node, T cells encounter many APCs. Those that do not recognise their specific antigen leave via the medulla and return to the lymph. T cells that recognise their specific antigen bind firmly to the APC and are activated through the TCR to become armed effector T cells (Butcher and Picker, 1996; Kersh and Allen, 1996).

The differentiation of naive CD4 T cells into armed effector cell types is influenced by cytokines elicited by the pathogen. The nature and amount of ligand presented to a CD4 T cell during primary stimulation can also determine its functional phenotype (Kersh and Allen, 1996).

CTLs and TH1 cells migrate to the periphery where they can activate macrophages and other accessory cells at the site of infection. TH2 cells, however, interact with B cells in lymphoid tissues. The initial encounter takes place at the margin of the T-
and B-cell areas in lymphoid tissue and triggers proliferation of B cells in contact with TH2 cells, resulting in some isotype switching. The activated B cells then migrate to primary lymphoid follicles where they proliferate rapidly to form a germinal centre under the influence of trapped antigen on follicular dendritic cells and TH2 cells. In some instances (after infection with immunodeficiency virus (HIV) and hepatitis B virus in humans, or lymphocytic choriomeningitis virus in mice) the B cells which will go on to produce virus-neutralising antibodies are themselves eliminated by specific CTLs (Planz et al., 1996a,b).

The antibody-secreting plasma cells that develop in lymphoid tissues remain in the medullary cords of lymph nodes and the red pulp of spleen. Other B cells leave the germinal centre as pre-plasma cells, migrating to the bone marrow where they finish developing into plasma cells. IgA-secreting pre-plasma cells usually begin in Peyer's patches and travel via efferent lymphatics to mesenteric lymph nodes and into the blood. The blood then distributes them to those surfaces and secretory areas where IgA can be exported across epithelial layers (Husband et al., 1996).

The effect of a successful primary adaptive immune response to infection is to clear the primary infection and to provide protective immunity against re-infection by the same pathogen. Protective immunity consists of pre-formed immune reactants, such as antibody molecules or armed effector T cells, and immunological memory.

Immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens that had already been encountered in the past; the phenomenon is long-lived, sometimes lasting a lifetime, even if with diminishing effectiveness (Miller, 1996b). It reflects the pre-existence of a clonally expanded and differentiated population of antigen-specific lymphocytes. The generation of secondary antibody responses from memory B cells is distinct from the generation of the primary antibody response. The primary response usually consists of antibody
molecules from a relatively large number of different precursors of relatively low affinity with few somatic mutations; the secondary response comes from fewer, high affinity precursors, perhaps coming from a distinct precursor pool (Linton, Decker and Klinman, 1989; Linton et al., 1992; Linton, Rudie and Klinman, 1991), whose receptors show significant somatic mutation and which have undergone substantial clonal expansion; thus activation produces fewer B cells that generate a more intense and effective response. The affinity and amount of antibody increase with repeated antigenic challenge (immunisation). The increase in affinity (affinity maturation) is evident mainly in IgG (as well as IgA and IgE, to a lesser extent), produced by mature B cells that have undergone isotype switching and somatic hypermutation; a minor degree of affinity maturation takes place in the primary response (Ahmed and Gray, 1996).

Antigenic challenge generates effector T cells and long-lived memory T cells. Most of the effector T cells derived from antigen-stimulated naive T cells are relatively short-lived, dying either from antigen overload or the absence of antigenic stimulus. Some may become long-lived memory T cells, which may also differentiate directly from activated T cells. Although there is evidence to the contrary (Dirosa and Matzinger, 1996), continuing stimulation by retained antigen may be required for these cells to persist; the antigen is probably retained as immune complexes bound to follicular dendritic cells in lymphoid follicles. Many cell surface molecules alter their expression on memory T cells. The changes increase the adhesion of the T cell to APCs and to endothelial cells; they also increase the sensitivity of the memory T cell to antigenic stimulation (Ahmed, 1996; Ahmed and Gray, 1996; Sprent and Tough, 1994; Tough, Borrow and Sprent, 1996).
2.3.7.3 Intrinsic regulation of immune responses

At the crudest level, immunological homeostasis is maintained by negative feedback involving antigen. The presence of antigen triggers a response, resulting in reduction/elimination of antigen, resulting, in turn, in a reduction of the immune response. The immune system, however, does not return to the pre-infection state, as memory T and B cells remain, together with some antigen. The combined effect has several elements to it: antibody suppresses further naive B cell activation by cross-linking the specific B cell antigen receptor to FcγRII, and without having the same effect on memory B cells; thus memory B cells can still be triggered to produce antibody, allowing the secondary antibody response; memory CD8 T cells can regain CTL activity rapidly enough to kill the APCs that might activate naive CD8 T cells, thereby inhibiting their activation (Amigorena et al., 1992; Fridman, 1993; Uhr and Moller, 1968).

The immune system is also able to regulate itself, through interactions between the innate and adaptive responses and through the actions of T cells. The essence of innate immunity is the detection of molecules that are unique to infectious organisms. This allows the innate immune system to guide the selection of antigen by B and T cells, and the secretion by CD4 T cells of cytokines that promote an appropriate host response to the infection (Fearon and Locksley, 1996). In the most significant interaction, C3d of complement acts as an adjuvant which can reduce the threshold for an adaptive response by a factor of $10^4$ (Dempsey et al., 1996).

Each of the two subsets of CD4 T cells produces cytokines that can negatively regulate the other subset. Activated $\text{TH}_2$ cells secrete IL-10 and TGF-$\beta$, both of which inhibit activation and growth of $\text{TH}_1$ cells. For their part, activated $\text{TH}_1$ cells secrete IFN-$\gamma$ which inhibits $\text{TH}_2$ cell proliferation (Gajewski and Fitch, 1988;
Ruegemer et al., 1990; Sher et al., 1992). The nature of a TH1/TH2 immune response may also be influenced by the time of day. Diurnal rhythmicity in plasma cortisol and possibly melatonin regulates diurnal variations in the IFN-γ/IL-10 ratio (Petrovsky and Harrison, 1997). In addition, TH1 cells can suppress the activation of B cells by TH2 cells, so that no antibody production is evident (DelPrete et al., 1991). CD8 T cells can also regulate responses by secreting cytokines that suppress T cell subsets and APCs (Damle and Engleman, 1990; Powrie and Coffman, 1993).

In order that the interactions described here can proceed, the right numbers of the right types of cells have to be in the right locations. The size of lymphocyte populations is maintained by a balance between cell proliferation and cell death through apoptosis (Boise and Thompson, 1996; Steller, 1995; Thompson, 1995b). At the same time, regulated trafficking of lymphocytes, by a process termed homing, disperses the immunological repertoire, directs lymphocyte subsets to the specialised microenvironments that control their differentiation and regulate their survival, and targets immune effector cells to sites of antigenic challenge (Bagnolini, 1998; Butcher and Picker, 1996).

2.3.8 Inappropriate immune responses

2.3.8.1 Immunodeficiency

Immunodeficiency disease arises whenever one or more elements of the immune system fail to function normally. The most common cause of human immunodeficiency is malnutrition (Chandra, 1979, 1981), usually confined to inhabitants of undeveloped countries and people, such as those in many Australian Aboriginal communities (Beck, 1985), living in Third World conditions within developed countries. The malnutrition-immunodeficiency link has also been
established for wild populations of other mammalian species (Borg, 1970, 1975; Degabriele, 1989; Porter et al., 1984). Indeed, the interplay between nutrition and immune status appears to be bidirectional (Husband, 1995), to the extent that malnutrition might be reduced if childhood infectious diseases are controlled (Black, 1991). In developed countries, most immunodeficiency diseases are inherited.

The most common form of inherited immunodeficiency is termed late-onset hypogammaglobulinaemia. In this condition the deficiency is in circulating B cells and/or B cells with IgG surface receptors. Such individuals are unable to produce antibody-secreting plasma cells in response to antigen (Atlas, 1995).

A B cell defect will limit the immune response to the extent that antibody production is reduced or absent. A T cell defect, however, prevents the development of any protective immunity, leading to severe combined immunodeficiency (SCID). This phenotype can be generated by a number of genetic deficiencies (Atlas, 1995; Chapel and Sewell, 1990; Schwarz et al., 1996).

The age-related decline in the protective immune response that occurs irrespective of genotype is largely a consequence of changes in T cell populations. Age leads to the replacement of naive T cells by memory T cells and to the accumulation of cells with signal transduction defects (Miller, 1996b).

Defects in complement components cause defective humoral immune function and persistence of immune complexes. Defects in the early components of the alternative pathway lead to susceptibility to extracellular pathogens; defects in the early components of the classical pathway affect removal of immune complexes via complement receptor 1; defects in membrane attack components are associated with susceptibility to different strains of *Neisseria* spp. (Phimister and Whaley, 1990).
Most of the acquired immunodeficiencies have obscure pathogenesis, while a few are caused by known agents such as drugs or irradiation that damage lymphocytes, or by infection with human immunodeficiency virus (HIV). The main effect of HIV infection is the destruction of CD4 T cells. As the CD4 T cell count diminishes, the person becomes progressively more susceptible to opportunistic infection by intracellular microbes. Eventually, most HIV-infected individuals develop acquired immune deficiency syndrome (AIDS) and die; some people (3-7%), however, remain healthy for many years with no apparent ill effects, perhaps because of a mismatch between HIV and the co-receptors found on CD4 T cells (Clapham and Weiss, 1997; Moore, 1997) or because of a block on viral replication within the T cell (Kaiser, 1997).

\[\text{2.3.8.2 Allergy and autoimmunity}\]

While failure to function normally leads to immunodeficiency, normal but inappropriate immune responses can lead either to allergy if the trigger is harmless foreign substances or to autoimmunity if the trigger is self tissue antigens.

Allergic reactions, also termed hypersensitivity reactions, occur on secondary and subsequent contact with an antigen, termed an allergen in this context (Gura, 1996). A hypersensitivity reaction may have a rapid or even explosive onset, or may develop slowly (Colten, 1994). The hypersensitivities have been grouped into four types.

In type I, or anaphylactic, hypersensitivity, the first encounter between the antigen and the immune system leads to the production of IgE. The antibody molecules attach to mast cells by their Fe region, with the Fab regions protruding outwards. The mast cells are now said to be sensitised. When the sensitised mast cells
encounter the same antigen on a subsequent occasion, the antigen binds to IgE and
the immediate degranulation and mediator release that follow lead to the generalised
reaction of anaphylactic shock or to a localised reaction such as urticaria. Thus, type
I hypersensitivity is also termed immediate hypersensitivity (Lewis, 1986;
MacDonald et al., 1995). Common localised type I reactions include hay fever,
allergic asthma, urticaria, and the diarrhoea of food allergy (Bazan, 1995; Foster et
al., 1996; van Herwerden et al., 1995; Vardaxis, 1995).

As maladaptive as this mechanism may appear, it seems to have evolved in order to
trigger protective host inflammatory responses against pathogens (Echtenacher,
Männel and Hültner, 1996; Galli and Wershil, 1996; Malaviya et al., 1996) or as an
adaptation to cold temperatures (Parker, 1991). Anaphylaxis can also be triggered
independently of IgE (Oettgen et al., 1994), perhaps relying on IgG instead (Takai et
al., 1996). Irrespective of the class of immunoglobulin involved, antibody-mediated
type I reactions ultimately rely on a Th2 cell first step (Holt, 1996; Holt and
Sedgwick, 1987; McMenamin and Holt, 1993; McMenamin et al., 1994;

Type II hypersensitivity is mediated by antibodies directed against the cell surface or
against cell surface receptor molecules. Thus, it is referred to as cytotoxic or cell
receptor hypersensitivity. Antigen presented on the cell surface stimulates and binds
antibody (IgG/IgM) by its Fab region. Phagocytes may then become involved in the
destruction of the cell in three possible ways. First, the complement system may be
activated by attached antibody, allowing the phagocyte to adhere to the cell;
complement also has the ability to lyse the cell directly. Second, phagocytes may
bind to the cell through their Fc receptors. Third, NK cells, which also exhibit Fc
receptors, are able to lyse the cell by antibody-dependent cell-mediated cytotoxicity
because of the IgG coating the cell (Lewis, 1986). When the IgG is directed against
the cell surface, erythrocytes and platelets are the most commonly affected cells. The
most common manifestations are transfusion reaction, autoimmune haemolytic anaemia, and allergies to such drugs as penicillin. When the IgG targets cell surface receptors, it alters signalling, leading to such conditions as Graves' disease or myasthenia gravis (Janeway and Travers, 1996; Vardaxis, 1995).

Type III reactions are termed immune complex-mediated hypersensitivity. When soluble antigen and antibody (usually IgG) are deposited in tissue together as an immune complex, activation of complement can lead to tissue damage. The inflammatory mediators that are produced will increase vascular permeability, thus increasing deposition of immune complexes, and attract neutrophils. These cause further tissue and vascular damage which causes more mediators to be produced. Typical local manifestations of type III reactions include farmer’s lung and glomerulonephritis. Systemic manifestations include serum sickness and systemic lupus erythematosus (SLE) (Lewis, 1986; Sylvestre and Ravetch, 1994).

The type IV reaction is termed delayed-type hypersensitivity. The delay refers to the inflammatory response that develops in a previously sensitised person 24-48 hr after contact with antigen; i.e. expression of the reaction induced by the second stimulation takes time. During that time period, injected antigen is processed by local APCs, then primed TH1 cells recognise the antigen and release vasoactive cytokines, and finally an inflammatory cell infiltrate dominated by macrophages accumulates at the site. An alternative inflammatory response is dominated by CTL activity. At this stage the lesion becomes apparent. Thus, in contrast to the first three types of hypersensitivity, type IV reactions are mediated by T cells rather than antibody. Common type IV reactions include contact dermatitis and the reaction to mosquito and flea bites (Janeway and Travers, 1996; Lewis, 1986; Vardaxis, 1995).

Autoimmune diseases are mediated by sustained adaptive immune responses specific for self antigens. Each autoimmune disease has a characteristic pathogenesis that
involves one or more of the mechanisms involved in hypersensitivity reactions type II to IV; IgE responses to self tissues have not been demonstrated. The specific antigen or group of antigens against which the autoimmune response is directed and the mechanism by which the antigen-bearing tissue is damaged together determine the pathology of the disease.

Thus, autoimmune type II hypersensitivity can lead to autoimmune haemolytic anaemia, Graves' disease or myasthenia gravis. Chronic generation of immune complexes (a type III reaction) causes the tissue damage of SLE. T cell mediated type IV autoimmune reactions can manifest as insulin-dependent diabetes mellitus, or rheumatoid arthritis, or multiple sclerosis (Atkinson and Maclaren, 1990; Streit and Kincaid-Colton, 1995).

Autoimmunity develops following a loss of self-tolerance. Many explanations for the phenomenon have been proposed, but the majority of autoimmune diseases have a multifactorial etiology (Keech, Gordon and McCluskey, 1996) which is not explained by any single theory.

2.3.9 Tolerance and rejection

The normal but inappropriate immune responses characteristic of allergy and autoimmunity are based on inappropriate immune recognition. The significance of appropriate immune recognition can be seen in the context of phenomena such as: self and non-self tolerance; male/female recognition; pregnancy; and transplant rejection/acceptance. Some of these have already been discussed in detail and so will only be mentioned briefly here.
Tolerance to self is a normal state that is maintained chiefly by clonal deletion of developing cells, and clonal or deletion inactivation of mature peripheral cells. In addition, some antigens appear to be ignored by the immune system, many being present in so-called immunologically privileged sites. A mechanism has been proposed whereby presentation of potential auto-antigens can lead to clonal deletion or inactivation (Kurts et al., 1996, 1997). Tolerance has already been described in detail in the context of B and T cell selection.

Male/female immune recognition is not entirely reciprocal, even when donor and recipient tissues are closely matched immunologically and genetically. Males seem to tolerate transplanted female tissues well but females sometimes reject transplants from males. The difference in recognition is due to the presence on the Y chromosome of a gene (Smcy) for a male-specific histocompatibility antigen, named H-Y (Pennisi, 1995; Scott et al., 1995; von Boehmer, 1995; Wang et al., 1995). No such recognition difficulties exist with fertilisation, however (Belton and Foltz, 1995).

Having achieved fertilisation by a process that is loosely analogous with immune recognition, the semiallogeneic zygote must now develop in a potentially immunologically hostile environment. In fact, during pregnancy the foetus/embryo survives despite the presence of maternal T cells specific for paternally inherited histocompatibility antigens. Survival is favoured by a number of mechanisms. Firstly, maternal T cells acquire a transient state of tolerance for paternal alloantigens (Tafuri et al., 1995), a type of temporary immune privilege (Streilein, 1995). Secondly, the outermost layer of the human placenta is devoid of both class I and class II antigens, preventing recognition by maternal T cells but leaving the way open to NK cell attack. However, trophoblast cells in direct contact with maternal tissues express the unusual class I molecule HLA-G, protecting them from
recognition by NK cells. This arrangement protects the placenta from maternal T cell and NK cell attack (Pazmany et al., 1996). Thirdly, demonstration of the persistence of foetal cells in maternal blood for as long as 27 years postpartum suggests that pregnancy may establish a long-term, low-grade chimaeric state in the human female (Bianchi et al., 1996). Fourthly, and on the other hand, the mother appears to be able to eliminate migrating foetal cells without eliminating the foetus (Bonney and Matzinger, 1997).

While the foetus can be thought of as a natural allograft that is tolerated, the same cannot be said for transplants. They are neither natural nor normally tolerated. The acceptance or rejection of a transplant is determined largely by the genetic similarity or difference, respectively, between the donor and the recipient. Matching at the MHC loci improves the outcome significantly although rejection can still occur due to differences at other loci known as the minor histocompatibility antigens (the H-Y antigen is one example). When rejection does occur, it is more or less a standard immunological response mediated primarily by T cells. In spite of the difficulties, transplantation of certain tissues and organs is performed routinely and with considerable success. This is possible because of several factors: a high level of surgical skill, precise tissue typing, and the use of potent immunosuppressive drug therapy. Some of the current effort in transplantation research has been directed at the T cell. Attempts have been made to manipulate lymphocyte activation (Duchosal et al., 1996; Trucco and Stassi, 1996), antibody recognition (Coghlan, 1996; Lazarovits et al., 1996), or the initiation and amplification stages of the T cell response (Larsen et al., 1996).
2.3.10 Clonal selection and defence?

The roles of the innate and adaptive immune responses are usually considered to be different and to take place sequentially. Innate immunity has been characterised as providing rapid, incomplete host defence until the slower, more specific adaptive immune response develops. More recently, however, it has been proposed that mammalian innate immunity, in fact, dictates the conduct of the adaptive response (Fearon, 1997; Fearon and Locksley, 1996). The adaptive response recognises peptides but cannot distinguish between potential pathogens and innocuous substances, while the innate immune system recognises carbohydrates that are unique to infectious organisms. Therefore, various elements of innate immunity influence the adaptive response in order to overcome its apparent lack of specificity.

Firstly, dendritic cells and macrophages, acting as APCs, determine which antigens will activate CD4 T cells. Secondly, C3d protein of the complement system, when attached to microbial carbohydrate antigen, binds to CD21 on the surface of B cells, reducing the threshold for an antibody response. Finally, the nature of the TH response is mediated, in part, by the innate system. Thus, activation of tissue macrophages causes secretion of IL-12 which induces differentiation of TH1 cells. The development of TH2 cells is stimulated by IL-4 that can be induced by APCs expressing B7.2 or CD30 ligand (Dempsey et al., 1996; Fearon and Locksley, 1996; Medzhitov, Preston-Hurlburt and Janeway, 1997). This proposal, then, remains consistent with clonal selection but describes a network of interactions that weave the innate and adaptive systems into a unified response.

Moving on from a consideration of the immune response to an examination of the biology of the virus-host interaction (Marrack and Kappler, 1994) has led to a re-evaluation of some of the key parameters of the immune response - specificity,
memory and tolerance (Zinkernagel, 1996). This view is based on the (biologically self-evident) premise that a degree of co-evolutionary balance has been reached between viruses and their hosts whereby both survive.

It has been proposed, therefore, that specificity is best defined operationally (Zinkernagel, 1996). Antibody specificity depends on the extent of neutralisation of antigen. CD4 T cell specificity can be tested by the switch from IgM to IgG production, or by macrophage activation. The specificity of CD8 T cells is reflected in the balance between, on the one hand, a reduction in virus titres and, on the other, protection from host cell damage in solid organs.

The important primary biological function of immunological memory is the adoptive transfer of memory antibodies from mother to offspring, based on an accumulated maternal antibody experience of at least 13 years in humans (Zinkernagel, 1996), that has been further enhanced by female hormones (Fox, 1995; Schalk and Forbes, 1997; Schuurs and Verheul, 1990). Consequently, immunological memory is said to be indistinguishable from low-level, continuing, normal immune responses (Arala-Chaves, 1992; Bocci, 1992; Dirosa and Matzinger, 1996).

The notion of tolerance is also under question in this context of virus-host interactions. Thus, it is suggested that the mature immune system does not distinguish between self and nonself. Instead, B cells distinguish antigen patterns whereby they are triggered by antigen that is repetitive and rigidly ordered in a paracrystalline fashion, but not by antigen that is mobile, poorly organised or monomeric. It is also proposed that T cell responses depend on localisation, transport, and kinetics of antigen within lymphatic organs (Zinkernagel, 1996).

The necessity for self/nonself discrimination that has been questioned by Zinkernagel's biological approach is also under scrutiny by a series of studies based
on standard immunological techniques. The additional significance of these investigations is that they sought to replicate the experiments (Billingham, Brent and Medawar, 1953) that confirmed the mechanism by which self-tolerance develops (Burnet and Fenner, 1949).

Both neonatal and adult T cells have been activated by professional APCs such as dendritic cells, or rendered tolerant by cells that do not provide co-stimulation (nonprofessional APCs such as B cells). The dose was important in both cases. Thus the tolerance that occurred in the supposedly definitive early experiments (Billingham, Brent and Medawar, 1953) was not due to the neonate being inherently tolerisable but because an inoculum of spleen or bone marrow cells contained many cells that are unable to provide co-stimulation to naive T cells. Both neonates and adults could be rendered tolerant by such an overdose, bearing in mind that an overdose is a significantly larger amount for an adult than for a neonate (Ridge, Fuchs and Matzinger, 1996). Just as an adult could be rendered tolerant by a large enough overdose, a neonate could develop a protective CTL immune response if the initial viral dose was low enough (Sarzotti, Robbins and Hoffman, 1996). Finally, both TH1 and TH2 responses can be elicited in the neonate, depending on the type of adjuvant employed. Thus, neonates are said to be immunocompetent so that their CD4 T cell response does not differ essentially from that of adults (Forsthuber, Yip and Lehmann, 1996). Incidentally, it has been suggested that adjuvants function as they do by initiating an innate immune response which, in turn, facilitates an adaptive response (Brown, 1996a).

These three studies show that tolerance is not an intrinsic property of the newborn immune system. In fact, mature naive T cells can be immunised, rendered tolerant, or switched to TH1 or TH2 responses according to such factors as the dose of antigen, the type of adjuvant and the type of APC. The results are also consistent with the view that response to a peripheral antigen is based not on self/nonself
distinctions but on the conditions under which the antigen is introduced. The conditions have been seen as revolving around either antigen pattern and T cell dynamics (Zinkernagel, 1996) or danger signals (Matzinger, 1994).

The “danger” model suggests that the immune system does not discriminate between self and nonself but between dangerous and harmless encounters. The primary distinction is made by APCs (Epstein et al., 1995), which are activated to trigger co-stimulatory molecules only when induced by alarm signals from their environment (tissues undergoing stress or abnormal death, or microbial products) (Matzinger, 1994).

It is probably not a coincidence that the studies cited above, all of which challenge aspects of the clonal selection theory, are firmly based in biological principles. They describe integrated, interdependent processes occurring in a host in the context of its own life history stages, and in its dealings with the environment. They also provide additional support for the earlier concepts of self/nonsense (Vaz and Varela, 1978) and foreign-familiar/foreign-foreign recognition (Vaz, Martinez-A. and Coutinho, 1984), ideas that were developed in the context of an immune network (section 2.2).

The primacy of the clonal selection theory has also been challenged by a proposal that, at the same time, entrenches the importance of defence against infection. Cohen (1992a) has suggested that, in order to prevent “death from infection”, the immune system has to solve three problems. The first is the signal/noise problem which amounts to one of specificity. This issue has been addressed by Zinkernagel (1996) who defined specificity operationally, and by Fearon (1997; Fearon and Locksley, 1996) who proposed that the innate response confers specificity on the adaptive response. The second problem is one of context; it revolves around the appropriateness of self/nonself discrimination. Matzinger (1994) has suggested that the immune system responds in the context of danger, while Zinkernagel (1996) has
substituted antigen pattern and T cell dynamics in place of self/nonself. The third problem is the response problem, whereby the nature of the response gives effective meaning to the recognition. This problem is solved when the response repertoire (what the system can do) adjusts to the receptor repertoire (what the system can see). Such an adjustment is possible if the immune system is viewed as a cognitive system, one that contains internal information that precedes and imposes order on experience (Cohen, 1992a). The weakness in Cohen’s (1992a) proposal is that his idea of a cognitive system includes the element of intentionality, a suspect concept in biological terms. Perhaps clonal selection and defence belong together.

In fact, the clonal selection view of the function of the immune system revolves around surveillance, defence and attack in order to maintain the integrity of the organism. The recurring metaphor is one of a continuous war in which the defender (the immune system) is ruthlessly efficient and, just occasionally, gets out of control. Although this military perspective finds its most blatant expression in the non-scientific literature (e.g. Jaret, 1986; Jaroff, 1988), such reporting is representative of the view of some immunologists (e.g. Dwyer, 1989; Hayunga, 1989; Williams, 1996c) and put most plainly by Cohen (1992b):

“The immune system is not the kindest of hosts. It flat out hates strangers, indiscriminately killing both helpful and harmful visitors shortly after check-in. It also abuses its own kin, occasionally savaging a pancreas or a nerve cell for no good reason. Considering the nasty characters that drop in now and then, most people accept the dark side of these behaviors. But immunologists do not. They have long been using crude tools to try and modify the errant behavior.”

Thus, not only is the immune system involved in a war with strangers and the occasional battle with friends, it is also engaged in a meta-struggle with immunologists.
The most powerful military imagery is becoming associated with discussions of HIV, perhaps because of its significant medical and social impact. Macrophages shelter the invader which targets and destroys CD4 T cells. Thus, the immune system is attacked, the cellular soldiers are destroyed, self-defensive integrity is violated by rampant growth of opportunistic organisms (a secondary invasion), and AIDS-related death finally triumphs (Tauber, 1994). The war is lost.

This grim and distinctly unbiological form of explanation is perhaps a natural consequence of the reductionistic and deterministic nature of mainstream immunological thinking. Although early immunology was founded on Metchnikoff’s concerns for the whole organism in an evolutionary context, it was subsumed by the reductionist agenda of his detractors (Tauber, 1994). In fact, the “self-righteous zeal of the new ethos of scientific objectivity of reducing living processes to chemistry and physics blocked the development of other scientific method or conjecture” (Tauber, 1994), to the extent that one can now claim, almost without challenge, that organismic identity equates to the genome of the organism (Tauber and Sarkar, 1992, 1993). Furthermore, immunological identity is said to be “hard-wired” in the MHC genes (Watson et al., 1992), which are more completely understood than any other genetic region of similar size (Trowsdale, 1993). Despite the existence of this detailed genetic information, however, the widespread reductionistic/deterministic view of life in general, and immune function in particular, is being challenged.

While an individual’s genotype may provide the blueprint for action, the expression of that genotype happens in a life being lived (Levins and Lewontin, 1985; Lewontin, Rose and Kamin, 1984). Even a life that appears to be governed strictly by rules is not necessarily following a deterministic path. It is behaving rules reflecting arbitrary choices made during evolution, rather than the strict determinism of the physical laws of the universe (Monod, 1971; Rose, 1988). Thus the individual, including their immune system, is subjected to selection pressures whereby inherent
flexibility develops into functional stability (Burnet, 1965). In other words, the
development of the immune system is based on a capacity to learn, a form of
epigenetic development also seen in the nervous system (Kradin, 1995).

The importance of epigenetic development mechanisms is becoming evident
(Roemer et al., 1997; Strohman, 1993; Wasserstein, 1996) with the recognition of
indeterminacy as a key characteristic of natural phenomena (Hislop, 1991;
Prigogine, 1989; Rice, 1996; Sapolsky and Balt, 1995), based on the inherent
quantum uncertainty resulting from the small size of all cells (Hallett, 1989, 1997).
Formal recognition of the phenotypic plasticity inherent in epigenetic development is
found in state-dependent life history theory (McNamara and Houston, 1996).

What of the clonal selection theory and the defence metaphor now? Some of their
fundamental tenets are being questioned from within, and the
reductionist/determinist mode of enquiry may be generating less than the full picture.
Furthermore, it would seem to be operating at a rather rudimentary level of
explanation.

In an analysis of the (then) developing discipline of ethology, Tinbergen (1963)
identified several types of “explanation”, all of which are necessary for complete
explanations of organic phenomena (in ethology and beyond), and none of which is
usefully reducible to the others. They include: description (of both mechanisms and
proximate causes); ontogeny; evolution (the past); and, survival value (the future).
Description and ontogeny relate to “how” questions, while “why” questions are in
the realm of evolution and survival (Hunter, 1996). Like most of biomedical science,
the clonal selection framework and the defence metaphor (including
psychoneuroimmunology) deal almost entirely with the “how” questions. Perhaps
that is because these questions are not incompatible with reductionism and
determinism and because their pursuit has been very productive.
In the process of moving to a more holistic, organismic level of explanation (the "why" questions) it would be a mistake to ignore the reductionist approach. In fact, this approach has yielded the vast amount of mechanistic detail that permits a more complete understanding of larger-scale patterns and processes. While it is important that reductionist molecular biology should aim to scale up to the level of the whole organism, it is equally important that organismic investigations scale down to biochemical and molecular levels (Callahan, Pigliucci and Schlichting, 1997), so as to approach a complete explanation of the immune system.

2.4 The biology of immune function

The biology of the immune system, and indeed any body system, extends beyond anatomy and physiology. Nevertheless, the majority of mainstream treatments of immunology, including the highly regarded Immunobiology (Janeway and Travers, 1996), rarely go beyond anatomy and physiology (health), or pathophysiology (disease). Other aspects of the biology of the immune system usually receive a passing reference, if at all. In order to begin to redress the imbalance, therefore, this section explores the ecology of the immune response (section 2.4.1), and the ontogeny (section 2.4.2) and the evolution (section 2.4.3) of the immune system. These three areas are treated selectively and briefly, to provide a bridge between the immunocentric approach exemplified by section 2.3 and the holistic, organismic perspective that is developed in section 2.5. Perhaps this section would be more correctly entitled: "The rest of the biology of immune function". Furthermore, it should be borne in mind that the explanations contained in this section extend beyond clonal selection and the defence metaphor but remain consistent with both.
2.4.1 The ecology of the immune response

In ecological terms, the existence of a functional immune system in a mammal can be viewed from several perspectives. From the point of view of the mammal, the vast immunological repertoire represents a library of knowledge about the environment on a molecular level. This knowledge is part of the adaptive armoury that enables the mammal to find its place in the ecosystem, but at a molecular level (Kaplan, 1987). However, mammalian immunoglobulin covers limited specificities (about $10^{12}$), none of them being lipid, or molecules smaller than 250 daltons, or hydrocarbons polymerised as plastics (Kabat, 1957; Kaplan, 1987; Tonegawa, 1983). Thus, just as there are limitations to the ecological range of a mammal at the organismic level, so also at the molecular level.

From the perspective of the microorganism, a mammal is a reactive habitat in which the immune response of the mammalian host has the potential to convert it from being habitable to one that is potentially uninhabitable (Begon, Harper and Townsend, 1990; Paul, 1993). It is not surprising, then, that some microorganisms have evolved counter-adaptations to either evade the immune response or to exploit it as another host resource (Damian, 1987; Hayunga, 1989). The range of pathogen counter-adaptations is vast (Finlay and Cossart, 1997; Marrack and Kappler, 1994). The point can be made, however, by selecting a few categories.

Pathogens that can manipulate the cytokine environment can alter their host’s response in various ways. Several strains of vaccinia virus express a receptor which binds IL-1β, the major endogenous pyrogen in a poxvirus infection. In this instance, manipulating the availability of IL-1β allows the virus to suppress fever and attenuate the disease (Alcamí and Smith, 1996). Measles virus infection of primary human monocytes inhibits IL-12 production, leading to suppression of cell-mediated immunity (Karp et al., 1996).
*Trypanosoma cruzi*, the protozoan that causes Chagas' disease, produces acute immunosuppression of the T cell compartment and is able to persist in an infected host for decades, because it induces CD4 T cell death by apoptosis (Lopes et al., 1995). CD8 T cells have also been targeted, by both HIV and hepatitis B virus (Kersh and Allen, 1996).

Pathogens have also developed strategies aimed at another element of cell-mediated immunity, the processing and presentation of antigen by APCs. Epstein-Barr virus maintains its persistence and pathogenesis by interfering with MHC class I-restricted antigen processing (Levitskaya et al., 1995). Cytomegalovirus is able to selectively block antigen processing and presentation (Gilbert et al., 1996) by encoding proteins that trigger destruction of newly synthesised MHC class I molecules (Bonifacio, 1996; Wiertz et al., 1996). Then, in order to block NK cell attack of a cell not expressing MHC class I molecules, cytomegalovirus produces its own homologue of MHC class I molecules (Farrell et al., 1997; Kärre and Welsh, 1997; Reyburn et al., 1997).

Hitching a ride on an intermediate vector has enabled yet other pathogens to succeed. Avian influenza viruses, which supposedly are able to mutate to new pandemic strains of human influenza virus, use ducks as worldwide reservoirs (Parham, 1995). The bacterium *Vibrio cholerae* is the vector for a virus, named CTX, whose genome encodes cholera toxin. Thus cholera is not a bacterial infection, but a viral infection mediated by a bacterium (Brown, 1996b).

Bacterial virulence, the degree to which a bacterium can survive and multiply in hostile host environments, is regulated by specialised signal transduction systems that influence the transcription of virulence genes ( Cotter and Miller, 1996; Pettersson et al., 1996). Uropathogenic *Escherichia coli* exhibit adhesion molecules
on surface fibrillar structures termed P-pili. P-pilus-mediated attachment to host cells regulates virulence and the development of urinary tract infection (Zhang and Normark, 1996). *E. coli* is also able to enhance its virulence, but at the population level, by organising into colonies of differentiated non-clonal populations that undergo complex morphogenesis (Shapiro, 1988, 1995), in a behaviour reminiscent of slime moulds (Waddell and Vogel, 1985).

Ultimately, however, the survival of pathogens depends on the survival of susceptible hosts. Thus, the mammalian immune system and pathogens have co-evolved complementary facets (Marrack and Kappler, 1994; Zinkernagel, 1996), perhaps best illustrated by the benign relationship between a mammal and its normal bacterial flora, found largely in the gut lumen (Bocci, 1992). The complex and intimate nature of host-pathogen relationships can also be seen in the phenomenon of multiple viral pathogenicity. For example, measles virus is responsible for measles (an acute systemic infection), subacute sclerosing panencephalitis (a chronic disease limited to a single organ), and measles encephalitis (an autoimmune disorder in which the virus plays a hit-and-run role) (Sotelo, 1996).

### 2.4.2 Ontogeny of the immune system

This discussion of the ontogeny of the immune system through the life cycle of the individual (predominantly the human) is based on material from several sources (Adinolfi, 1993; Coleman, Lombard and Sicard, 1992; Lotzová, 1993; Marieb and Mallatt, 1997; Miller, 1996b; Turner, 1994). The immunological challenges faced in establishing a pregnancy and defending it from attack have already been described.

In the process of development the foetus and neonate progress through three major stages of immune competence. During the first two to three months the foetus
exhibits relative incompetence. During the middle trimester transitional competence emerges when the immune system is being formed and maternal antibodies become increasingly abundant. The final period, when differentiation of T and B cells is occurring and expression of independent immune status is increasing, is termed a time of progressive competence.

Maintenance of immune competence depends on an adequate number of cells, on their proper distribution into appropriate subsets, and on the intrinsic properties of the cells themselves. These characteristics are sustained throughout most of life, although aging introduces defects that can lead to immune failure. Throughout the first two months of development, all blood cells form in these blood islands, including both the blood stem cells that will last a lifetime and primitive nucleated erythrocytes. Late in the second month, circulating stem cells from the yolk sac reach and lodge in the liver and spleen which assume the blood-forming function and are the major haematopoietic organs until the seventh month. During this time they produce the first leukocytes, the first platelet-forming cells, plus nucleated and non-nucleated erythrocytes. The bone marrow receives stem cells and begins low-level haematopoiesis during the third month. It becomes the major haematopoietic organ during the seventh month and the only one from birth onwards.

The lymphatic system develops from a number of sources. Both lymphatic vessels and the main clusters of lymph nodes grow from lymphatic sacs, which are projections from the large veins of the embryo. The thymus originates as an outgrowth of the endoderm lining the embryonic pharynx, detaches from the pharynx, and migrates caudally into the thorax. In the foetal period, the thymus first receives immature lymphocytes that will become T cells. All the other lymphoid organs and tissues arise from mesodermal mesenchyme. Except for the spleen, these organs are poorly developed before birth. However, shortly after birth, they become heavily populated with circulating lymphocytes and start to develop their functional
properties, mediated by hormones secreted by the young thymus.

Expression of humoral immunity begins in the foetus at about 25 weeks with the production of IgM. Between that time and approximately one year after birth, there is progressive expression of IgG, IgD and IgA which continue to increase until adult antibody levels are reached, in the context of B cell differentiation and maturation. Expression of cellular immunity does not follow as clearly defined a pattern of progression and is dependent on T cell differentiation.

Expression of NK activity is low before birth, reaches a peak soon after birth, then declines to adult levels. At birth, macrophages are still inefficient at processing antigen, while neutrophils lack full bactericidal competence. The complement system begins to develop early in foetal life. Partial expression of the pathway is accomplished at birth and completed later. In addition, the complement proteins present at birth occur at only a fraction of the levels found in adults.

2.4.3 Evolution of the immune system

The evolutionary pressures leading to the development of an immune system are likely to have been twofold - the need for defence against pathogens, and the homeostatic need to monitor and maintain the internal environment in terms of alterations of the self such as cancer or degeneration (Cooper, 1993; Kolb, 1977). It would appear that the second pressure is the older one, since neoplasia and immunity are attributes of both invertebrates and vertebrates (Marchalonis and Schluter, 1994), and since a central characteristic of both neoplasia and immunity is supposedly the capacity to distinguish self from non-self (or altered self) and to display specific recognition (Marchalonis and Cohen, 1980).
In evolutionary terms, the oldest cells in the animal kingdom either are macrophages (Cooper, 1993; van Furth et al., 1982) or exhibit macrophage-like function (Beck and Habicht, 1996), an integral part of both the innate immune response and the cell-mediated adaptive response. Indeed, both phagocytosis (a characteristic feature of macrophages) and agglutination (an invertebrate precursor of opsonisation) are mediated by the same molecular mechanism (Ratcliffe et al., 1985; Renwrantz et al., 1981). Thus, the earliest immune responses (both innate and adaptive) in multicellular animals were cell-mediated (Cooper, 1993; Good and Papermaster, 1964), and initially directed at non-peptide antigens (Balk, 1995). In addition, the primary role of complement during the course of evolution was to promote phagocytosis rather than to mediate cell lysis (Hughes and Yeager, 1997), and the alternative pathway, which is not strictly limited to activation by immune complexes, represents the earliest form of complement activity (Adinolfi, 1993; Bertheussen and Seljelid, 1982; Koppenheffer, 1987; Nonaka, 1985; Ratcliffe et al., 1985).

Ancestral members of the immunoglobulin superfamily arose among unicellular organisms (Hildemann, 1977) and subsequently became committed to a number of recognition responses that are prerequisites for the development of multicellularity (Beck and Habicht, 1996; Matsunaga and Mori, 1987; Raftos, 1993). The responses initially took the form of heterophilic recognition between related molecules within an organism (Williams, 1987). They were then externalised to produce an immune system by the adaptation of apoptosis, so that recognition and killing became integrated, using internal cytotoxic cells as a starting point (Cooper, 1993). Given that NK function is almost as old as macrophage function (Savary and Lotzová, 1986), those early cytotoxic cells would have exhibited NK cell-like activity (Lotzová, 1993; Lotzová and Ades, 1989).
The primordial immunoglobulin domain that led to the vertebrate immunoglobulin superfamily may have been encoded by the Thy-1 gene before the emergence of the vertebrates (Cooper and Mansour, 1989; Marchalonis and Schluter, 1990; Raftos, 1993) and may have been based on neurohypophyseal-related precursors (Geenen et al., 1994). That was followed by the acquisition of mechanisms for DNA rearrangement, leading to the appearance of precursor MHC molecules (Cooper, 1993). The possible alternative selection pressures favouring the development of vertebrate MHC molecules include: (1) facilitation of cell-cell interactions, (2) peptide binding, (3) intracellular transport (Cooper, 1993).

Lymphocyte populations may have evolved from a common lymphocyte precursor in jawless fish, some 500 million years ago (Beck and Habicht, 1996). These lines may have then undergone divergent evolution, becoming distributed among various organs and eventually giving rise to T and B cells (Cooper, 1993). In fact, it seems that adaptive immune responses may have begun with the first vertebrates (Litman, 1996; Millar and Ratcliffe, 1994).

In overview, the primordial function of adaptive immunity in invertebrates is allorecognition, a process for which vertebrates have very little physiological need. However, as evolution is a conservative process, invertebrate allorecognition underwent adaptation to fulfil the requirements for pathogen surveillance that predominates among vertebrates (Coombe, Ey and Jenkin, 1984; Hultmark, 1994; Mäkelä, Koskimies and Karjalainen, 1976; Raftos, 1993; Rothenberg, 1978; Schwartz, 1985). A large part of the immune system continues to be directed against self, however, where natural antibodies carry out a physiological regulatory role that includes removal of altered cells or cell products (Marchalonis and Schluter, 1994).
2.5 Interactions of the immune system

"Research on isolated small building blocks of living organisms may result in a rapid accumulation of theories and hypotheses on these objects. However, we should consider that these small building blocks make up larger entities in an integrated fashion, and that these parts are coordinated by feedback loops in a synergistic and balanced organization. This integration of small parts makes it possible that large living systems are capable of performing functions that cannot be delivered by simply adding up the functions of the isolated small parts......We do not try to deny the necessity and usefulness of studying subcellular and molecular constituents of the living system. We assert, however, that if we intend to approach the problem of how organisms live, such study should derive its problems and targets from the integrated whole in order that essential internal coordinations not be forgotten.” (Pinter and Pinter, 1992).

Much of the information presented in section 2.3 is of the “small building blocks” variety; section 2.4 is a small step in the direction of the whole organism in as much as the focus is on the entire immune system. The present section places the immune system within the organism by examining interactions with the other two significant regulatory and integrative systems, the nervous system and the endocrine system. Indirectly, the outcomes of the interactions impact on other systems and, indeed, the whole organism. In fact, it has been suggested that “the immune system operates as a specific molecular (antigen)-sensitive mechanism within a spectrum of physiologic adaptive responses with which an organism reacts to perceived changes in its environment” (Ottaway and Husband, 1992) - the defence metaphor is not far below the surface of this statement.
The notion that the nervous system, the endocrine system and the immune system are functionally interconnected is now firmly established. There is anatomical, physiological, biochemical and pharmacological evidence of reciprocal communication and modulation among the three systems (Blalock, 1989; Bréard, Costa and Kordon, 1995; Divirgilio et al., 1996; Fuchs and Sanders, 1994; Haas and Schauenstein, 1997; Jankovic, 1989, 1994; Kropiunigg, 1993; Madden and Felten, 1995; Sgoutas-Emch et al., 1994). It has also been demonstrated, with some reservations (Cohen and Herbert, 1996), that this communication is of primary importance in health and disease (Biondi and Zannino, 1997; Black, 1994, 1995; Moynihan and Ader, 1996; Reichlin, 1993; Wilder, 1995). Communication includes the use of common signals and recognition molecules (De Souza and Appel, 1991; Friedman and Irwin, 1997; Pert et al., 1985). Thus, typical cytokines produced by immune cells, such as IL-1, IL-2 interferons and TNF, are also produced by neural cells (Fannon, 1991; Farrar, 1988; Frei et al., 1987). In an analogous manner, cells of the immune system have been shown to express neuroendocrine hormones such as pro-opiomelanocortin hormones, adrenocorticotropic hormone (ACTH), thyrotropin, growth hormone, growth hormone-releasing hormone, insulin-like growth factor I, prolactin and vasopressin (Auernhammer and Strasburger, 1995; Ballieux, 1994; Blalock, 1984; Dardenne and Savino, 1996; Smith and Johnson, 1989). The study of these interactions has been variously termed psychoneuroimmunology (PNI), behavioural immunology, neuroimmunomodulation (NIM), neuroendocrinimmunomodulation and neuroimmunoendocrinology. The label that seems to be emerging as the most widely used is PNI, a word coined by Ader (1981).

".....the problem that NIM investigators have to deal with is their ability to accept the end of the artificial system-specificity of messengers and to look at their specific patterns in physiologic and pathologic conditions" (Panerai, 1994). However, in
order to be able to adopt this holistic approach, it is necessary to first subdivide (dis-
integrate) the information into rather arbitrary, and overlapping, subsystems of the
system that is the organism. Beginning with stress (section 2.5.1), historically the
intellectual antecedent to PNI, the following discussion deals with immune
interactions with the endocrine (section 2.5.2) and nervous (section 2.5.3) systems.
The interplay among all the systems is then examined (section 2.5.4), leading to a
consideration of some pathological states (section 2.5.5). Finally, several therapies
are discussed in the light of the PNI framework (section 2.5.6).

2.5.1 Stress

Some of the earliest work on connections or interactions between body systems
revolved around the concept of stress. Experiences that later became known as
stressors were seen to lead to a distinct set of changes including adrenocortical
hypertrophy, atrophy of the thymus and other lymphatic structures, and bleeding
ulcers (Selye, 1935). Regardless of the nature of the stressor, the physiological
response is a characteristically non-specific activation of the hypothalamus-pituitary-
adrenal (HPA), termed the general adaptation syndrome (Selye, 1956). The non-
specificity of the response was subsequently shown to be largely due to the fact that
the subject perceived the experience as a stressor (Mason, 1968).

The conventional wisdom has been that stress alters immune function (via the HPA
axis (Bateman et al., 1989)), usually causing immunosuppression (Herbert and
Cohen, 1993) that may leave the subject susceptible to infectious disease (Cohen and
Williamson, 1991). The existence of such an interaction between stress and the
immune system has been known for some time (Kelley, 1980). As recently as 1982,
however, the nature of the mediating mechanisms was largely speculative (Locke,
1982), but became clearer relatively quickly (Blalock and Smith, 1985; Locke and
Hornig-Rohan, 1983). It also became evident that the interactions between the immune system and the HPA axis are complex and reciprocal (Blalock, 1984, 1989; Buzzetti et al., 1989; Griffin, 1989). Nevertheless, it is true to say that, as a gross oversimplification (Kusnecov and Rabin, 1994), any event that is perceived as stressful will result in activation of the HPA axis, with immune consequences (Khansari, Murgo and Faith, 1990; Ursin, 1994). Before moving on to consider some of the details of these interactions, it is worth noting that the word “stress” has been used so widely and so loosely that it can mean almost anything, becoming virtually meaningless. Indeed, it has given rise to what could be termed the stress industry (Chaitow, 1992; Kermani, 1992), complete with its detractors (Dantzer, 1993). Nevertheless, a number of useful working definitions of stress have been proposed, such as the following: “Stress, it is argued, can only be sensibly defined as a perceptual phenomenon arising from a comparison between the demand on the person and his ability to cope. An imbalance in this mechanism, when coping is important, gives rise to the experience of stress, and to stress response. The latter represents attempts at coping with the source of stress. Coping is both psychological (involving cognitive and behavioural strategies) and physiological. If normal coping is ineffective, stress is prolonged and abnormal responses may occur. The occurrence of these, and prolonged exposure to stress per se, may give rise to functional and structural damage. The progress of these events is subject to great individual variation” (Cox, 1978).

The links between stressful emotional processes and immune function have been explored in humans, but the results are far from clear (Irwin, 1991). In a review of earlier work, O’Leary (1990) noted that acute stressors elicit mixed effects: lymphocyte numbers increased in some studies, but decreased in others; the functional capacity of immune cells tended to be reduced. These divergent findings may have been due to activation of both the sympathetic and the adrenocortical stress systems (Bachen et al., 1995). On the other hand, both positive and negative
emotions can trigger the same immune change, a decrease in lymphocyte responsiveness, with negative emotions having the greater effect (Knapp et al., 1992). Perhaps the immune changes reflect the heightened arousal induced by the protocol rather than specific emotions (Dantzer and Mormède, 1995).

The acute stress of university examinations has been shown to generate a more predictable immune response, decreased activity of peripheral lymphocytes together with reduced cellular immune response to viruses (Glaser et al., 1987), bearing in mind that the perception of the examination as a stressor is more significant than the fact of the examination (Vedhara and Nott, 1996). These immune changes are also seen in response to chronically stressful situations such as the experience of marital difficulties (Kiecolt-Glaser et al., 1987, 1993; Mayne et al., 1997) or being the carer of a dementing spouse (Kiecolt-Glaser et al., 1991; McCann, 1991) or of a handicapped relative (Pariante et al., 1997). Even when the nature of the chronic emotional stress is not taken into account, the fact of being chronic can be significant. For example, chronic emotional stress can influence the progression of gingivitis and periodontitis by upsetting the balance between normal resident oral microorganisms and the host’s immune response (Breivik et al., 1996). In animal studies of chronic stress, the stress response has been shown to exhibit habituation (Pitman, Ottenweller and Natelson, 1988, 1990; Sandi et al., 1992; Sklar, Bruto and Anisman, 1981), sensitisation (Lysle, Cunnick and Rabin, 1990), or no change (Steplewski and Vogel, 1986). Despite some earlier methodological reservations (Pitman, Ottenweller and Natelson, 1986), the behaviourist concepts of habituation and sensitisation (Groves and Thompson, 1970; Thompson and Spencer, 1966), and classical conditioning are now being applied widely to a range of physiological processes (Dworkin and Dworkin, 1990).

Bereavement following the death of a spouse has also been shown to lead to immunosuppression (Bartrop et al., 1977, 1992; Irwin, Daniels and Weiner, 1987;
Schleifer et al., 1983). However, bereavement *per se* does not influence immune status; rather, depressive symptoms (Irwin et al., 1987), consequent upon the perception of life events as stressful (Schleifer et al., 1985; Seligman, 1992; Weisse, 1992), were shown to be inversely related to NK cell activity.

Having stated that an event is stressful when it is perceived to be so, the question remains - under what circumstances is an event seen as stressful? It appears that controllability and predictability (Mineka and Hendersen, 1985; Steptoe and Appels, 1989) are key elements. Thus, inescapable but not escapable electric shock has been shown to enhance tumour growth (Sklar and Anisman, 1979), and impair both tumour rejection (Visintainer, Volpicelli and Seligman, 1982) and lymphoproliferative response to lectins (Laudenslager et al., 1983) in rats; it also disrupts normal circadian body temperature rhythms (Kant et al., 1991), leading to potential immunocompromise (Minors and Waterhouse, 1981). Unpredictable but not predictable electric shock also reduces lymphoproliferative responses in rats (Mormède et al., 1988). A human study, however, has produced counter-intuitive results. Subjects who could control the stressor displayed lowered lymphocyte proliferation while those who could not control the stressor were not affected, in spite of increased anger and frustration (Weisse et al., 1990). Although these findings might obscure any underlying mechanism, they confirm the significance of controllability in the perception of stress. They also seem to confirm earlier findings that personality variables (trait characteristics) are important mediators of the effects of stress on immune function (Byrne, Steinberg and Schwartz, 1968; Gaines, Smith and Skolnik, 1977; Kobasa, Maddi and Courington, 1981; Kobasa, Maddi and Khan, 1982). Subjects with high internality (with respect to locus of control) and high stress have lower levels of salivary IgA than those with high internality and low stress. Thus, the effects of stress may be potentiated by the belief that one is directly responsible for (at least some) outcomes (Kubitz, Peavey and Moore, 1986). Indeed, it may be that only a reactive subset of individuals is susceptible to the negative
effects of stressors and adversity (Boyce et al., 1995; Sgoutas-Emch et al., 1994).

The extent to which a person perceives events as being a consequence of their own behaviour and therefore potentially under personal control is measured by locus of control (LOC) scales (Lefcourt, 1976). The most widely known scale, which measures generalised expectancies for LOC, was devised by Rotter (1966, 1975). A more specific scale, which measures locus of control of behaviour (LCB), has since been developed and used to monitor the efficacy of behaviour modification therapy (Craig, Franklin and Andrews, 1984). The LCB scale has also been employed to show that stressful but constructive experiences in general lead to greater maturity and more effective coping strategies, particularly in adolescence (Andrews, Page and Neilson, 1993).

Just as LOC has been shown to be a potent mediator of physiological responses to stress (Frankenhaeuser and Rissler, 1970) so also has the parallel concept of optimism/pessimism, whereby an optimist usually has a more robust immune status than does a pessimist (Kamen-Siegel et al., 1991; Seligman, 1992). Thus, the ability to cope with stress is a major determinant of whether, and to what extent, stress results in immunosuppression (Borysenko, 1984; Dantzer, 1989; Hislop, 1991). Furthermore, individual differences in immune reactivity may be predictors of the ability to cope with stress (Hessing et al., 1995). Thus, the relationship between immune activity and coping ability (a trait characteristic) is bidirectional.

The nature of the ability to cope with stress has also been modeled by Antonovsky (1979) in an apparently similar, but fundamentally different, way. Beginning with the premise that the human condition is stressful, the question posed is not how some “stressed” people manage to stay healthy, but how anyone stays healthy. Staying healthy relates to one’s sense of coherence (SOC). “The sense of coherence is a global orientation that expresses the extent to which one has a pervasive, enduring
though dynamic feeling of confidence that one’s internal and external environments are predictable and that there is a high probability that things will work out as well as can reasonably be expected” (Antonovsky, 1979). Any apparent resonance with the concept of LOC has been specifically rejected, on two grounds. Firstly, the usefulness of LOC is restricted to a particular culture, that of its proponents, who are themselves questioning its value (Brownell, 1991). Secondly, SOC does not rest on whether power to determine outcomes lies in one’s own hands. Rather, it is important that power is located where it is legitimately supposed to be. Thus, a strong SOC is not necessarily endangered by not being in control oneself (Antonovsky, 1979). Finally, SOC is characterised as a dispositional orientation (different from both state and trait characteristics), whereby the three components of SOC (comprehensibility, manageability, meaningfulness) develop in response to one’s pattern of life experiences (Antonovsky, 1988).

Coping can also be seen as a step in a process (a state characteristic) that begins with cognitive appraisal, followed by coping and producing an encounter outcome (Cohen, 1984; Folkman et al., 1986a,b). In this view, coping is process oriented, it is contextual, and its appropriateness is determined by the particular outcome. In addition, stress that produces immunosuppression is, by definition, unpredictable or uncontrollable (Vogel, 1986), and is more correctly termed distress (Hislop, 1991) as it has exceeded the coping capacity of the individual (Olff et al., 1995). Adopting the logic of this position, it ought to be possible to enhance the immune status of distressed subjects by alleviating their psychological distress. Yet, just such an attempt has produced equivocal results. While a stress management program did improve psychological well-being, some immunological parameters showed improvement and others declined (Blenkhorn et al., 1992). Thus, although psychological interventions have an effect on immune function, the nature of the effect is not clear.
A number of recent studies has confirmed the significance of the impact of unpredictable and/or uncontrollable stress on immune status and health. During the recent Persian Gulf War ("Operation Desert Storm"), the civilian population of Israel was exposed to both unpredictable and uncontrollable acute stress from exposure to missile attacks. As expected, there was an increase in the incidence of acute diseases but, surprisingly, psychological distress was lower than in peacetime (Soskolne et al., 1996), while immune status was enhanced (Weiss et al., 1996); perhaps wartime provides a unifying focus for Israeli society. An earthquake can also produce unpredictable and uncontrollable stress. Its effects on the immune status of adults correlate with appropriate coping with the realistic degree of life stress (Solomon et al., 1997). In young children, it does not alter their immune reactivity to more predictable events (Boyce et al., 1993). The chronic stress of working as a hospital consultant in the British health service is itself a health hazard. It leads to an increased prevalence of both psychiatric morbidity and burnout, with job satisfaction having a mitigating effect (Ramirez et al., 1996). By the same token, highly controlled psychological stress can have an immunostimulatory effect. An air traffic controller’s time spent at work results in elevation of both salivary cortisol and salivary IgA (Zeier, Brauchli and Joller-Jemelka, 1996). The stress produced by a first-time recreational parachute jump in tandem with an instructor has been shown to lead to a significant increase in NK cells and their cytotoxic activity, as a result of secretion of noradrenaline (Schedlowski et al., 1993b). A similar outcome has been demonstrated in response to another controlled psychological stress, a short mental arithmetic task. Rapid immune changes include the release of CD8 T cells and NK cells into circulation in adults, with only NK cell activity increasing in younger subjects (Naliboff et al., 1991).

Social stressors have been investigated in animal studies (for obvious ethical reasons). Early work established the adverse effects of social isolation (Vessey, 1964) and crowding (Edwards and Dean, 1977) on immune responses. More recent
work has focused on social status and disruption of attachment.

Subordinance in rats or mice is associated with decreased cellular (Raab et al., 1986) and humoral (Vessey, 1964) immune responses. The time course of the response is dynamic and includes elements of habituation, repression and denial (Henry, 1992). Over time, chronic social stress among male rats also results in a decrease in plasma corticosteroid binding globulin levels. This leads, in turn, to greater access of free corticosterone to type II (glucocorticoid) receptors in the spleen than is typically present in rats under basal or acute stress conditions, suggesting a mechanism by which chronic stress may have a greater impact than acute stress on splenic immune function (Spencer et al., 1996).

Until recently, the immune cost of subordinance was thought to be a widespread phenomenon. However, recent studies of mice (Barnard, Behnke and Sewell, 1996) and of animals in their natural environments have shown that stress hormone levels (chiefly corticosteroids) are more likely to be situation-dependent, both between and within species. For example, social dominance is stressful and immunologically costly in dwarf mongooses and in African wild dogs, as is subordinance in olive baboons; the situation varies in rhesus monkey groups, depending on the stability of the particular group (Creel, Creel and Monfort, 1996; Davies, 1996; Morell, 1996). It would appear that the immunological cost of dominance in both the dwarf mongoose and the African wild dog is outweighed by the benefits, such as preferential access to resources such as, for example, food, habitat and mates. At the other end of the social scale, an individual denied optimal resources may be better able to manage due to lower stress levels and an uncompromised immune system.

Peer separation of pairs of infant monkeys results in reduced lymphocyte function in both, followed by an increase after reunion (Reite, Harbeck and Hoffman, 1981). Both infant pigtail macaques and infant rhesus monkeys separated from their
mothers exhibit reduced lymphoproliferative responses (Coe et al., 1992; Laudenslager, Reite and Harbeck, 1982), while maternal separation of infant squirrel monkeys results in a decreased capacity to mount a humoral response to a virus (Coe et al., 1988; Coe, Rosenberg and Levine, 1988). Even the prenatal period is a time when stress can alter immune function, at least in rats (Klein and Rager, 1995).

Although there is a strong relationship between stress and immunity, stressors do not always alter immune activity. Context is important. For example, male rats undergoing social regrouping, but in the presence of females, do not exhibit changes in immune function, in spite of increase in adrenal size, involution of the thymus, increased plasma corticosterone levels and loss of body weight (Klein et al., 1992).

The strain of the animals also has a bearing on the immune effects of stress (Starec et al., 1994). For example, inescapable shock has an immunosuppressive effect on some strains of mice but not others (Irwin and Livnat, 1987; Lysle, Cunnick and Rabin, 1990). In addition, large differences in lymphocyte reactivity have been described in both rats and mice selected on the basis of behavioural reactivity (Granger et al., 1997; Sandi et al., 1991; Vidal, 1996), suggesting that immune response may sometimes co-vary with behavioural traits (Dantzer and Mormède, 1995).

The effects of stress on immunity also depend on the time at which the stressor is applied with respect to the time course of the immune response. For example, the extent of a secondary antibody response following immunisation differs, depending on whether the stressor is applied before or after immunisation (Esterling and Rabin, 1987; Moynihan et al., 1990; Okimura and Nigo, 1986). The immune response during the course of a chronic stressor is different again. Several significant changes in immune function occur in a time-dependent differential pattern involving both immunosuppression and immunoenhancement (Van Raaij et al., 1996).
The animal studies described above have been discussed primarily as models of the stress/immunity interaction. They have included models of physical stress, restraint, electric shock and social stress (groups, dyads, isolation). They have also included humoral, cell-mediated or functional immunological parameters. Nevertheless, it is still extremely difficult to draw general conclusions (Koolhaas and Bohus, 1989, 1995). Naturally, other animal studies have been conducted in which the focus is the animal, rather than the animal as model; some of these studies are discussed below.

An animal whose nutritional needs are not being met could be said to be under stress and immunocompromised. The nutritional inadequacy could be due to an inability to extract nutrients from a source of food: for example, old koalas during a drought (Degabriele, 1989) or old roe deer in a severe winter (Borg, 1975); it could be due to an increased demand for nutrients: for example, white mice and wild deer mice responding to an environmental toxicant (Porter et al., 1984) or live sheep being exported by sea at different ages and seasons (Higgs, Norris and Richards, 1991); it could be due to an exacerbation of normal seasonal metabolic factors and appetite, a combination of physiological and behavioural elements: for example, live sheep being exported by sea at different seasons (Richards et al., 1991); it could be due to food deprivation: for example, the removal of food from domestic fowls (Jones, 1989).

Simply changing an animal’s environment can be stressful and can be manifested in both immunological and behavioural changes. Introducing grazing sheep to a feedlot leads to a doubling in locomotor activity measured in an arena test (Fell et al., 1991), while moving sheep from grazing to individual indoor crates produces both an increase in active behaviours and a significant decrease in plasma cortisol (Fordham et al., 1991). A change in the thermal environment of pigs (exposure to cold) also results in large increases in plasma cortisol levels (Baldwin and Stephens, 1973),

Transportation can also be stressful for domestic animals. Pigs respond via the HPA axis (increased levels of circulating cortisol), the adrenal medulla (adrenaline levels increase), and alterations in lymphocyte migration patterns (Dalin et al., 1993). The involvement of both the adrenal cortex and the adrenal medulla is also found in cattle being transported. The magnitude of the adrenocortical response varies with breed in calves (Fell and Shutt, 1986), whereas the overall response in adult beasts begins with the HPA axis and grades into a sympathetic-adrenal-medulla phase (Mitchell, Hattingh and Ganhao, 1988).

It is perhaps not surprising that various surgical procedures that are performed routinely on livestock are stressful in various ways. Surgical castration of calves produces a significantly higher short-term salivary cortisol response than does the application of a rubber ring (elastrator) which is still, nevertheless, significantly stressful (Fell, Wells and Shutt, 1986). The pattern is different, however, in the lamb in which the rubber ring is considered to be more distressing than surgery because surgery leads to the development of stress-induced analgesia (Shutt et al., 1988a). The modified mules operation performed on sheep causes a marked elevation in plasma cortisol (the stress response) and β-endorphin (stress-induced analgesia), together with behavioural changes (Fell and Shutt, 1989); pre-treating the sheep with corticotropin-releasing factor increases the hormonal response without affecting behaviour, suggesting that analgesia is insufficient to inhibit either the cognitive response to the procedure or the perception of post-operative soreness (Shutt, Connell and Fell, 1989).

A number of the studies cited above have also included behavioural observations. In fact, the relationship between stress and behaviour has been investigated in a number
of species: the tonic immobility response to chronic stressors in the domestic fowl (Jones, 1989); the behavioural response to a change of environment in sheep (Fordham et al., 1991); the behavioural response by sheep to docking and castration (Shutt et al., 1988a), to mulesing (Fell and Shutt, 1989) and to being penned in a feedlot (Fell et al., 1991); and the behaviour of calves following castration (Fell, Wells and Shutt, 1986). A model for assessing the impact of behavioural stress on domestic animals has also been proposed (Moberg, 1987). The other side of the stress/behaviour coin involves manipulation of behaviour to assess the subsequent stress response. Thus, escape avoidance conditioning in pigs raises the level of circulating plasma corticosteroids, though to a lesser extent than does exposure to cold (Baldwin and Stephens, 1973).

This consideration of stress may have begun on a somewhat negative note, with the slightest hint that, sometimes, stress can be immunostimulatory. In order to put things in perspective it seems appropriate to finish on a positive note. A recent review of stress across the animal kingdom (Ottaviani and Franceschi, 1996) has concluded that, for several reasons, stress is a fundamentally positive response. It is a consistently similar response in different species and taxa in spite of the variety of stressors. The basic mechanisms and molecules involved in the stress response are also fundamentally similar and conserved throughout animal evolution. Furthermore, the primitive cellular basis of the response can be identified in such immune cells as invertebrate haemocytes, producing a range of cytokine-like molecules, simultaneously capable of producing hormone-like and neuropeptide-like molecules, and mounting a protostress response (the release of biogenic amines). Thus, stress “can be seen as the most important and complex body reaction to ensure survival” (Ottaviani and Franceschi, 1996).
2.5.2 Immune-endocrine interactions

The discussion in this section will be restricted to interactions between elements of the immune system and the hormones that are products of the classical endocrine glands.

MHC class I molecules, expressed on most cell types, usually present peptides derived from endogenous proteins (virus or altered self) to CTLs. They also have some "non-immune" functions, the most striking being their interactions with receptors for several hormones, neurotransmitters and a cytokine (Cremașchi and Sterin-Borda, 1994). The hormone receptors include those for insulin and luteinising hormone. Although both of these hormones induce cell growth in their respective target tissues, the precise physiological nature of these associations is unclear. Nevertheless, they are an indication of the depth of the interaction between the immune and the neuroendocrine systems.

Immune-endocrine interactions can also have an impact on nutritional status which, in turn, is implicated in autoimmunity (section 2.3.8.1). The "endocrine-immune gradient" responds to environmental stressors, including microbes, by influencing metabolic processes (Husband, 1995). In fact, the interactions are likely to be psychoneuroimmunological, given the link between psychoneural stress and abnormal gastrointestinal function (Berin and Perdue, 1997).

Both the humoral and cell-mediated immune responses are more active in females than males. This immunological sexual dimorphism appears to rely on gonadal, thymic and pituitary hormones (Grossman, 1990). The effects on the immune system of the sex hormones, especially oestrogen and testosterone, are mainly related to changes in migration and circulation of lymphocytes. These hormones alter the
localisation of B lymphoblasts within the gut, uterus and lachrymal glands. They also regulate T lymphoblast entry into mammary gland and the T cell content of lachrymal glands (Ottaway and Husband, 1992). In female rats, short-term exposure to elevated oestradiol and low progesterone decreases resistance to tumour metastasis by altering the activity of large granular lymphocytes and NK cells (Ben-Eliyahu et al., 1996).

Cytokines, for their part, are also able to regulate endocrine function, chiefly by acting on the hypothalamus and/or the pituitary during infection or stress (McCann et al., 1994). The cytokines involved in this interaction include IL-1, IL-2, IL-6, TNF-α, IFN-γ and thymosin α1. Their combined effect is to increase the release of ACTH, prolactin and growth hormone and to suppress the release of thyrotropin, luteinising hormone and follicle stimulating hormone (Haour et al., 1994; McCann et al., 1994). The actions of the cytokines in this context are themselves modulated by antagonists. For example, alpha melanocyte stimulating hormone inhibits a number of cytokines, including IL-1, IL-6, TNF-α and IFN-γ (Catania et al., 1994).

The interplay between cytokines and immune cells such as NK cells is under hormonal control. The ontogeny of NK cells and their function involves a sequential, reciprocal interaction between hormones and cytokines (Provinciali, Di Stefano and Fabris, 1994). In a younger individual, thyrotropin enhances the sensitivity of NK cells to IL-2 which stimulates the early differentiation of new NK cells from precursors; this capacity is maintained throughout life. In an older individual, thyroid hormone sensitises NK cells to IFN which promotes maturation and activation of NK cells; this capacity declines with age but can be overcome by administration of thyroid hormone. Thus the hormone/cytokine time sequence in NK cell function is thyrotropin/IL-2/thyroid hormone/IFN (Provinciali, Di Stefano and Fabris, 1994). NK cytotoxicity can be impaired by high levels of oestrogen (oestradiol) during endometriosis, such that endometriosis is characterised by higher serum levels of
oestradiol together with decreased NK cell activity (Di Stefano et al., 1994; Garzetti et al., 1993). In contrast, the catecholamines (adrenaline and noradrenaline) induce an increase in NK cell numbers and cytotoxicity, and antibody-dependent cell-mediated cytotoxicity (Schedlowski et al., 1993a). Adrenaline has also been shown to promote the migration of T cells from spleen and lymph nodes into the lungs and the movement of CD8 T cells from the lungs to the blood (Rodberg and Kradin, 1994). Noradrenaline can induce the brain (hypothalamus and amygdala) to release arginine vasopressin which contributes to the activation of the HPA axis (Raber and Bloom, 1996).

The usual trigger for activation of the HPA axis is acute stress, whether physical or psychological. The hypothalamus responds by releasing corticotropin releasing factor which stimulates the anterior pituitary to release ACTH which activates the release of glucocorticoids from the adrenal cortex (Brown and Blalock, 1990; Lightman, 1995). Corticotropin releasing factor-binding protein regulates the activity of corticotropin releasing factor, which also has cognitive-enhancing effects (Behan et al., 1995), and which is one of a family of related peptides found, to date, in fish, amphibians and mammals (Vaughan et al., 1995).

It has been known for some time that immunoregulation involves an inverse relationship between cytokines, especially IL-1, and glucocorticoids whereby immunosuppression is characterised by elevated levels of glucocorticoids and reduced levels of cytokines, and immunostimulation exhibits a reversal of the ratio (Besedovsky, del Rey and Sorkin, 1981; Besedovsky et al., 1986). At the tissue level, glucocorticoids promote the redistribution of leukocytes from the bloodstream to immune compartments such as lymph nodes, Peyer's patches, bone marrow, lungs, skin, mucosa and spleen (Dhabhar et al., 1995).
While the anti-inflammatory effects described above relate to acute glucocorticoid exposure, the response to chronic excessive levels of glucocorticoids includes damage to the hippocampus, a structure vital to learning and memory, and possessing high levels of glucocorticoid receptor. This long term effect of glucocorticoids has been implicated in major depression, Cushing’s syndrome and post-traumatic stress disorder. It also raises questions about possible neuropathological consequences of glucocorticoid medication regimes used to control many autoimmune and inflammatory diseases (Sapolsky, 1996).

The effects of acute stress can be completely counteracted by melatonin, a pineal hormone (Maestroni, Conti and Pierpaoli, 1988), whose actions are mediated by endogenous opioid peptides (Lissoni et al., 1994). It is now becoming apparent that melatonin may play a significant role in many interactions between the endocrine and immune systems (Liebmann et al., 1997).

The thymus occupies a pivotal position in terms of neuroendocrine-immune interactions. It is both an organ of the immune system and an endocrine gland in its own right, which responds to stress in an independent manner (Coe and Hall, 1996). The thymus is the site of the earliest neuroendocrine-immune interactions (Geenen et al., 1994; Mocchegiani, Santarelli and Fabris, 1994) which are progressively disrupted as the thymus involutes with age (Goya et al., 1994). The range of hormones that the thymus either responds to or produces is extensive and incompletely documented to date. The thymus is sensitive to growth hormone, prolactin, ACTH, oxytocin and arginine vasopressin, which it also produces, and other hormones produced elsewhere; it also produces follicle stimulating hormone, luteinising hormone and thyrotropin (Dardenne and Savino, 1994a,b). This vast hormone repertoire may explain the dual role the thymus performs in both positive and negative T cell selection (Geenen et al., 1993).
2.5.3 Immune-nervous interactions

Although the focus in the preceding discussion (section 2.5.2) was on immune-endocrine interactions, it should be obvious that the nervous system is often involved at the same time. Thus, there are neuroendocrine elements to thymic function. Positive and negative T cell selection involves "cryptocrine" signaling between thymic epithelial cells and thymic nurse cells on the one hand, and pre-T cells on the other, mediated by an array of hormones and neuropeptides (Geenen et al., 1993, 1994). These include thymosin (Hall et al., 1985) which also appears to be both hypophysiotropic and neurotropic (Goya et al., 1994), and calcitonin gene-related peptide which selectively inhibits inappropriate or premature activation of TH1 cells in the thymus (Wang, Millet and Vignery, 1992).

Calcitonin gene-related peptide-positive nerve fibres have been found in the thymus (efferent vagal fibres) and in lymph nodes (Bulloch et al., 1991), while calcitonin gene-related peptide binding sites occur in lymph nodes, spleen and bone marrow (Henke et al., 1987; Nakamuta et al., 1986; Popper et al., 1988). At the cellular level, thymocytes, T cells, mast cells, macrophages and dendritic Langerhans cells express functional receptors for calcitonin gene-related peptide. Although the details are still not clear, the overall effect of calcitonin gene-related peptide is to inhibit an immune response (Bulloch et al., 1994).

The immunomodulatory effects of the neuropeptide α-melanocyte stimulating hormone arise out of its action as an antagonist of IL-1 (Catania et al., 1994). α-melanocyte stimulating hormone blocks neutrophil accumulation at sites of inflammation in response to IL-1, producing anti-inflammatory and antipyretic effects (Mason and Van Epps, 1989). The anti-inflammatory effects are seen in
acute, chronic and systemic inflammation (Lipton et al., 1994), while the lateral septal area, a forebrain area providing input to the hypothalamus, is the site of action of the antipyretic effects (Greenberg et al., 1992). Changes in the balance between melatonin and α-melanocyte stimulating hormone in the pineal may also be reflected in age-related changes in immune function (Pierpaoli, 1994; Pierpaoli and Lesnikov, 1994).

The enteric nervous system is richly supplied with peptidergic nerves. Therefore, potential local interactions between neuropeptides and lymphocytes have been examined intensively in this tissue, even though the same neuropeptides are also found in many other tissues. Two of the most common enteric neuropeptides are vasoactive intestinal peptide (VIP) and substance P (Ottaway and Husband, 1992). It should be remembered, however, that about 20 different neuropeptides have been identified in enteric nerves, with only some demonstrating immunomodulatory effects, namely, calcitonin gene-related peptide, VIP, substance P, cholecystokinin, enkephalins, neuropeptide Y and somatostatin (Stanisz, 1994).

The effects of VIP are tissue specific and dependent on the resident cell populations within the lymphoid tissue and on the local microenvironment. Effects of VIP on immune function may result from indirect effects on secretory cells, endothelial cells, and smooth muscle cells in blood vessels, ducts and airways. The influences of VIP on immune function may also vary depending on the presence of other signal molecules. Finally, the activational state of target cells may influence the type and level of VIP receptor expression (Bellinger et al., 1996).

VIP has both stimulatory and inhibitory effects on phagocytic cells and lymphocytes, depending on the local circumstances (Calvo, Pozo and Guerrero, 1996; De la Fuente, Delgado and Gomariz, 1996; Ganea, 1996; Robberecht et al., 1996; Weinstock, 1996). It can influence adherence capacity, mobility and chemotaxis,
phagocytosis and free radical production in phagocytes, either positively or negatively. VIP inhibits NK cell cytotoxic activity, but can either inhibit or stimulate lymphocyte adhesion, migration, proliferation, or the production of cytokines or antibodies (De la Fuente, Delgado and Gomariz, 1996). VIP directly inhibits IL-2 production by TH1 cells, leading to inhibition of both T cell proliferation and IL-4 production by TH2 cells. VIP also directly prevents TH2 cells from producing IL-10 (Ganea, 1996). VIP stimulates immunoglobulin production in activated B cells directly, in an isotype-nonspecific way. It promotes IgA1 and IgA2 production in the intestinal tract by isotype switching, but inhibits IgE and IgG4 production in the respiratory tract (Kimata, 1996).

As well as being a neuropeptide, VIP is produced endogenously by both leukocytes and phagocytic cells. Thus, it is also an endogenous autocrine modulator of immune function, perhaps a cytokine (Leceta et al., 1996).

Of the various neuropeptides that have been implicated in immunomodulation, substance P has received the most attention. It is produced by sensory neurons, under the influence of T cells and IL-1β (Stanisz, 1994). Its other physiological effects include contraction of intestinal smooth muscle, arteriolar vasodilation, increased salivary gland secretion, and alteration of microvascular permeability (Pernow, 1983). In the immunological context, substance P activates lymphocytes, macrophages, neutrophils and mast cells (Bozic et al., 1996; Stanisz, 1994).

The effects of substance P on lymphocytes are widespread. It increases B cell differentiation, IgA synthesis, lymphocyte proliferation and NK cell activity. It also increases lymphocyte traffic in the gut mucosa and in lymph nodes, but in a time-dependent way, whereby CD4 T cell output is depressed early (the first hours) but enhanced later (within a day) (Ottaway and Husband, 1992; Stanisz, 1994). Substance P promotes chemotaxis and phagocytosis in neutrophils, and chemotaxis
in macrophages (Ottaway and Husband, 1992). On the basis of its effects on neutrophils, substance P, together with growth hormone and prolactin, has been implicated as a mediator of the immunity-boosting effects of moderate exercise (Smith et al., 1992). Substance P is the only neuropeptide able to cause degranulation of mast cells, resulting in the release of histamine at mucosal surfaces (Stanisz, 1994). In the case of allergic rhinitis, substance P is released from nasal nerve terminals and triggers an increase in nasal epithelium secretions (Gauci et al., 1993a). Thus, substance P is able to fine tune mast cell-dependent events such as IgE-dependent hypersensitivity and anaphylaxis (Stanisz, 1994).

The overall effect of a number of the actions of substance P is to enhance lymphocyte mobility through changes in vascular perfusion and permeability, through release of inflammatory mediators, and perhaps directly and selectively through cell surface receptors on lymphocytes (Ottaway and Husband, 1992). In fact, the contrast between the proinflammatory effects of substance P and the tendency of VIP to inhibit mucosal immunity is reflected in the distribution of their respective receptors on T cells. CD4 T cells express more substance P receptors and fewer VIP receptors than do CD8 T cells (Moore, Spruck and Said, 1988; Payan, Brewster and Goetzl, 1984; Payan et al., 1984). Thus, these two neuropeptides may act antagonistically in controlling mucosal immunity (Stanisz, 1994).

Substance P has also been shown to have a role in the preoptic area of the anterior hypothalamus in the modulation of the febrile response to circulating pyrogens such as lipopolysaccharide (LPS), via a mechanism that is yet to be clarified (Blatteis, Xin and Quan, 1994). Indeed, the link between LPS and fever is still unknown (Dantzer et al., 1993b), despite the finding that the prostaglandin E2 that is produced in response to LPS is synthesised within the preoptic area, so that LPS and prostaglandin E2 are found either side of the blood-brain barrier (Sehic et al., 1996).
The peripheral immune challenge provided by LPS has been shown to stimulate catecholaminergic and serotonergic neurotransmission at multiple brainstem nuclei which show differential responsiveness (Molina-Holgado and Guaza, 1996). The main effects of LPS are seen in the nucleus of the tractus solitarii, an important station in visceral sensory information processing, where levels of noradrenaline, dopamine and serotonin increase significantly. A lesser effect is seen in the locus coeruleus, the site of the greatest number of noradrenaline cell bodies in the CNS, where both noradrenaline and dopamine levels rise. On the other hand, the midbrain raphe nuclei, in which serotonergic projections represent the major afferent neurotransmitter system of the hippocampus, show no response to LPS stimulation (Molina-Holgado and Guaza, 1996). Thus, the transmission of immunological information through the CNS includes a brainstem pathway involving differential activation of a number of centres and their particular neurotransmitters.

The interaction between the catecholamines and the immune system is intimate and reciprocal. Immune stimuli trigger the release of catecholamines which, in turn, influence immune function (Basso et al., 1994). Furthermore, the immune system is able to modulate the α1-adrenergic sensitivity of the brain. Biologically active molecules contained in a thymic extract termed TME have been shown to induce a significant increase in the density of α1-adrenergic receptors in brain cortex. This action shows some specificity in that β-adrenergic receptors are not modified (Basso et al., 1994). Here is another example of the major role played by the thymus in maintaining the network of neuroendocrine-immune interactions.

One of the interactions, which has behavioural outcomes, involves stress, the glucocorticoids and the catecholamines. Dopamine is the major neurotransmitter of the mesolimbic system (Willner et al., 1991), a system that is powerfully activated by stimuli that predict imminent delivery of reward (Bozarth, 1991; Scheel-Krüger and Willner, 1991). It is preferentially activated by appetitive stimuli (Mirenowicz
and Schultz, 1996), the effects of which can be inhibited by IL-2 (Anisman, Kokkinidis and Merali, 1996). Although aversive stimuli can also lead to the desirable outcome of avoidance of, or escape from, a stressful situation (D'Angio et al., 1988), inappropriate avoidance or escape linked to mesolimbic activation presents as depression (Zacharko and Anisman, 1991). Under more normal circumstances, the effects of stress on behaviour are mediated by dopamine neurons under the influence of the glucocorticoids released during stress (Piazza et al., 1991). Thus, the effects of glucocorticoids released during stress range beyond direct immunological changes (section 2.5.2) to include behavioural responses mediated by the mesolimbic system.

In a similar fashion, noradrenaline and dopamine released centrally in response to an immune challenge (LPS) influence the mesolimbic system in an interactive way. Activation of β-adrenergic receptors for noradrenaline results in enhanced dopamine activity at the level of D2 receptors; this inhibits the excitatory action of hippocampal input to the ventral striatum. On the other hand, suppression of α-adrenergic receptors for noradrenaline results in enhanced dopamine activity at the level of DA1 receptors; this inhibits the excitatory action of amygdaloid input to the ventral striatum. Thus, while dopamine plays a permissive role in behaviour, noradrenaline performs a gating function on dopamine (Cools et al., 1991).

The increase in dopamine activity in the ventral tegmental area, in response to stress, is mediated by the endogenous opioid enkephalin (Zacharko and Anisman, 1991). Thus the endogenous opioids are also involved in the stress response, as a consequence of the release of corticotropin releasing factor following activation of the HPA axis. One of the direct effects of corticotropin releasing factor is to stimulate lymphocytes (Kavelaars et al., 1990; Shutt, Connell and Fell, 1989) and the CNS (Dunn, 1995) to release β-endorphin, an opioid which has been described, occasionally, as immunosuppressive (Hemick and Bidlack, 1989), and, more usually,
as antagonistic to HPA axis activity (Hadjipetrou-Kourounakis et al., 1989; Maestrini, Conti and Pierpaoli, 1988; Odio and Brodish, 1990). These seemingly opposite effects of β-endorphin may be a consequence of variations in expression of receptors by lymphocytes from day to day, depending on the current immune and endocrine status of the individual (Brown and Blalock, 1990; Janeway et al., 1985). The significance of opioids in immunomodulation extends beyond β-endorphin. Firstly, enkephalins have been shown to act as regulators/modulators of immune potential and induce positive and negative deviations of immune reactions depending on the dose used (Jankovic, 1994; Plotnikoff et al., 1985). Secondly, the ripple-out effect to the rest of the individual could be pivotal, given the variety of activities mediated by opioids: learning and memory (Schulteis et al., 1990); gastrointestinal function (Baile, McLaughlin and Della-Fera, 1986; Duranton and Buéno, 1983, 1984; Maas, Van Duin and Van Miert, 1986; Ruckebusch, Bardon and Pairet, 1984); and maternal behaviour (Mann, Pasternak and Bridges, 1990).

The arcuate nucleus of the hypothalamus is the major neuronal source of β-endorphin (Sibinga and Goldstein, 1988), an opioid with a range of direct effects on immune function (Bréard, Costa and Kordon, 1995). β-endorphin can alter expression of the CD4 determinant on T cells and HLA-DR determinants on mononuclear cells (Puppo et al., 1985) and, together with met-enkephalin, it can enhance IFN-γ production by mononuclear cells (Brown and Van Epps, 1986). It has been suggested, therefore, that β-endorphin plays a role in defining lymphocyte traffic and distribution patterns (Ottaway and Husband, 1992). Since the various endorphins and ACTH can also be produced by lymphocytes (Lolait et al., 1986; Smith and Blalock, 1981), it appears that lymphocytes themselves could influence local IFN-γ production and regulate the migration of other lymphocytes (Ottaway and Husband, 1992).
The release of endogenous opioids, when it leads to stress-induced analgesia, is a characteristic of uncontrollable pain; a reduction in the perceived level of uncontrollability leads to decreased opioid levels, which leads to a perception of control (Arntz and Schmidt, 1989; Dantzer, 1989; Steptoe, 1989). Thus the suppression of NK cell cytotoxicity seen in these circumstances (Maier, Laudenslager and Ryan, 1985; O'Donnell, 1992; Shavit et al., 1984) is a perceptual rather than a physiological consequence of stress, mediated by opioids (König et al., 1996).

In an intact individual, the rewarding properties of opioids are mediated by a dopaminergic substrate in the mesolimbic dopamine system, leading to increased locomotor activity (Bozarth, 1991). The opioid system stimulates some ventral tegmental area dopamine neurons indirectly, by disinhibition, through μ and δ receptors; others acting on κ receptors are inhibitory. Thus different opioids, acting on different receptors, produce differential degrees of activation and different behavioural outcomes (Gates et al., 1992; Scheel-Krüger and Willner, 1991).

Immune-nervous interactions are not only mediated by molecules synthesised by neuronal tissue. A significant array of interactions involves cytokines (Dunn, 1995). In terms of immunological effects on neural function, the cytokines have been implicated in the pathophysiology of certain neuropsychiatric disorders (examined separately in section 2.5.5) (Malek-Ahmadi, 1996), on the basis of several observations: brain tissue, both glial cells and neurons, is capable of synthesising cytokines (Breder, Dinarello and Saper, 1988; Konishi et al., 1994); cytokine receptors that mediate central actions of cytokines have been identified in various regions of the brain (Farrar et al., 1987; Lapchak et al., 1991); systemic use of cytokines can cause neuropsychiatric side-effects in psychiatrically healthy individuals (Denicoff et al., 1987; Fent and Zbinden, 1987; Niranen et al., 1988;
Renault et al., 1987; Triozzi and Rinehart, 1990; Walker et al., 1996); some cytokines have been used therapeutically in neurologic disorders such as multiple sclerosis (Benveniste, 1995; Malek-Ahmadi, 1996); some psychotropic agents are capable of altering the production and release of cytokines (Beyaert et al., 1992; Boukhris, Kouassi and Revillard, 1988; Gallicchio, Chen and Watts, 1984; Kleinerman et al., 1989; Wicher and Evans, 1990); cytokines released during inflammation of the central or peripheral nervous systems induce transient electrophysiological dysfunction (Köller, Siebler and Hartung, 1997).

Rather than attempting a comprehensive review of the neuronal actions of cytokines (Bréard, Costa and Kordon, 1995), this discussion will deal with some of the functions of IL-1 and IL-2, so as to illustrate the impact of cytokines on central nervous activity.

IL-1 is taken up directly by neural cells (Brown et al., 1993b), where IL-1/IL-1 receptor interactions are involved in central signals that initiate changes in immune responsiveness (Haour et al., 1994). Two different, but convergent, immunosuppressive pathways are activated. One operates through ACTH and glucocorticoids (Weiss, Quan and Sundar, 1994) and includes the induction of Fos, a marker of neural cell activation (Morgan and Curran, 1991; Sheng and Greenberg, 1990), in the brain (Ceccatelli et al., 1989; Sharp et al., 1991). In the other, neural sympathetic (noradrenergic) signals (Dunn, 1988; Smagin, Swiergiel and Dunn, 1996) influence splenic lymphocyte and macrophage function (Greenberg et al., 1992), and also mediate an increase in cerebral Fos (Bing et al., 1991, 1992; Gubits et al., 1989; Stone and Zhang, 1995). The Fos response to IL-1 requires an intact noradrenergic innervation (Swiergiel, Dunn and Stone, 1996). The pyrogenic effect of LPS is also mediated by centrally-active IL-1. Fever is induced by IL-1 inhibiting the cerebellar gamma-aminobutyric acid response (Pringle, Gardner and Walker, 1996).
The neuroregulatory functions of IL-2 were reviewed recently by Hanisch and Quirion (1996). IL-2 exhibits powerful effects on neural cells, including activities relating to cell growth and survival, transmitter and hormone release, and the modulation of bioelectric activity. IL-2 may also be involved in the regulation of sleep and arousal, memory function, locomotion, and the modulation of the neuroendocrine axis. IL-2 and/or IL-2 receptor molecules are found mainly in the frontal cortex, septum, striatum, hippocampus, locus coeruleus, cerebellum, pituitary, and tracts such as the corpus callosum; they are expressed on both neurons and glial cells, especially oligodendrocytes. Finally, the ability of IL-2 to easily cross the blood-brain barrier suggests that it could regulate interactions between peripheral tissues and the CNS. In fact, it has been hypothesised that the multiorgan dysfunctions that follow the physical disruption of the CNS by spinal cord injury are mediated by cytokines, chiefly IL-1 and IL-2 (Segal, 1993).

Immune-nervous interactions are also evident at the cellular level, whereby cell types that are considered to belong primarily to one system play a role in the other.

Mast cells are found in various tissues throughout the body, including the brain. They leave bone marrow as immature cells and mature under local microenvironmental conditions (Galli, 1990). Brain mast cells enter the brain during development via penetrating blood vessels with which they remain associated (Lambracht-Hall, Dimitriadou and Theoharides, 1990). Brain mast cells, which are ultrastructurally distinct from connective tissue mast cells (Dimitriadou et al., 1990; Ibrahim, Al-Wirr and Bahuth, 1979), have been shown to contain histamine, cytokines, proteolytic enzymes (Galli, 1993) and heparin (Pang et al., 1996). They have been localised in the leptomeninges (Edvinsson et al., 1977), the thalamus (Goldschmidt et al., 1984) and the hypothalamus (Dropp, 1976; Edvinsson et al., 1977; Ibrahim, 1974; Pang et al., 1996; Pollard et al., 1976) where, in broad terms,
they regulate vascular and blood-brain barrier permeability and inflammatory cell entry in brain parenchyma (Lutz et al., 1978; Pang et al., 1996; Persinger, 1977; Rosenblum, 1973; Theoharides, 1990).

It has been proposed that interactions between brain mast cells and neurons (Stead and Bienenstock, 1990; Undem and Weinreich, 1989) may be significant in neuropeptide regulation of hypersensitivity (Foreman, 1987; Payan, Levine and Goetzl, 1984). The fact that brain mast cells are activated by immobilisation stress (Spanos et al., 1994) is also significant in that they are localised close to nerve fibres positive for corticotropin releasing factor (Theoharides et al., 1995) which triggers mast cell degranulation (Boucher et al., 1995), one of the steps in type I hypersensitivity. Brain mast cell activity has also been implicated in multiple sclerosis (Krüger et al., 1990; Rozniecki et al., 1995; Theoharides, 1990). Thus brain mast cells, which both reside in, and traffic through, the brain providing targeted delivery of neuromodulators to specific regions of the brain (Silver et al., 1996), may act as neuroimmunoendocrine master players, initiating the first response to stressful stimuli (Pang et al., 1996).

Microglia are brain macrophages that have a central role in CNS-immune interactions (Fricchione, Bilfinger and Stefano, 1996; Htain, Leong and Ling, 1995), especially in terms of CNS response to injury (Gehrmann, Matsumoto and Kreutzberg, 1995). They invade the brain and spinal cord from the bone marrow during late embryonic development, becoming permanent residents of the CNS. Resting microglia constantly monitor the health of cells around them (Perry and Gordon, 1991); they become activated in response to various types of injury, such as infection, trauma and autoimmune inflammation (Zielasek and Hartung, 1996). Activation is associated with increased expression of genes involved in immune responses (Streit and Kincaid-Colton, 1995), resulting in fully competent APCs (Matsumoto, Ohmori and Fujiwara, 1992). The end result of this microglial
activation can be either further tissue destruction or the removal of debris and eradication of pathogens (Banati and Graeber, 1994; Ford et al., 1996).

Microglia that have been activated by either LPS or IFN-γ produce IL-10, with several CNS immunoregulatory consequences. IL-10 down-regulates HLA-DR expression by microglia, inhibiting their APC function (Williams et al., 1996); it inhibits microglial stimulation of CD4 T cells (Frei et al., 1994; Williams et al., 1996); it inhibits the development of experimental allergic encephalomyelitis (Rott, Fleischer and Cash, 1994). During the response to experimental allergic encephalomyelitis, microglia are also activated by secretoneurin, a neuropeptide that also recruits macrophages across the blood-brain barrier to the site (Storch et al., 1996); this combined microglia/macrophages response is seen in brain that has been damaged by various causes, including stroke (Arvin et al., 1996). LPS can also stimulate microglia to produce IL-1 and IL-6 but, in this case, the process is inhibited by IFN-γ (Loughlin and Woodrooffe, 1996). IFN-γ can also act synergistically with β-amyloid protein, a major component of the senile plaques characteristic of Alzheimer’s disease, to activate microglia to produce TNF-α and nitric oxide, perhaps leading to further neuronal degeneration (Meda et al., 1995). At low levels TNF-α will mediate the inflammatory response, but at high levels it is neurotoxic (Arvin et al., 1996). Microglial activation can also be induced by epidermal growth factor, the receptor of which is up-regulated during pathological conditions in the brain, and complement factor C5a, one of the complement proteins released at pathological sites (Ilischner, Nolte and Kettenmann, 1996; Nolte et al., 1996).

It would appear that the significance of microglia in CNS-immune interactions cannot be overstated. Thus “under no conditions where neurons are dying or regrowing are microglia not involved, and control of microglia is likely to be just as important in regeneration as providing a favourable environment for neurons to
grow. In short, microglia cannot be seen merely as cells of a certain type within the brain, possessing certain functions, but instead must be regarded as a concept that shapes the approaches taken to nervous system development, cell death, disease and trauma, and nervous system regeneration" (Moore and Thanos, 1996).

Olfaction, a neuronal activity based on molecules, has also been shown to influence immune function. Exposure of mice to stress odour pheromones suppresses cell-mediated immunity and enhances humoral immunity in a process mediated by glucocorticoids (Moynihan et al., 1994).

Immune-nervous interactions are not confined to the CNS. When myelinated peripheral nerves are injured, their Schwann cells produce TNF-α which has a pathogenic role in Wallerian degeneration. TNF-α may also be involved in the hyperalgesia associated with neuropathic pain (Wagner and Myers, 1996). Peripheral nerves will also express MHC class I molecules, but only when they are functionally impaired, rendering themselves susceptible to CTLs (Neumann et al., 1995). Macrophages that are phagocytic at a site of peripheral nerve damage also aid regeneration by growth-supporting activity, the inhibition of which in the CNS impairs central nervous regeneration (Lazarov-Spiegler et al., 1996).

2.5.4 Psychoneuroimmunology

It should be clear by now that the immune system does not interact with any one of the systems in isolation from the others. Cross-references have been necessary. This section, then, abandons such divisions and adopts a more holistic view under the banner of PNI whereby the systems themselves are not the focus of interest, and comparisons are readily made among species (at least among mammalian species).
PNI is a large, diverse, rapidly expanding field of investigation which will not be covered comprehensively in this section. Rather, several topics are examined, with varying degrees of detail, as exemplars of PNI, bearing in mind that many of the interactions that have already been discussed could have also been included in this section.

One area of interest in PNI has behaviour at its centre. Broadly speaking, immune function influences, or is influenced by, behaviour (Ader, Felten and Cohen, 1990; Maier, Watkins and Fleshner, 1994). The impact of the immune response is perhaps most evident when it results in sickness behaviour (Dantzer et al., 1993a), while the reciprocal effects are being uncovered by the use of Pavlovian conditioning (Cohen, Moynihan and Ader, 1994).

The cytokines that mediate the febrile response to pathogens (IL-1 in particular) also trigger sickness behaviour in a separate, direct pathway (Dantzer et al., 1993b; Kent et al., 1992). Thus, IL-1 released peripherally during infection acts centrally to induce sickness behaviour (Bluthé, Dantzer and Kelley, 1997) which is typified by increased sleep (Krueger et al., 1990; Obal et al., 1990), decreased social behaviours (Bluthé et al., 1991, 1995, 1996; Dantzer, Bluthé and Kelley, 1991), changes in exploration of novel environments (Spadaro and Dunn, 1990) and weight loss (Kent et al., 1996). The behavioural effects of IL-1 are also modulated by the environmental context, so that sickness behaviour develops in a quiet setting while agitation results in response to a threatening stimulus; the behavioural outcome also depends on motivational state (Friedman, Reyes and Coe, 1996), supported by the mesolimbic system (Willner and Scheel-Krüger, 1991). Changes in the behaviour of sheep after parasite infection or re-infection appear to represent a less clear-cut form of sickness behaviour. When observed in a classic approach/avoidance conflict situation known as arena testing (Fell, 1992), the sheep exhibited inappropriate approach behaviour, degraded flocking behaviour and increased locomotor activity.
(Fell et al., 1991; Gates et al., 1992). While sickness behaviour is thought of as an adaptive host response to infection or injury (Hart, 1988), it is not clear if these sheep behaviours, mediated by endogenous opiates, favour the sheep or the parasite (Gates et al., 1992).

Behaviourally conditioned immunosuppression was first demonstrated, in the rat, by Ader and Cohen (1975). It has since been shown that various aspects of immune function can be conditioned, leading to a deeper understanding of the response (Ballieux, 1994). In the rat, for example, enhanced heart allograft survival can be conditioned (Schedlowski et al., 1992), as can: the induction of sublethal anaphylactic shock (Jankovic, 1994); enhanced effects of immunosuppressive drug therapy for adjuvant arthritis (Klosterhalfen and Klosterhalfen, 1992); the antipyretic effects of α-melanocyte stimulating hormone; and, perhaps, LiCl and the pyretic effects of LPS (Bull et al., 1992; Gauci et al., 1992), and antibody production in response to specific antigen (Husband et al., 1993). In general, it appears that conditioning can influence immune cell distribution and corticosterone secretion in the rat (Buske-Kirschbaum et al., 1996). It should be noted, however, that the timing and magnitude of lymphocyte suppression consequent on HPA axis activation is different in different strains of rats (Shurin et al., 1995).

A similar picture is emerging from mouse studies. For example, it has been possible to condition both immunosuppression of murine SLE (Ader and Cohen, 1982a, b) and enhanced pharmacological effects of immunosuppressive drugs for the condition (Klosterhalfen and Klosterhalfen, 1992). Factors that can influence the nature of the interaction in the mouse include the strain of mice being studied (Starec et al., 1994), brain and/or behavioural lateralisation (Neveu et al., 1994), and the age of the mouse (Spector et al., 1994).
Although an attempt to demonstrate conditioned alteration to an allergic skin test response in humans has been unsuccessful (Booth, Petrie and Brook, 1995), expression of allergic rhinitis has been behaviourally conditioned, so that associative learning triggers the release of mucosal mast cell mediators usually only released after exposure to allergen (Gauci, Husband and King, 1992). This observation points to CNS involvement in the mast cell degranulation characteristic of allergic rhinitis (Gauci et al., 1993a), and suggests that psychological manipulations may be useful in managing allergic disorders (Gauci et al., 1993b). Behavioural conditioning in humans can also increase NK cell activity (Buske-Kirschbaum et al., 1992) and enhance mucosal IgA levels (Gregerson, Roberts and Amiri, 1996).

It is but a small step to go from behaviour to the psychosocial context. Some of the psychosocial aspects of PNI have already been introduced in the discussion of stress (section 2.5.1), and others are better discussed in terms of either pathological conditions (section 2.5.5) or therapies (section 2.5.6). The significant influence of psychosocial variables on immune function has been elucidated by many animal studies, chiefly dealing with nonhuman primates.

For example, the importance of normal maternal care means that, in both primates (Boccia et al., 1997; Coe, 1993) and rodents (Von Hoersten et al., 1993), maternal deprivation or separation lead to immunosuppression and altered behaviour in the young. The response to maternal separation can be mitigated, however, by strong social affiliation or support from a peer group (Boccia et al., 1997; Coe, 1993; Laudenslager and Boccia, 1996). Disruption of social relationships, whether by separation or social reorganisation, can lead to depressed cellular immunity and increased levels of circulating cortisol (Gordon et al., 1992; Line et al., 1996; Reite, Harbeck and Hoffman, 1981). These responses are further modulated by the level of individual behaviours, such as fearfulness or aggression, within a group (Coe, 1993;
Line et al., 1996). In general terms, critical times for nonhuman primates seem to be those when social relationships are in the process of change (Coe, 1993).

In an attempt to provide a theoretical basis for these studies, Rotenberg, Sirota and Elizur (1996) have proposed that every behaviour that includes search activity prevents psychoimmunological disorders while renunciation of search is associated with a predisposition to these disorders. This proposal would appear to be no more than a re-statement of the concept of locus of control.

The findings in nonhuman primates can be extrapolated to humans. Thus, psychosocial stressors and/or interventions can modulate immune status sufficiently to lead to changes in health (Kiecolt-Glaser and Glaser, 1995; Mestel, 1994). For example, a study of human pregnancy outcomes has shown that social competence is a positive determinant of health and health behaviour, mediated by the immune system (Hagoel et al., 1995).

Exercise is another category of behaviour with connections to immune function and, ultimately, health (Sharp and Parry-Billings, 1992). Moderate exercise seems to be beneficial to health while severe exercise can be detrimental (Nehlsen-Cannarella et al., 1997).

Moderate exercise can confer psychological benefits similar to those produced by meditation (Harte, 1992). The immunostimulatory effects are mediated by the actions of growth hormone, which primes neutrophil microbicidal activity (Weidemann et al., 1993), noradrenaline, β-endorphin, corticotropin releasing factor (Harte, 1992), prolactin and substance P (Smith et al., 1992). Moderate exercise is being used to lessen the risk of lifestyle-associated diseases such as heart disease, and as an adjunct therapy in cases of AIDS or cancer (Mackinnon, 1994). It has also been shown to attenuate the stressor-induced psychological and immunological
changes in HIV-positive individuals (Laperriere et al., 1994, 1997).

It is now commonly accepted that athletes have an increased susceptibility to infection during intense training and competition (Mackinnon, 1994). In fact, sports competition can be regarded as a psychological stressor that suppresses mucosal immunity whereby daily intense training and major competition lead to cumulative reductions in both resting and post-exercise IgA levels. This situation then leads to an increased risk of upper respiratory tract infections (Mackinnon, Ginn and Seymour, 1992). Exercise at or beyond 65% of maximum capacity triggers the release of ACTH, leading to an increase in glucocorticoids and opioids, and immune suppression. While acute immune suppression may be advantageous in preventing exercise-induced inflammatory injury, chronic immune suppression increases the risk of infection (Smith et al., 1992). Coaches, as well as athletes, are subjected to competition stress. The acute psychological stress of a match involving their team activates non-specific humoral immune functions, but only transiently (Kugler et al., 1996).

Moving from conscious activities to those that are more autonomic leads to a consideration of sleep. The purpose of sleep is unknown, despite the proliferation of theories attempting to explain it (Rechtschaffen, 1998). The reason for sleep has been variously attributed to synaptic remodelling, memory consolidation, glycogen replenishment, energy conservation, adaptation to environmental pressures, and combinations of some or all of the above (Beardsley, 1996: Holmes, 1997; Maquet et al., 1996, 1997). The theory that may be relevant here was proposed by Willner (1991) who linked REM (rapid eye movement) sleep with the physiological response to uncontrollable stress. Thus, the REM/non-REM (NREM) sleep cycle is mediated by a reciprocal interaction between groups of cholinergic and noradrenergic neurons in the brain stem. During waking, the same interaction is responsible for the behavioural and hormonal transition from an active to a passive mode of stress.
control. On that basis, Willner has suggested that the underlying neuroanatomy may have evolved to facilitate adaptation to prolonged stress, with REM sleep as an accidental consequence. In a further development of this theory the diurnal sleep-wake rhythm is seen as the result of oscillatory mechanisms involving brain IL-1 and the HPA axis (Krueger and Obál, 1993).

Sleep can be promoted/induced by IL-1, TNF, IFN (Toth, 1995), growth hormone releasing hormone, growth hormone, somatostatin and melatonin (Moldofsky, 1995a, b); it is inhibited by corticotropin releasing factor, ACTH, α-melanocyte stimulating hormone and glucocorticoids (Moldofsky, 1995a, b). Peripherally released IL-1 can communicate with the brain via the vagus nerve which has been shown to exhibit IL-1 receptors in the gut and the spleen (Brown, 1997); IL-1 is also produced directly in the CNS (Brown et al., 1993b). Substances of bacterial or viral origin (muramyl peptide) also appear to be involved in regulation of normal sleep (Bauer et al., 1995a; Brown et al., 1992; Bull et al., 1993; Toth, 1995), so that the relationship between sleep and bacteria (and body temperature regulation) may represent a finely-tuned endosymbiotic process (Krueger and Karnovsky, 1995).

While the levels of somnogenic cytokines vary in a circadian pattern (Toth, 1995) in which IL-1 regulates sleep-wake activity by promoting NREM sleep (Opp and Kreuger, 1994a, b), the nature of the pattern is unclear. On the one hand, it has been suggested that IL-1 activity is related to the sleep-wake cycle, but not specifically to clock time (it increases at night during undisturbed sleep), with consequent changes in peripheral immune and endocrine responses driven by IL-1 levels (Moldofsky, 1995a). On the other hand, given that different immune functions exhibit circadian, circaseptan or circannual rhythms (Knapp, 1992), it seems that circadian rhythms in immune parameters are associated most closely with the circadian rhythm of cortisol (Kronfol et al., 1997).
There is general agreement on the immunological efficacy of sleep. NREM sleep is an integral component of the immune response; it promotes disease resistance and aids in recovery from infection (Brown et al., 1992; Krueger and Karnovsky, 1995; Toth, 1995). In fact, disorganisation or loss of the sleep-wake system is accompanied by altered immune, neuroendocrine and thermoregulatory functions, and contributes to pathological processes (Cover and Irwin, 1994; Moldofsky, 1995b).

It is ironic that a recent “theory” that claims to explain PNI as a system would disintegrate the organism and would separate humankind from the other mammals. This “networking organ system theory” of Vishwanath (1996) appears to be based on several doubtful premises: the integrated functioning of the whole organism requires independent immune and nervous systems; neuroimmune networks are absent in “submammalian” vertebrates; and, human consciousness is required to establish a “psyche/central nervous system/organs’ network”. The level of anthropomorphism embodied in this “theory” makes it biologically suspect.

Finally, a word on the effects of age. Apart from the direct effects of age on T cells (section 2.3.8.1), age has an indirect effect mediated by neuroendocrine age-related changes (Mosley, 1996; Pariante et al., 1997).

2.5.5 Pathologies

Before considering the immune interactions involved in particular pathological states, some generalisations can be made. Firstly, it is biologically self-evident that the adaptive benefits conferred on other species by sickness behaviour (section 2.5.4) also apply to humankind (Nesse and Williams, 1994). Secondly, the PNI framework has been useful in making sense of behavioural psychotherapy, a collection of techniques that reduce stress and improve coping skills, thereby enhancing immune
function and promoting quality of life (Baum, Herberman and Cohen, 1995; Cottraux, 1993). Finally, PNI has contributed to exploration of both the role of the unconscious in healing (Dossey, 1991) and the very function of illness itself (Davis, 1996).

“The belief that cancer might be related to temperament or distress has been emphasized throughout the history of medicine”, yet a “causal relationship between psychosocial stress and the development of cancer has not been proven” (Fife, Beasely and Fertig, 1996). Nevertheless, many associations, correlations and connections have been established.

The risk of developing cancer has been associated with a number of factors. Healthy women with one or more first degree relatives with cancer and/or with high levels of distress exhibit lowered NK cell cytotoxic activity which, in turn, may contribute to an increased cancer risk (Bovbjerg and Valdimarsdottir, 1993). The distress associated with loss and grief may activate neoplasia and/or impair immune surveillance so that breast cancer develops (Biondi, Costantini and Parisi, 1996). In fact, a variety of psychosocial stressors has been shown to either modulate endocrine processes directly related to tumour growth or decrease immune regulation of tumour development and metastasis (van der Pompe et al., 1994).

Studies of intrinsic predisposition to cancer have produced the type C personality profile. This biobehavioural cancer risk pattern is characterised by denial and suppression of emotions, especially anger, by “pathological niceness”, and by “anti-emotionality” (Baltrusch, Stangel and Titze, 1991). The validity of this construct has been challenged, however, by the notion that repression could be a response to the cancer diagnosis, aimed at keeping anxiety at a tolerable level (Kreitler, Chaitchik and Krietler, 1993). Yet, expression of negative affect (the opposite of type C behaviour) correlates positively with NK cell activity, negatively with β-endorphin,
and is prognostically favourable in cases of early breast cancer (O’Donnell, 1992).

Psychosocial factors are important components of assessment and management of cancer patients (Walker and Eremin, 1996). For example, host anti-tumour defences that determine the effectiveness of chemotherapy can undergo neuroimmunomodulation by stress, reducing the effectiveness of the drugs (Giraldi et al., 1994). By the same token, relaxation training can enhance immune parameters in cancer patients, even if the training takes place during chemotherapy (Lekander et al., 1997). The role of placebo chemotherapy in enhancing immune status emphasises the significance of psychological factors (Lekander et al., 1994). Indeed, patients involved in a chemotherapy regime can display anticipatory immune changes (Lekander et al., 1995), a type of conditioned immune response. Additional stressors, such as immigration, also make cancer patients more vulnerable (Baider et al., 1996). The immune impairment seen in breast cancer (elevated basal cortisol together with normal range basal ACTH) leads to a compromised response to behavioural challenge; cortisol levels decrease and the ACTH increase is relatively minor (van der Pompe, Antoni and Heijnen, 1996).

Surviving cancer may be a result of successful IL-2 activation of the anticancer response that consists of an increase in total lymphocytes, T cells, NK cells, eosinophils and macrophage activity, especially when it is enhanced by the presence of melatonin (Lissoni et al., 1994). The risk of psychological and behavioural morbidity for cancer survivors can be improved with psychological intervention (Andersen, 1994). At the other end of the spectrum, patients with malignant brain tumours generate an illusion that enables them to cope by combining reality with protection/hope that they had created using various cognitive manoeuvres (Salander, Bergenheim and Henriksson, 1996).
While psychotherapy may be useful in modifying the cancer-immune system interaction, immunological changes also correlate with psychiatric disorders. The group of disorders termed depression has been the subject of many studies with a PNI focus. The HPA axis becomes deranged in a number of psychiatric disorders including anxiety, anorexia nervosa and major depression (Maes et al., 1995b; Merola et al., 1994), such that various indexes of immunocompetence are lowered in proportion to the severity of depressive symptomatology (Weisse, 1992). For example, systemic autoimmune disorders are more prevalent in treatment-resistant depression than in other forms of depression or in the general population (Scott, Hickie and Lovric, 1993). In addition, the overall severity of major depression correlates with plasma concentrations of haptoglobin which, in turn, correlates with psychomotor disorders, anorexia/weight loss, and insomnia (Maes et al., 1993a), and which is significantly related to activation of cell-mediated immunity (Maes et al., 1993b).

A number of immune parameters are known to change in association with depression. The inhibition of NK cell activity by depression (Bauer et al., 1995b) does not appear to be caused by reduction in either number or percentage of NK cells in peripheral blood (Maes et al., 1994). Moreover, NK cell numbers rise in depressed subjects responding to a stressor, but in proportion to the severity of the depression rather than the stressor (Ravindran et al., 1996). The immune response accompanying major depression includes significantly higher levels of IFN-γ, IL-1, IL-2, IL-6 and TNF (Bauer et al., 1995a; Maes, 1995; Maes et al, 1995a,b).

IL-1 and IL-6 have been implicated in the associated sickness behaviour (Maes, 1995) which may be a common feature of a number of neuropsychiatric syndromes (Hickie and Lloyd, 1995). IL-6 may also contribute to the pathophysiology of major depression (Maes et al., 1995a). Also, the hypersecretion of IL-1, IL-2 and IL-6 seen
in both major depression and schizophrenia is thought to augment the disorder, possibly by an influence on the catecholaminergic system (Muller, 1995). Finally, the increased levels of IL-1 and IL-6 may be a consequence of the decreased serum activity of dipeptidyl peptidase IV that accompanies major depression. Dipeptidyl peptidase IV is a serine protease that has IL-1 and IL-6 as possible substrates (Maes et al., 1997).

A recent review of the association between the immune system and major depression (Anderson, 1996) has identified several outstanding issues and problems. Firstly, some of the apparently contradictory findings may be due to inadequate psychiatric diagnostic specificity. For example, immune dysfunction may be confined to older, hospitalised, more severely depressed patients (Hickie and Hickie, 1992). Etiologically different subgroups of depression can result in either activation or suppression of immune function (Muller et al., 1993). Both positive and negative changes in mood state influence some immune parameters in the same way (Futterman et al., 1994). Secondly, the vast majority of immune parameters has been measured in peripheral blood which does not necessarily reflect events in other parts of the immune system (Ottaway and Husband, 1992). Thirdly, the temporal element is rarely considered, despite its importance (Knapp, 1992). Finally, the link between depression-related immune changes and increased incidence of physical illness is not well established (Hickie, Hickie and Bennett, 1993).

Moving on to a consideration of HIV involves shifting from psychiatric disorders with immune involvement to an immune disorder with psychosocial involvement. The progression of HIV disease appears to be accelerated by such factors as distress, denial, low adherence to behavioural interventions (Evans et al., 1997; Solomon et al., 1994), and feelings of depression, anxiety and hopelessness (Goodkin et al., 1993; Rayner-Brosnan, 1993). Furthermore, cortisol (reflecting stress) and circulating HIV-derived products acting synergistically seem to be involved in HIV
progression, by inhibiting NK cell activity (Nair and Schwartz, 1995), despite the contrary idea, now no longer held (Vedhara et al., 1997), that the relationship between stress and HIV progression may be equivocal (Nott, Vedhara and Spickett, 1995).

On the other hand, active coping strategies can decrease the clinical progression of HIV, possibly mediated by greater compliance with medical treatment (Mulder et al., 1995a). However, irrespective of the type of psychosocial intervention program, which could generate hope and empowerment (Rayner-Brosnan, 1993), the resultant decrease in stress is related to increases in CD4 T cell counts (Mulder et al., 1995b).

Several other illnesses have been examined from the PNI perspective, usually because they include an obvious immunological element. One which may not be so obvious is coronary artery disease, in which the macrophage plays a key role (Fricchione et al., 1996). Hyperlipidaemia, leading to oxidised low density lipoprotein, together with hostility-associated stress, leading to neurochemical changes, suppress the activation of macrophages to the extent that atherogenesis is more likely to develop (Adams, 1994). Later, vasoconstriction of atherosclerotic coronary arteries may result from a similar suppression of μ3 opioid receptor (which mediates nitric oxide release from macrophages and endothelial cells), leading to further reduction in nitric oxide mediated vasodilation. Paradoxical vasoconstriction may then worsen an unstable coronary artery syndrome through pathological responses to catecholamines, serotonin and thrombin. These substances are associated with plaque rupture, which is further facilitated by the presence of lipid-laden macrophages (Levine, Keaney and Vita, 1995).

The remaining illnesses to be considered here all have clear immune involvement. Chronic fatigue syndrome is an illness in which the combination of immune dysfunction and neuroendocrine changes suggests dysregulation of the interactions
between the nervous and immune systems (Farrar, Locke and Kantrowitz, 1995). Thus, “an individual with elevated stress and poor coping skills has associated immune perturbations, which in conjunction with an external insult such as a viral infection, lead to a prolonged illness” (Krupp and Pollina, 1996). The generalised immune dysfunction includes mild perturbation of humoral activity, disturbance of cellular immune response, and cytokine abnormalities (Krupp and Pollina, 1996). Both the physical and the psychological symptoms of chronic fatigue syndrome may be a result of HPA axis dysfunction (Demitrack, 1994), which has also been implicated in Cushing’s syndrome, a condition of excess glucocorticoid production (Kronfol et al., 1996).

Multiple sclerosis, an inflammatory and demyelinating disease of the CNS, involves an immune-mediated process in genetically susceptible individuals who have been exposed to critical environmental triggers such as infections. In such instances, viral molecular mimicry may activate T cells that then cross-react with myelin basic protein in an autoimmune fashion (Johns, 1997). Stress is significant in multiple sclerosis. On the one hand, it has been associated with immune dysregulation in multiple sclerosis patients. On the other hand, stress increases the risk of upper respiratory tract infections which can act as triggers for multiple sclerosis flare-ups (Coyle, 1996).

Dysregulation of nervous-immune interactions is also evident in other autoimmune diseases. In general terms, a person’s psychosocial status influences disease progression in rheumatoid arthritis (Lindberg et al., 1996; Rimón and Laakso, 1985; Rogers and Fozdar, 1996; Scott, Hickie and Lovric, 1993), while the state of the illness has a bearing on the psychological level of a sufferer of SLE (Rogers and Fozdar, 1996; Scott, Hickie and Lovric, 1993).
The nature of the alteration in immune function in rheumatoid arthritis is not clear. One hypothesis suggests the presence of extravascular immune complexes, while another proposes that anti-joint, activated T cells trigger a targeted response (Rogers and Fozdar, 1996). Recent evidence, however, indicates that there need not be a joint-specific antigen, but rather a breakdown in general mechanisms of self-tolerance that result in systemic self-reactivity (Kouskoff et al., 1996).

2.5.6 Therapies

Having dis-integrated the interactions that involve immune function, in order to examine them in some detail, it is appropriate to conclude by re-integrating them into a person-centred approach. One way to achieve this integration is to explore the immunological element of several therapies.

An early review of the use of hypnosis to modify immune responses, in the context of the "emerging field of psychoneuroimmunology", made a number of claims (Hall, 1983). It was suggested that hypnosis could lead to: a reduced allergic response; recovery from skin conditions such as urticaria and ichthyosiform erythrodermia; inhibition of the Mantoux reaction; remission of cancer; and enhancement of the immune status of healthy subjects, mediated by unknown neural processes. A decade later, however, it would appear to be premature to be making inferences about the benefits of hypnosis in the treatment of immune related diseases. Studies of the effects of hypnosis on immune function have revealed inconsistencies related to methodology and subject characteristics (Zachariae et al., 1994).

Nevertheless, hypnotherapy has been successful in curing patients of warts, suggesting a mind-body connection of the type that underpins psychoneuro-
immunology (Noll, 1994). It can also reduce skin sensitivity to histamine, whereby the change in weal size correlates with mood and physiological variables, but not hypnotisability (Laidlaw, Booth and Large, 1996; Laidlaw et al., 1994).

Self-hypnosis, even when practised by children, can be used to increase salivary IgA levels in the short term (Olness, Culbert and Uden, 1989). When used in a stress management plan, self-hypnosis led to long term improvement in psychological well-being, but less clear-cut immunological improvement (Blenkhorn et al., 1992).

Nursing, a multidisciplinary and holistic therapy, has begun using the PNI framework in examining relationships between behaviour and biological phenomena and their effects on health outcomes (Zeller et al., 1996). The PNI perspective has informed the areas of: cancer nursing (Bauer, 1994); psychiatric nursing (Hayes, 1995); HIV/AIDS nursing; childbirth stress; wound healing; perimenstrual stress; therapeutic touch; and the nursing back rub (Zeller, McCain and Swanson, 1996).

Therapeutic touch has been described as a "treatment modality based on an exchange of energy between healer and patient" (Macrae, 1979). It is said to benefit sufferers of so-called endogenous disorders resulting from: deficient immune responses; a "sluggish lymphatic system"; psychological stress; low self-esteem; or "an unproductive lifestyle" (McCormack, 1991). During therapeutic touch the practitioner is in a state of steady concentration, termed healing meditation, while the patient is deeply relaxed (Krieger, Peper and Ancoli, 1979). The outcome for the patient includes decreased state anxiety, increased positive affect, decreased negative affect, and a decrease in CD8 T cells, while the practitioner experiences an increase in positive affect and a decrease in negative affect (Quinn and Strelkauskas, 1993). Perhaps the practitioner's mesolimbic dopamine system has been activated (Willner and Scheel-Krüger, 1991). A review of some twenty years of research and publications on therapeutic touch has concluded that the theoretical basis has
remained unchanged despite an inability to confirm the theory (Richardson, 1995). How solid a theory can it be?

The nursing back rub is a practice which, unlike therapeutic touch, actually involves touching the patient. Even in healthy adults, a 10 min nursing back rub will increase salivary IgA levels, irrespective of relatively unchanged state anxiety scores (Groër et al., 1994).

Moving from the particular to the general, massage therapy produces several effects, including lower anxiety levels, lower stress hormone (cortisol and noradrenaline) levels, and better mood. The effects are the result of alteration of a number of processes or functions, including: growth and development; pain; attentiveness; stress/anxiety; sleep; neuroendocrine balance; and immune function. The common underlying mechanism seems to be increased parasympathetic activity (Field, 1995).

The medical counterpart of these nursing therapies has been termed mind body medicine (Goleman and Gurin, 1995), while the self-help version is referred to as autogenic training (Kermani, 1992).

Acupuncture, a therapy that is older than Western nursing or medicine, also has effects on immune function. It has been shown to directly enhance both cell mediated and humoral immunity, with different manipulations producing different results. The effect peaks on the fifth day, then returns to normal. Acupuncture also produces analgesia at specific sites, unlike the generalised analgesia induced by stress. The pituitary and the adrenals can also be stimulated directly by acupuncture, releasing ACTH and/or cortisol (Bensoussan, 1991). Persistent repetition of acupuncture treatment leads to lasting changes by a process of supposed physiological relearning (Bensoussan, 1994), perhaps akin to a conditioned immune response.
2.6 Immune networks

"The most characteristic structures of mammalian immune systems are the variable regions of Igs and T-cell receptors (TCRs). The structural diversity of these molecules is greater than that of all the other molecules in the vertebrate body put together....unique to the immune system....refer to such structures as variable region molecules (VRMs”) (Stewart, 1992). The structure and synthesis of VRMs has already been described in terms of the antibody molecule (sections 2.3.1 and 2.3.3.3), the B cell receptor (section 2.3.3.3) and the TCR (section 2.3.5.3). The fact that VRMs recognise each other led to the idea of an idiotypic network (section 2.2) which has been developed further into the concept of second generation immune networks that also account for activity associated with clonal selection. “...a clonal as well as a network organization coexist in the immune system, which derives some of its properties from the complementarity in the characteristics of both” (Coutinho, 1989).

The following description of the immune system is an overview that forms the basis of subsequent discussion.

In a normal, antigen-free immune system, 10-20% of splenic lymphocytes show autonomous activity, while the majority conform to clonal selection (Pereira et al., 1986). Autoreactivity (autoimmunity) is evident and is physiologically normal (Guilbert, Dighiero and Avrameas, 1982). However, some autoreactive clones are eliminated (sections 2.3.3.1 and 2.3.5.1). Thus, autoreactivity should be considered with respect to global lymphocyte connectivity with all the molecular patterns (somatic and immune) available to the immune system (Stewart, Varela and Coutinho, 1989; Vaz, Martinez-A. and Coutinho, 1984). Lymphocyte activity is
characterised by three partly overlapping bell-shaped response curves to receptor occupancy (with high dose inhibition). One curve describes low level interactions that determine lymphocyte survival in the resting state. A second curve describes intermediate values that determine the expression of effector functions and proliferation. The third curve depicts the highest values that determine proliferation with no effector function (Coutinho, 1989). Lymphocyte activity is facilitated by high turnover rates (Freitas, Rocha and Coutinho, 1986) of both B cells (section 2.3.3.1) and T cells (section 2.3.5.1).

The immunoglobulins of a newborn are produced by B-1 B cells (section 2.3.3.2); they exhibit high level connectivity but are not very autoreactive or multireactive (Holmberg, Ivars and Coutinho, 1984; Kearney, Vakil, and Nicholson, 1987). They initiate the network, then become self-tolerant (Holmberg et al., 1986). They are also mitogenic for other B cells. Consequently, the network determines its own metadynamics by preselecting the generation of available repertoires (Davidkova et al., 1997; Vakil et al., 1986). It also contributes to the survival and/or proliferation of B cells. The loop is closed because the levels of autonomous lymphocyte activity correlate with the levels of immunoglobulin connectivity (Varela, Sánchez-Leighton and Coutinho, 1988). Hence, initial development of the immune system is T cell independent (Forni, Heusser and Coutinho, 1988; Malanchere et al., 1995). The proliferation and differentiation of the first T cells selects for threshold affinities and against high affinities to self MHC complexes (section 2.3.5.1). Physiological T cell tolerance is ensured by assertion (positive reactivity) rather than by ignorance (absence of reactivity following deletion) of self (Coutinho and Bandeira, 1989). Finally, VRM repertoire (immunoglobulins, B cell receptors, TCRs) selection contributes to defining the boundaries and fine specificity of that repertoire (Pereira et al., 1989); the converse also happens as the whole process is recursive (Coutinho, 1989).
The result is a network of VRMs produced by activated lymphocytes and all the other molecules available in the internal environment that find VRM complementarities in the individual’s repertoire. This network has structure (connectivity), dynamics (Stewart and Varela, 1989), and metadynamics (cognitive functions) (Varela et al., 1988); it is termed the central immune system (CIS). In addition, there is a set of resting lymphocytes that produce VRMs with reactivities below activation thresholds to all molecular shapes in their environment, including the network VRMs (Coutinho, 1989). The resting lymphocytes, capable of a reflexive, specific clonal response to antigen, constitute the peripheral immune system (PIS) (Huetz et al., 1988).

Having established the outline of the immune network framework, the remainder of section 2.6 deals with the details, and re-visits and re-examines earlier topics that have been examined from within the clonal selection/defence framework. Specificity and connectivity (section 2.6.1) are the crux of the network which, in turn, provides for autoimmunity and tolerance (section 2.6.2) as normal, active processes. Emergent characteristics of the functioning network include immunological identity and memory (section 2.6.3). The network perspective provides for different interpretations of both the ontogeny and the evolution of the (mammalian) immune system (section 2.6.4). A more holistic view of the immune network is one in which the dynamics and metadynamics are considered (section 2.6.5). Finally, interactions among the immune network and other body systems acquire new meanings in this framework (section 2.6.6).
2.6.1 Specificity and connectivity

If any one characteristic of VRM interactions could be seen as pivotal, it is
costivity, the basis for both the existence and the nature of the interactions. As
VRM interactions are based on recognition, some degree of specificity is involved.
Thus, this more detailed discussion of immune networks begins with a consideration
of these two concepts.

Immunoglobulins from newborn mice that had not been artificially stimulated with
antigens are reactive with a variety of unrelated molecules, with each other, and with
many self components (Berneman, Ternynck and Avrameas, 1992; Dighiero,
Guiibert and Avrameas, 1982; Holmberg et al., 1984; Holmberg, Ivars and Coutinho,
1984). The determinants responsible for the interconnectivity of natural
immunoglobulin resemble those on MHC molecules (Forsgren et al., 1984;
Holmberg et al., 1984). Moreover, this level of multispecificity to self is not found in
Ig of adult mice (Holmberg et al., 1986), but is evident in newborn humans (Ayoubia,
Peltre and Coutinho, 1996; Lydyard et al., 1990). A comparable level of self-directed
activation has also been observed in both CD4 and CD8 T cells (Bereta, Ermonval
and Larsson, 1986; Bianchi, Bril and Benner, 1983; Pereira et al., 1985, 1986; Sredni
and Schwartz, 1980). Hence, in the neonate, there is an endogenous network of
immunoglobulins and T cells involved in spontaneous immunological activity, best
described as degenerate polyreactivity (multispecificity) (Vaz, 1996). This
multispecificity is of two kinds - on the one hand, a combining site on an
immunoglobulin molecule can bind to a range of antigens, with different degrees of
affinity; on the other hand, an immunoglobulin can display several different
combining sites (Hajela and Lee, 1996; Jerne, 1984).
A neonatal immune system as described above has components that inherently carry the potential to generate internal connectivity. Significantly, the "stimulus" for the initiation and maintenance of these connections is the organism itself, since the immunoglobulins produced react with each other and other self components (Vaz and de Faria, 1990).

The meaning of this type of immune network connectivity has been explored in considerable detail by Stewart and Varela (1989) with computer simulations based on real-life affinity matrices. They found that the physical range of connectivities (based on stereochemistry) gives rise to a matrix that then facilitates immunological behaviours including recognition, memory and tolerance. The behaviours are connected, non-linear and fluctuate according to the degree of connectivity, but do not collapse or explode.

If a silent clone couples with its external antigen, clones with which it has affinity, and which are the "internal image" of the antigen, are activated and persist even when the perturbation falls to zero; this persistence is a form of dynamic short term immunological memory. Antigenic coupling to a poorly connected clone leads to explosive growth of the clone. Coupling to a well connected clone that retains its specificity will decrease its activity, leading to a type of active, maintained tolerance.

These simulation-based findings led Stewart and Varela (1989) to propose the existence of at least four categories of B cell clones, some of which have subsequently been observed (Kazatchkine and Coutinho, 1993; Mouthon et al., 1995a; Nobrega et al., 1993). The first category is a multi-affinity group containing all the auto-affinities and affinities for most other Igs; this is the group that starts and maintains the immune system. The second and third categories are two mirror groups with no within-group but only between-group affinities; they exhibit cyclic
fluctuations in autonomous dynamics and are centrally involved in emergent properties such as memory, tolerance and cognitive activity. These three categories are elements of the central immune system. The fourth category is a group with low affinities, only with clones of the first group; this group may not be connected to the network. It is a component of the peripheral immune system.

Subsequent mathematical models of immune networks that explored connectivity (Stewart and Varela, 1991) relied on the concept of "shape space" (Perelson and Oster, 1979), based on the assumption that a specified list of parameters can locate an individual molecule in an objective, referential shape space. The usefulness of this concept has been questioned more recently, however. Shape complementarity cannot adequately account for the specificity of idiotypic interactions (Carneiro and Stewart, 1994), and the original real affinity matrices (Stewart and Varela, 1989) may prove to be no less useful than theoretical shape space (B-Rao and Stewart, 1996).

### 2.6.2 Autoimmunity and tolerance

Normal, antigenically-unstimulated individuals usually carry natural IgM, IgG and IgA auto-antibodies (Berneman, Ternynck and Avrameas, 1992; Lacroix-Desmazes et al., 1995; Mouton et al., 1995a; Nobrega et al., 1993; Ronda et al., 1994). Encoded by germline genes with very few mutations, natural auto-antibodies are characteristically multispecific and do not undergo affinity maturation in normal individuals, resulting in a restricted repertoire that is stable throughout life (Coutinho, Kazatchkine and Avrameas, 1995; Lacroix-Desmazes et al., 1995). Hence, natural autoimmunity, the result of natural tolerance, is a normal state that is quite distinct from autoimmune disease (Willenborg, Staykova and Miyasaka, 1996). In spite of recent theoretical refinements (Lafferty and Gill, 1993), clonal deletion
and anergy (sections 2.3.3.1 and 2.3.5.1) do not adequately account for natural tolerance (Coutinho, 1995b; Coutinho et al., 1992).

T cell mediated natural tolerance is developmentally-acquired, thymus-dependent and established by positive selection of regulatory cells. It is an additional mechanism whose operation precedes both deletion and anergy and that continues into adulthood (Le Douarin et al., 1996; Modigliani, Bandeira and Coutinho, 1996; Modigliani et al., 1995, 1996; Thomas-Vaslin et al., 1995). Thus, normal adults are protected from autoagression by regulatory T cells. They are CD4 T cells characterised by differential levels of expression of CD45 isotypes (Powrie et al., 1994), and by the expression of high levels of IL-4 and IFN-γ and low levels of IL-2 (Coutinho et al., 1994).

An idiotypic network of B cells and immunoglobulins will spontaneously generate natural tolerance to auto-antigens, if the level of connectivity is appropriate (Calenbuhr et al., 1995). The repertoire will then organise itself so that most auto-antibodies are included within the network (Takumi and De Boer, 1996), following V region dependent selection (Viale et al., 1993). At the start of development, newly formed B cells in the bone marrow make up the potential repertoire; they mature into the peripheral, immunocompetent, resting B cells of the available repertoire; the actual repertoire consists of high-rate immunoglobulin-secreting B cells (section 2.2). The repertoires in normal adults are the result of continuous processes of positive and negative B cell selection, mediated by the self molecular environment. Thus, the potential repertoire exhibits the initial high level of self-multireactivity, but extensive deletion (by IgM-receptor ligation) eliminates some 50% of all B cells, predominantly those that are multireactive. Of the 50% that became the available repertoire, the vast majority (about 49%) are deleted by apoptosis. However, because selection proceeds in the absence of antigen, the 1% of B cells that become the actual repertoire are predominantly self-related. Those that are multireactive, and so
participate in the generation of tolerance, had not been positively selected but are the clones that have escaped inactivation or deletion (Grandien et al., 1994a,b).

Having established that autoimmunity is a natural state and that self-tolerance is an active process in an immune network, the next step is to examine autoimmune disease (section 2.3.8.2). It is instructive, however, to pause and consider explanations of autoimmune disease that invoke linear idiotypic cascades rather than a true network. They either refer to this “perplexing enigma” in defeat (George, Levy and Shoenfeld, 1996), or invoke a viral interaction in an obscure explanation that seems to amount to an antigen-driven change in connectivity (Kennedy, 1995).

In the context of immune network theory, however, autoimmune disease can result from reduced connectivity in the central immune system (Arala-Chaves et al., 1994; Dietrich et al., 1993; Lacroix-Desmazes et al., 1996; Vilanova et al., 1994), disruption of the B cell repertoires (Arala-Chaves et al., 1992; Forsgren et al., 1991; Ronda et al., 1994), or T cell mediated autoimmune disease under the influence of natural antibodies (Andersson et al., 1994).

2.6.3 Immunological identity and memory

At the most basic level, the immunological self refers to the autonomous activity of the central immune system which persists in the absence of environmental contacts (Coutinho et al., 1984). In this respect, the immune system, especially the central immune system, can be described as a closed network of processes of cellular and molecular production. It is organised in such a way that it regenerates the processes that produced its components. At the same time it continually undergoes structural changes triggered by interactions with the environment. Significantly, the outcome of the changes is determined by the structure of the system at the time, and not by
the structure of external interacting elements (Vaz and Carvalho, 1993).

Therefore, interaction with antigens does not alter the organisation of the immune system. It may alter structure, however, by selecting components that may then follow a different outcome in the individual. Essentially, these alterations are no different from changes brought about by the constant turnover of components of the immune system. Thus, the identity of the immune system consists of its history to date, a sequence of structural modifications. It is defined by the collection of lymphocyte clones that are activated at that time, the actual repertoire (Capra, 1996; Coombe, Ey and Jenkin, 1984; Kazatchkine and Coutinho, 1993; Mouthon et al., 1995a; Nobrega et al., 1993; Tauber, 1991, 1994; Vaz and de Faria, 1990; Vaz, Martinez-A. and Coutinho, 1984; section 2.2).

Although individual components of the immune system do not separately “know” about anything, the network that is the actual repertoire has, as one of its emergent characteristics, a cognitive domain. That is, the central immune system, as a system, has a domain of potential interactions in which it can sensibly engage. In this way, identity and cognitive function are related dynamic characteristics of the immune network (Capra, 1996; Varela et al., 1988; Vaz and Varela, 1978). Thus, the immune system is an autonomous (auto Poietic; Mingers, 1991) cognitive network (Joshi, 1996) that is responsible for molecular identity.

The significance of a retained memory of a previous encounter with an antigen lies in the ability to mount a faster, more effective subsequent response, so that immunological memory amounts to vaccination. This idea has already been explored in the context of the clonal selection framework (sections 2.3.3.2 and 2.3.7.2).

Early speculation about memory in the context of immune networks suggested a distinction between static memory, residing in resting, long-lived memory B cells,
and dynamic memory, an emergent property of network activity (Perelson, 1989; Stewart and Varela, 1989). This distinction may be inappropriate. At one level, memory is a clonal property since memory B cells are functionally disconnected from the network. At another level, however, the persistence of memory may be a network-dependent, cognitive activity since, of all the disconnected clones participating in a primary response, the only ones that don't die after antigen clearance are those with minimal affinity to self - the memory B cells (Chowdhury, Deshpande and Stauffer, 1994; Coutinho, 1989; Hsu, Sercarz and Miller, 1989).

In order to investigate memory capacity, a model immune network has been proposed, with the following characteristics. Responses to antigens are localised so that unrelated antigens elicit uncorrelated responses; memory about one antigen does not influence the cell populations responsible for maintenance of memory of other antigens. When network connectivity is low, memory capacity is proportional to the square root of the maximum number of clones. Exceeding that capacity leads to memory loss and/or failure to vaccinate (Weisbuch and Oprea, 1994). When network connectivity is random and affinity is distributed continuously, memory capacity is essentially zero (Boutet de Monvel and Martin, 1995). Thus, the most important factors to influence network memory are affinity and connectivity (Joshi, 1995).

### 2.6.4 Ontogeny and evolution

The nature of the operations of the immune network develops over time, so much of the broader picture of the ontogeny of the immune system has already been dealt with. Ontogeny with respect to the clonal selection/defence framework is discussed in section 2.4.2, while aspects of immune network development are covered in sections 2.6 and 2.6.1. Therefore, the following information is largely additional material, presented from within the immune network framework.
The transfer of maternal immunoglobulins, either through the placenta or by lactation, exerts a significant influence on the active development of the new immune system (Vaz and de Faria, 1990). Since the transferred immunoglobulins react both with each other and with the immunoglobulins appearing for the first time in the newborn (Holmberg, Ivars and Coutinho, 1984; Holmberg et al., 1986; Vakil and Kearney, 1986), the initial conditions for network development are being set. This is quite different from the passive transfer of protective anti-infectious antibodies. Thus, maternal antibodies add another layer of connectivity on the developing immune system, conveying the immunological history of contemporaneous adults of the species (Cooper-Willis et al., 1985; Kindred and Roelands, 1974; Martinez-A. et al., 1986; Vaz and de Faria, 1990). The neonatal immunoglobulin repertoire, comprising self-antigens, differentiates in the first years of life and remains relatively constant thereafter (Mouthon et al., 1995b, 1996).

At the other end of the ontogenetic spectrum, the increase in autoreactive immunoglobulins seen in old age is due to an increase in specificity of pre-existing autoreactive immunoglobulins, resulting from heavy selection from among the small number of B cells expressing autoreactive immunoglobulins (Grandien, Coutinho and Andersson, 1993; Gueret et al., 1993; Nobrega et al., 1996). Old age is also accompanied by progressive immunodeficiency due to less diversified antibody repertoires because of reduced precursor B cell production and increased peripheral selection (Viale et al., 1994).

The evolution of the immune system has already been discussed (section 2.4.3), in a manner consistent with the defence metaphor. The immune network perspective alters that picture in a small but significant way. The primordial function of VRMs may not have been defence against external attack, since the evolutionarily most primitive VRM is likely to have been the γδ TCR (section 2.3.5.2). The first VRMs
functioned as membrane-bound receptors, enabling lymphocytes to interact with each other and so control their own growth. This contributed to the integration of the internal molecular environment. Subsequently, some VRMs (the peripheral immune system) were redeployed to trigger pre-existing defence mechanisms, to convert an innate response into an adaptive one (Stewart, 1992; Stewart and Coutinho, 1996).

2.6.5 Dynamics and metadynamics

The immune system has a structure, it exhibits dynamic patterns and has a metadynamic level of operation (section 2.6). The network is structured around matrices of connectivity of VRMs, B cell receptors and TCRs. Network dynamics relate to the changes in concentrations of the structural elements over time, and the factors involved in those changes. The continuous production of novel VRMs, together with the high lymphocyte turnover rate, leads to constant changes in the dynamics of the system, resulting in network metadynamics. Thus, emergent properties, such as cognition, tolerance and memory are products of the metadynamics of the system. Many of these have already been discussed. Therefore, this section is concerned with aspects of dynamics and metadynamics that may not fall easily into the topics already covered. It concludes with a consideration of two global models of the immune system.

Until recently, the majority of the mathematical models of immune function dealt only with interactions involving immunoglobulins and B cells. The underlying assumption has been that T cell activity is not limiting. Models have dealt with such things as methodology, simplifying assumptions, immune/tolerant states, and issues of chaos.
The replicator equation has been shown to be useful for studying antigen-B cell networks (Stadler, Schuster and Perelson, 1994), while a refinement of the bell-shaped function for idiotypic interactions (section 2.6) has called into question results obtained with earlier versions (De Boer et al., 1996). The limitations of the shape space model (section 2.6.1) have been approached by substituting the random graph model, and by developing an improvement termed “continuous shape-space” (Noest, Takumi and De Boer, 1997).

Various simplifying assumptions have been challenged. For instance, in B cell activation by ligand-induced immunoglobulin cross-linking, activation functions might not be identical for all ligands (Faro and Velasco, 1993). Local rules may need to be more generalised and based on good experimental evidence (Faro, Carneiro and Velasco, 1997). Indeed, the assumption that T cell functions are not limiting has also been questioned (Detours et al., 1994).

The stability of immune and/or tolerant localised states has been shown to depend on the ratio of antibody to B cell lifetimes and on the rate of antibody complex removal (Anderson, Neumann and Perelson, 1993). The immune state loses stability if B cell and antibody concentrations change on different time scales (De Boer, Perelson and Kevrekidis, 1993a). Tolerance is only achieved if a stable fixed dynamic and metadynamic point exists (Detours et al., 1994) and with large-scale production of anti-idiotypic (Sulzer and Weisbuch, 1995). At high rates of antibody complex removal, sustained oscillatory/chaotic behaviour persists (De Boer, Perelson and Kevrekidis, 1993b). In fact, the entire idiotypic network system has been described as either chaotic (Hirayama, Nishimura and Fukuyama, 1996) or on the edge of chaos (Bernardes and Zorzenon dos Santos, 1997).
In a relatively rare instance of a model of T cell function, it appears that the key feature underlying the regulation of CD4 T cell differentiation pathways is the population dynamics of the lymphocytes themselves (Carneiro et al., 1995).

The severity of the limitations of models that deal only with immunoglobulins and B cells has been demonstrated convincingly by the appearance of a model that includes T cells. In this model, which consists of a series of differential equations, B cell activation is dependent on T cell help and immunoglobulin-TCR engagement down-regulates activated T cells. The resulting normal, non-immunised immune system exhibits one of two modes, depending on the degree of idiotypic connectivity. Low connectivity produces the immune response mode in which T and B cell clones grow exponentially. High connectivity produces the tolerant mode in which T cell clones are controlled by inclusion of all TCRs in the repertoire of an idiotypic B cell network (Carneiro et al., 1996a, b).

The model can also simulate metadynamic recruitment of T cells from the thymus and B cells from the bone marrow. The CIS and PIS modes are able to co-exist in the immune system that develops over time. This happens when enough founder antigens couple in a tolerant mode, so that isolated antigens that are first presented once development is complete will then couple in an immune response mode. This is the first model of a possible mechanism that could give rise to the CIS/PIS distinction as an emergent, self-organising property of the immune system. Furthermore, self/nonself now becomes CIS/PIS engagement (Carneiro, Coutinho and Stewart, 1996a, b).

The second model to be discussed here is, in some ways, a major departure from the model of Carneiro and colleagues and, indeed, from the immune network framework which it seems to fail to acknowledge altogether. Booth and Ashbridge (1992a, b;
have developed a descriptive model, a theory concerning psychoimmune relationships. Their formal proposal states:

"that the relationships among the psychological, neurological, and immunological levels of a living individual can be better explained and understood through a unifying principle of "teleological coherence" as defined in the following two premises:

# The relationships/interactions between psychoneural and immune systems arise as a consequence of the shared goal to establish and maintain a self-identity.

# The nature of these relationships is governed by the necessity for "harmony of purpose", or coherence, among all levels of the organism involved in establishing and maintaining a self-identity." (Booth and Ashbridge, 1993b).

This amounts to a model of immune structure and function based on the process of organismic self determination that is also embedded in the context of autopoiesis. It also asserts that the immune self and the psychological self are mutually generative domains that are not independent constructs. However, Booth and Ashbridge caution that mutual influence does not necessarily mean direct correspondence between processes at different levels of the system hierarchy, but rather symbolic or isomorphic (relating to parallel characteristics) correspondence.

The exploration by Booth and Ashbridge of the consequences of teleological coherence crystallised into the following postulates:

"1. A model in which immunological and psychological self-determination processes are interdependent, mutually generative, and directed by the requirement for teleological coherence implies that psychological variables are not a class of separate entities that may influence immune behavior but instead may be integral components of various modules of the immune system.

2. The coherent experience of illness at all levels of the hierarchy, not just at the immunological level, may be necessary for the development of a coherent
psychoimmune self-image.

3. Immune responses can be considered as factors in the overall encoding of memory, learning, and behavior, and so there should be situations where the nature of an immune response is radically affected by the psychosocial state of the individual.

4. Disturbances of the self/nonself boundaries of one system (immunological, psychological, or social) may affect the boundary determinations of other systems in various ways, depending on such factors as the nature of the disturbance and age of the person.

5. Psychologically associated immune changes should be buffered by "coherent", "sturdy", or "engrained" psychological self-image.

6. A corollary of the previous postulate is that appropriate cognitive-behavioral therapies may alter the course of chronic immunological disorders by promoting a harmonization of the psychoimmune self-image and by recreating a coherent sense of purpose or meaning in a person's life in relation to his/her situation and to other people." (Booth and Ashbridge, 1993b).

Out of the published reactions to this theory, the two that are perhaps the most enlightening come from diametrically opposite positions. Langman and Cohn (1993) argue that Booth and Ashbridge are entirely wrong. Their criticism is couched in purely reductionistic terms, precisely the characteristic that Booth and Ashbridge identify as a major weakness of PNI to date (section 2.3.10). Perhaps this exchange confirms the limitations of a purely reductionist approach.

von Uexkull, Geigges and Herrmann (1993), on the other hand, argue that teleological coherence characterises all living systems. In doing so, however, they explicitly equate teleological coherence with self organisation, and harmony of purpose with emergence. This inadvertently removes what could be seen as a major weakness in the theory by replacing intentionality with consequence, a biologically
more acceptable concept. Therefore, if Booth and Ashbridge were to talk about self organisation instead of teleological coherence, and emergence instead of harmony of purpose, the substance of their theory would not necessarily be diminished. They would also establish the (currently hidden) connections between their model and the immune network framework (sections 2.3.6.1 and 2.3.6.3). Interestingly, another common point is their reference to degrees of connectedness (or coherence) in the context of health and illness (section 2.6.1). Coherence has also appeared as a characteristic of the integrated internal molecular environment mediated by VRMs (Stewart, 1992). Finally, the “teleological coherence” of Booth and Ashbridge may well have isomorphic correspondence to the “sense of coherence” of Antonovsky (1979; section 2.5.1).

2.6.6 Immune network interactions

The idea that tolerance and immunisation are two aspects of essentially the same process (sections 2.6.2 and 2.6.3) can be verified by examining interactions between the immune network and the digestive system (Vaz, 1979). The interactions are significant from the beginning of life, when the maternal influence continues beyond the intra-uterine period (section 2.6.4). Lactation is important in the development of gut associated lymphoid tissue in the newborn both directly, due to the nature of dietary antigens, and indirectly, because of the nature of the bacterial flora being added to the gut (Vaz and de Faria, 1990).

Indeed, the significance of the interaction, which is mediated by the CNS (Berin and Perdue, 1997) and a local paracrine/autocrine hormonal network (Shanahan, 1997; Wang, Whetsell and Klein, 1997), cannot be overestimated, for several reasons. The gut mucosa is the largest area of a mammal that is exposed to its environment. In addition, processing and presentation of peptides to T cells can be thought of as
digestive activities (Vaz and Carvalho, 1994). Finally, in the absence of gut bacterial immunostimulation (Bocci, 1992), dietary macromolecules are responsible for most lymphocyte activity in the gut (Vaz and Carvalho, 1994). That activity can either lead to immunisation, as in the development of CTL-mediated autoimmune diabetes (Blanas et al., 1996), or tolerance, which can prevent hypersensitivity reactions in the gut (Mowat, 1987).

Although the phenomenon of oral tolerance can be predicted by immune network theory in terms of changes in connectivity (sections 2.6.1 and 2.6.2), the mechanisms producing it cannot. Orally administered antigen has been shown to induce tolerance not only by active suppression and clonal anergy, but also by extrathymic deletion of antigen-reactive CD4 T cells. Thus, multiple mechanisms of oral tolerance have evolved to prevent the host from mounting a harmful immune response to ingested antigen (or peptide/protein nutrient). The mechanism that is triggered depends on the amount of antigen encountered by the mucosal immune system (Chen et al., 1995b). Could this be an explanation for the differential recognition of foreign-familiar and foreign-foreign (Vaz, Martinez-A. and Coutinho, 1984; section 2.2)?

The immune network view of PNI is also evident in studies of its interactions with the psychoneural system, especially in the context of the Booth and Ashbridge model discussed in section 2.6.5. Although some studies examined the effect of stressful situations, the majority appear to be looking for positive outcomes.

Beginning with stressful situations, while day-to-day psychological load may bear no particular relationship to a range of immunological parameters (Jabaaij et al., 1993), it does add to the immunosuppressive effect of additional experimental stress (Brosschot et al., 1994). Furthermore, psychological stress reduces antibody formation after low-dose hepatitis B vaccination, but not after a higher dose (Jabaaij et al., 1996).
Emotional self-disclosure regarding a traumatic or stressful experience has been shown to have health-enhancing effects. For instance, it can enhance antibody formation following hepatitis B vaccination (Petrie et al., 1995). It can lead to increased NK cell activity, which is then further enhanced if the subject displays a high level of cynical hostility (Christensen et al., 1996). Its health-promoting effects may include buffering of day-to-day immune variations (Booth, Petrie and Pennebaker, 1997).

Mood can also buffer immune activity. While negative mood correlates with lower levels of NK cell activity, positive mood following earlier negative mood buffers the earlier effect so that NK cell activity increases (Valdimarsdottir and Bovbjerg, 1997). Mood has a significant modifying effect on the type I hypersensitivity skin reaction. Specifically, the more lively the subject, the smaller their allergic response (Laidlaw, Booth and Large, 1994). Skin sensitivity to histamine could also be reduced by a cognitive-hypnotic technique (Laidlaw, Booth and Large, 1996). The general theme of these studies is that there is a "harmony of purpose" relating immunological and psychological behaviour, whereby creating a more robust psychology has the effect of strengthening the immunology (Booth, Petrie and Pennebaker, 1997). Would it not be more in keeping with immune network theory to suggest that a psychological improvement enhances self organisation so that immunological improvement becomes an emergent property?

2.7 Conclusion

This review of the literature has shown that the immune system consists of the central immune system and the peripheral immune system. For historical reasons, the ascendency of the clonal selection/defence framework has meant that a
considerable amount of detailed information has been discovered concerning the peripheral immune system. Significantly less is known about the central immune system, most being at the descriptive or theoretical levels. The discrepancy is reflected in the information presented in this review. An understanding of the interactions between the immune system and other systems in the organism indicates the connectedness of the systems into a whole organism. Finally, autopoiesis has been shown to be a valuable integrating concept for the interactions within the organism. Consequently, the following speculative summary statement can be made.

The network of interactions within an autopoietic organism consists of a series of connected sub-networks, such as the immune system, the nervous system and the endocrine system. Each sub-network can be identified by a group of interactions with a level of mutual connectivity that is greater than connectivity with other elements of the organismic network. The activities of each sub-network generate emergent characteristics that are identifiable with that system. Thus, the emergent characteristics of the immune system include molecular identity (the result of a history of structural coupling), a cognitive domain of action, memory and tolerance. The greater network that is the organism also has higher order emergent characteristics that identify a particular individual of a particular gender of a particular species, and so on.

Therefore, concurrently with the immune response, the organism is maintaining homeostasis and autopoietic processes by means of the activity of other sub-networks reflected in such things as behaviour and endocrine responses. Assuming that the sub-networks are not operating independently, then, how much and what kind of connectivity is there between them? The existence of the connectivity is evident in the PNI literature, most of which (section 2.5) does not operate within the network/autopoiesis paradigm; a relatively small number of recent studies (section 2.6.6) acknowledge network biology. Nevertheless, concurrent changes have been
observed in a variety of variables that could be said to represent aspects of different sub-networks. In addition, changes representing one system (sub-network) have been documented following the imposition of a stressor (perturbation) known to result in a response (compensation) from another system (sub-network).

It can be seen, therefore, that PNI investigations can be approached differently if the network/autopoiesis paradigm is adopted. Indeed, even if the same kind of experimental design as used by others is adopted, it may be possible to delve deeper into an understanding of an integrated homeostatic system by viewing it as organisationally closed, as a network of sub-networks. It should be acknowledged, however, that it is difficult to measure the organisation as a whole, so that selecting variables whose changes can be interpreted holistically becomes important. It may then be possible to apply different treatments and measure different variables that are more likely to reflect the organisation as a whole, for example, concentrating on the components of the CIS. Thus, it might not be possible to test the network/autopoiesis idea experimentally in its entirety; rather, one is looking for evidence that could either strengthen or weaken it.

Bearing in mind that each sub-network (like the entire network) may operate self-referentially so that external influences are perturbations to which it compensates and that the sub-networks may be more or less closely inter-connected in achieving this, the following research questions form the basis of this investigation:

(1) are there immune changes that accompany endocrine and psychoneural changes in response to a perturbation and, if so, what trend is indicated - does it suggest a connection between immune and psychoneural systems?

(2) are these changes influenced by other factors and, if so, does the relationship suggest a connection between immune and psychoneural systems?
2.8 Experimental plan

Broadly speaking, testing the research questions posed in this investigation requires situations in which: changes can be observed in endocrine, immune and psychoneural systems in response to a stressor (a perturbation); possible connections among the changes can be sought; and the influence of other factors on one or more systems is likely and may further elucidate possible connections. The first step is to establish and explore an animal model in which the stressor is known to have adverse effects. This is then to be followed by a human situation that is believed to be stressful, but with the expectation that the effects of the stress can be overcome, at least by most participants.

In this way, the effects of the stressor on the endocrine, immune and psychoneural systems can be monitored so that possible connections may emerge. In addition, the differences in the expected effects of the stressors may generate different outcomes that could shed light on the research questions. Finally, varying the length of the treatment on the sheep, as well as comparing the humans who overcome their stressor with those who don’t, also has the potential to lead to different outcomes, the differences being amenable to analysis.

In both instances, stress can be monitored by measuring cortisol levels since, regardless of the nature of a stressor, the physiological response is non-specific activation of the HPA axis leading to an increase in circulating cortisol, a phenomenon termed the general adaptation syndrome by Selye (section 2.5.1).

The psychoneural status of sheep is perhaps most easily monitored by measuring changes in behaviour. Therefore, a pilot study of a stressful situation will hopefully establish or confirm, perhaps even refine, a protocol that will yield meaningful behaviours. The outcome will then inform a definitive experiment. By contrast,
measuring the psychoneural status of humans is comparatively straightforward so an array of standard psychological tests for both state and trait characteristics will be used.

The methodology associated with measurements of both endocrine and psychoneural function sits easily in either PNI paradigm - the clonal selection/defence paradigm and the network/autocephaly paradigm. However, the influence of the paradigm is most significant in immunological aspects of the experimental plan. Therefore, this is the system that should be approached in ways that are consistent with the network/autocephaly paradigm. In particular, the CIS is said to show network organisation; it is part of the autopoietic organisation and function of the whole organism. Therefore, theoretically, changes in the state of the CIS would be reflected in changes in the degree of connectivity among the variable region molecules (VRMs - section 2.6).

The connectivity of the CIS can be defined by a matrix of affinities between all possible pairs of VRMs (Stewart and Varela, 1989). The process of determining affinities is technically difficult and extremely time consuming, so that a limited number of studies using only a limited number of self antigens have examined only the affinities for IgG and IgM (Berneman, Ternynck and Avrameas, 1992). Consequently, later studies have used changes in populations of cells involved in CIS activity as surrogate end points for changes in connectivity (Arana-Chaves et al., 1994; Vilanova et al., 1994). Therefore, it would be reasonable to adopt a similar enumerative approach in the present investigation, especially in view of the substantial number of published enumerative studies (for example: Benschop et al., 1994; Booth, Petrie and Pennebaker, 1997; Boyce et al., 1993, 1995; Brosschot et al., 1992; Cacioppo et al., 1995; Kiecolt-Glaser et al., 1993; Lekander et al., 1997; Naliboff et al., 1991; Olff et al., 1995; Solomon et al., 1997). At the same time, given their widespread use in PNI studies, functional measures of the immune
system had to be considered as well.

Functional measures would appear to be predominantly predicated on observing functional responses (by the PIS) to antigenic challenge, or else are elicited by antigenic challenge-based assays that clearly reflect a PIS response in the assay. Thus, although functional measures may be technically more precise than cell counts they would also seem to be narrower in the scope of the aspects of immune function they reveal. They are also consistent with the clonal selection/defence paradigm. Indeed, the reliability of lymphocyte proliferation assays has been questioned, in the context of PNI (Fillion et al., 1994). Finally, the relationship between lymphocyte proliferation and changes in lymphocyte populations is far from clear (Bachen et al., 1992). Therefore, on balance, the decision was made to study the immune system in this investigation by measuring changes in populations of circulating lymphocytes.

The enumerative approach allows the observations to be based on non-antigenic perturbations. Thus, while immune system changes would have resulted from a composite of CIS and PIS activity, they could reasonably be assumed to be largely the work of the CIS. Furthermore, the usefulness of counting circulating lymphocytes has been confirmed by a recent study of the response to a non-antigenic challenge (Booth, Petrie and Pennebaker, 1997), in which no other blood cell populations showed any changes. Therefore, the lymphocytes being counted in this investigation include T cells (only in the sheep study) and B cells (two of the three categories of VRMs, together with Ig), as well as NK cells, whose activity is not clonally selected.
CHAPTER 3

MATERIALS AND METHODS

The initial experiment, termed the confinement stress study in the present Chapter, enabled the development of a suitable methodology which would generate both physiological stress (measurable as cortisol levels) and observable behavioural changes. Both confinement and a startle stimulus were evaluated as potential stressors, while the usefulness of changes in naturalistic behaviour was also examined. This study is reported as Chapter 4.

The confinement stress study led to the more definitive experiment reported as Chapter 5 and termed the isolation stress study in the present Chapter. In that instance, physiological stress, induced by isolating individual sheep, was again measured through cortisol level, behavioural changes were evaluated with the arena test, and the immune response was monitored by counting lymphocyte sub-types in whole blood.

The sheep in these two experiments came from the same flock; some of the methods were common to both studies; other details differed. The similarities and differences are reflected in the detailed descriptions that follow.

A high stress situation with the likelihood that the effects of the stress could be overcome was provided by Army recruit training. That investigation, reported as Chapter 6, is termed the Army recruit study in the present Chapter. In that case, the
stressor was the recruit training process. Once more, cortisol gave an indication of stress and lymphocyte numbers reflected the immune response. The psychoneural element of the response was measured with several standard psychological tests.

3.1 Experimental animals

The sheep were cross-bred Merino non-lactating ewes, drawn from a flock in a paddock at Elizabeth Macarthur Agricultural Institute (EMAI). As far as could be ascertained, they had not been the subjects of previous studies or experiments. During the study the sheep were housed indoors and were provided with ad lib. drinking water and pelleted food which were checked daily. Food consisted of Monaro Special High Fibre Stock Mix, a complete, all purpose, fibre-based feed (15% crude protein, 3% crude fat, 27% crude fibre, 0.5% salt, 10.2 MJ.kg⁻¹).

At the start of each experiment, the appropriate number of sheep was taken from the flock. The wool on their necks was shorn to facilitate blood collection by jugular venepuncture and identifying numbers were sprayed on them with scorable branding dye. Blood was collected in EDTA using 10 mL vacutainers and 18G needles.

3.2 Plasma cortisol determination in sheep

The blood was centrifuged at 2000 g for 15 min, immediately after collection, and the plasma pipetted into sterile 5 mL sample tubes and frozen until required for cortisol assay.
Plasma cortisol was measured by radioimmunoassay, using the method supplied with the Orion Diagnostica Cortisol [125I] Radioimmunoassay Kit. Radiation in the pellets produced by the assay process was measured for 1 min using an LKB Gamma Counter connected to a computer and printer with RIA software. Standard curves and results (in nmol.L\(^{-1}\)) were printed automatically.

### 3.3 Experimental design - confinement stress study

Twenty sheep were taken from the flock, their necks were shorn and each was numbered on both sides of the body. Throughout the six weeks of the experiment the sheep were housed in an animal house containing other animals (sheep, cattle, goats) and with daily human activity. For the first two weeks they were kept together in a pen measuring 4.5 m x 4.7 m. They were then divided into two groups of 10 sheep each (groups A and B); sheep were allocated so that the groups contained similar mixes of sheep, matched for height at the shoulder.

The two groups spent the remaining four weeks in different animal houses, with sheep housed individually in adjoining pens measuring either 1.0 m x 1.5 m (group A) or 1.2 m x 1.6 m (group B). During weeks three and four, the two groups received the same treatment - physical separation from conspecifics, which did not prevent visual, olfactory or auditory communication. The treatment continued through weeks five and six for group A. Group B received an additional treatment during weeks five and six. They were subjected daily to a stimulus which evoked a startle response.
Behaviour was recorded for one hour from 0900 h every day on videotape, to be watched and scanned sampled at a later date. Blood samples were collected on 21 of the 40 days of the study, at 0900 h for group A and 0915 h for group B, for determination of plasma cortisol levels.

3.4 Startle stimulus - confinement stress study

A 55 L brown plastic garbage bin was connected to a rope leading up to the inside of the animal house roof, then away and down to a location at some distance from the pens housing the group B sheep. The following procedure was carried out on the mornings of sampling days in weeks five and six.

The video camera was switched on. The garbage bin, which was standing on the metal mesh floor adjacent to the group B sheep, was raised slowly on its rope as high as possible. The rope was then released, allowing the bin to fall quickly and noisily on the mesh floor. Blood samples were collected 15 min after the startle, with video recording continuing for a further 45 min.

3.5 Behavioural observations - confinement stress study

A Panasonic VHS video camera was set up so that the field of view included an entire treatment group of sheep. During weeks one and two, one camera was used to record the behaviour of the single group of 20 sheep. During the remaining four weeks, two cameras were used, one to record group A and the other for group B.
At about the same time each morning (0900 h), the video camera/s was/were switched on immediately after collection of jugular blood samples and switched off one hour later. This routine applied for all treatments in the first four weeks and group A in weeks five and six. The record of the behaviour of group B in weeks five and six included their response to the startle stimulus (15 min), their being blood-sampled, and the subsequent 45 min.

When the videotape was being viewed later, it was stopped at the end of every minute of elapsed time and the number of sheep in each category of behaviour was recorded. The categories of behaviour were:

- head angle - up, down, horizontal
- head orientation - left, right, forward
- activity - lying on the floor, feeding, drinking, mouthing objects.

These categories are not necessarily mutually exclusive. For each category of behaviour, the scores were converted to percentage of time spent in each component of that behaviour, aggregated for each treatment period.

3.6 Experimental design - isolation stress study

At the start of each experiment, eight sheep were taken from the flock, their necks were shorn and each was numbered on the head. Four of the sheep were housed in a group in an animal house containing other animals (sheep, cattle, goats) and with daily human activity. The other four sheep were housed individually in total isolation from other animals, their only contact being a brief daily maintenance visit by an attendant.
A week later, a second group of eight sheep was added to the experiment, their necks were shorn, identifying numbers were applied and they were allocated either to a group of four or to isolation. This was also the start of two weeks of behavioural testing and blood sampling. Thus each replicate experiment lasted three weeks and the treatments were termed three weeks isolation, two weeks isolation, three weeks group, two weeks group (Table 3.1).

Behavioural tests involved observing the sheep in their treatment groups of four in an open field test for 10 min, between 0900 and 1000 h, on days 1-4 and 9-12 from the start of sampling. Jugular vein blood was collected daily on each sampling day between 1000 and 1100 h. The percentage of cells marked for CD4, CD5 and CD8 was determined by flow cytometry. Plasma cortisol was determined by radioimmunoassay.

3.7 Behavioural tests - isolation stress study

Behavioural testing involved observing the sheep in groups of four in a motivational-choice open-field test, termed an arena test (Fell and Shutt, 1989), for 10 min on each sampling day. The groups were tested in a random order. The group was admitted to the arena through a gate at a point midway between the two ends of the rectangular pen and allowed to move around freely inside the arena for this period. Hessian walls and quiet surroundings reduced the chance of any disturbance to the sheep from extraneous stimuli. The floor was concrete and free of any grass or other objects that would distract the attention of the sheep. The arena was 12 m long and 3 m wide and was marked out in 1 m squares. At 15 sec intervals the position of each sheep (the square in which its front legs were situated) was recorded by an observer in a hide above the arena.
Table 3.1

Time spent by sheep in the isolation stress study after they had been taken from pasture and kept indoors for either 12 days (2 week) or 19 days (3 week) either in individual isolation or in pens in groups of four sheep. Sampling days 1 - 4 and 9 - 12 were days 8 - 11 and 16 - 19, respectively, of the experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days in the experiment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Isolated 3 week</td>
<td>*</td>
</tr>
<tr>
<td>Isolated 2 week</td>
<td>#</td>
</tr>
<tr>
<td>Grouped 3 week</td>
<td>*</td>
</tr>
<tr>
<td>Grouped 2 week</td>
<td>#</td>
</tr>
</tbody>
</table>
An approach-avoidance conflict was established by the presence of the remainder of the experimental sheep in a small pen just outside the arena at one end and an unfamiliar human standing quietly inside the arena at that same end. There was clear visibility through the fence between the two groups of sheep, but the width of the arena was such that the test sheep could not approach the other sheep without violating their normal flight distance from the human who stood quietly facing the sheep throughout the test. The sheep’s natural flocking tendency was pitted against their fear of humans (Lynch, Hinch and Adams, 1992) in a motivational-choice situation superimposed on an open-field test (Figure 3.1).

Three behavioural measures were derived from the data collected. These were called: (i) approach distance - the mean distance of individual sheep from the end of the arena where the other sheep and the human stood; (ii) motor activity, or the distance travelled by individual sheep during the test; and (iii) inter-sheep distance, or spread - the mean distance between the sheep for that group throughout the test.

3.8 Lymphocyte assay - isolation stress study

3.8.1 Total white blood cell count

Using freshly collected whole blood, 0.05 mL of blood was added to 1 mL of 2% acetic acid coloured with a few drops of crystal violet or methyl violet. After the red blood cells had lysed, the suspension was run under the coverslip on an Improved Neubauer Haemocytometer. White blood cells were counted with a microscope and converted to cells \( \times 10^9 \text{L}^{-1} \).
Figure 3.1 Plan of arena used to test behaviour in sheep after they had been taken from pasture and kept indoors for either 12 days (2 week) or 19 days (3 week) either in individual isolation or in pens in groups of four sheep.
3.8.2 Lymphocyte histochemistry

Monoclonal antibodies were used to bind to different types of T cells. The antibodies were then fluorescent-labelled. Finally, excess buffer was used to remove unbound antibodies by washing.

100 μL of whole blood was added to each labelled tube, the amount actually added depending on the total white cell count:

<table>
<thead>
<tr>
<th>Cells/L⁻¹</th>
<th>Amount Added (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 x 10⁹</td>
<td>100</td>
</tr>
<tr>
<td>6-10</td>
<td>75</td>
</tr>
<tr>
<td>10-20</td>
<td>50</td>
</tr>
<tr>
<td>20+</td>
<td>25</td>
</tr>
</tbody>
</table>

Monoclonal antibody (mAb), which had been prepared and supplied by Dr. Mal Brandon of the University of Melbourne (with no accompanying details), was added to each tube, as appropriate - anti-sheep CD4 was added to one tube, anti-sheep CD5 was added to the second tube, and anti-sheep CD8 was added to the third tube. The amount added depended on the total white cell count:

<table>
<thead>
<tr>
<th>Cells/L⁻¹</th>
<th>Amount Added (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>50 μL for whole blood</td>
</tr>
<tr>
<td>37</td>
<td>75 μL for whole blood</td>
</tr>
<tr>
<td>50</td>
<td>100 μL for whole blood</td>
</tr>
</tbody>
</table>

Two control tubes were also required - blood only, and blood plus anti-mouse fluorescein isothiocyanate (FITC) only (FITC-conjugated goat F(ab')₂ fragment against mouse IgG, Organon Teknika Corporation).

Each tube was flicked to mix the blood and mAb, then incubated 20 min in the dark in an icebath.
Two mL of PBS/Az (0.1% sodium azide in phosphate-buffered saline, pH 7.5) was then added to each tube and the tubes were centrifuged at 1100 g at 4°C for 5 min. The supernatants were discarded and the process was repeated.

Anti-mouse FITC diluted 1/200 was added to each tube, the amount depending on the amount of whole blood used:

- 25 μL for 50 μL whole blood
- 37 μL for 75 μL whole blood
- 50 μL for 100 μL whole blood

Each tube was flicked to mix, then incubated 20 min in the dark in an icebath.

100 μL of 7-8% formalin in PBS was added to each tube which was mixed and left for 1-2 min. One mL of reverse-osmosis distilled water was added to each tube which was mixed and placed in a 37°C waterbath for 3-5 min. If necessary, the tubes were returned to the waterbath for up to 10 min so that all the red cells had lysed.

Two mL of PBS/Az was added to each tube which was mixed, then centrifuged at 1100 g at 4°C for 5 min. The supernatant was discarded and this last step was repeated.

To each tube was added 0.2 mL of FACS-Fix (1% paraformaldehyde in PBS, Becton Dickinson), the cells were resuspended, then the tubes were covered and stored at 4°C until ready for flow cytometry analysis.
3.8.3 Flow cytometry

The percentages of lymphocytes labelled for CD4 or CD5 or CD8 were determined one to two days after staining using a Becton Dickinson FACScan/LYSYS II system, which is a dual-component flow cytometer. It is comprised of (1) a wet unit that includes a laser excitation source, fluidics, and emission detectors, and (2) a computer with Lysys II software for data acquisition and analysis.

After setting up Lysys II for data acquisition and storage, according to the instrument manufacturer's instructions, each sample was placed on the FACScan uptake stage, its fluid contents were drawn into the laser beam and the emissions were counted. The computer was on-line and converted the count into a FSC (forward scatter)/SSC (side scatter) dot plot which was then stored.

The first step in the analysis phase was to set a gate around the region of interest on the dot plot. This enabled the construction of a FL1/cell number histogram (the FL1 channel detects light emitted at 530nm by FITC as it passes through the 488nm argon laser excitation beam). Visual inspection of the control histograms permitted determination of the region containing positively fluorescent cells. The computer then calculated a range of arithmetic (linear) histogram statistics, including the percentage of lymphocytes carrying the appropriate marker (CD4 or CD5 or CD8).

3.9 Research design - Army recruit study

Recruit training at Kapooka is organised into three stages. Stage 1 (referred to as Red tab) is concerned with developing the soldier's individual skills. Stage 2 (Blue tab) is spent building on stage 1 and developing physical endurance, field skills and
expertise in using weapons. In stage 3 (Gold tab) the acquisition of individual skills and abilities is completed and group skills are developed, both in drill and a major field exercise.

The participants in this study were 55 male Australian Regular Army recruits, drawn from several platoons. Their ages at the start of recruit training ranged from 18 to 25 years. Those who successfully completed recruit training became the control group while those who did not complete the course, for whatever reason, became the study group. Allocation to the respective group was done a posteriori.

The study overlapped the period when the duration of recruit training was reduced from 12 weeks to 10 weeks. However, the content of the three stages of training remained substantially unchanged. The total time spent in training continued to be divided into three roughly equal periods.

The psychological status of the subjects was evaluated using three measures of state characteristics: the Beck Anxiety Inventory - BAI (Beck and Steer, 1993a), the Beck Depression Inventory - BDI (Beck and Steer, 1993b), the Profile of Mood States - POMS (McNair, Lorr and Droppleman, 1992), and one measure of trait characteristics: the Locus of Control of Behaviour Scale - LCB (Craig, Franklin and Andrews, 1984). Physiological stress levels were determined by assaying cortisol concentration in saliva. Immune status was measured by counting white blood cells, B cells, NK cells, CD3 T cells, CD4 T cells and CD8 T cells in blood. The general health of each subject was monitored by keeping a record of their history of infectious episodes.

Blood and saliva samples were collected on four occasions: at medical induction time on arrival at Kapooka and at the conclusion of each of the three stages of training. In order to minimise the effect of diurnal rhythmicity in both endocrine and
immune function (Petrovsky and Harrison, 1997), samples were collected before 0900 h on all occasions. The measures of state characteristics (BAI, BDI, POMS) were administered on the same four occasions, while the measure of trait characteristics (LCB) was applied on the second and fourth occasions only. Soldiers who were about to leave the course prematurely were asked to provide the next samples and measures that would have been collected.

3.10 Psychological measures - Army recruit study

The application of the three measures of state characteristics used in this study is restricted to registered psychologists. Therefore, the tests were delivered directly by the suppliers to the Officer Commanding 17 Psychology Unit at Kapooka, who was a registered psychologist. He administered these tests, analysed the results and sent me the outcome of the analyses.

The LCB Scale, however, is in the public domain. It consists of a 17-item Likert-type scale which, when scored appropriately, measures the extent to which subjects perceive responsibility for their personal behaviour. More importantly, a reduction in LCB score indicates a change in the subject toward internality (increased vulnerability) and an increase or no change in the score suggests a change toward externality (reduced vulnerability). The LCB Scale was also administered by the Officer Commanding 17 Psychology Unit, but sent to me for further analysis.

In general terms, a high score on the LCB Scale indicates externality. Ten of the items relate directly to externality so their scores were added together. The scores for the seven items relating to internality were transposed so that 5 becomes 0, 4 becomes 1, and so on. After transposing these seven items the test was scored by adding the scores for all 17 items.
3.11 Salivary cortisol determination - Army recruit study

Each subject provided their own saliva sample in a disposable sample jar. The sample was collected immediately after waking up (0600 h) on the days of blood collection. The samples were stored frozen at Kapooka, then transported to EMAI where they were centrifuged at 1600 g for 10 min so that cortisol assay could be performed on the clear supernatant fraction.

The assay method was essentially the same as that used to measure cortisol in sheep plasma except that 50 μL of saliva was used for each assay; the method has been described in detail in section 3.2, above.

3.12 Lymphocyte assay - Army recruit study

Blood samples were collected before 0900 h, by the medical and nursing staff at Kapooka. The samples were air freighted as soon as possible from Wagga Wagga to Sydney and delivered directly to the South Western Area Pathology Service (SWAPS) laboratory at Liverpool Hospital.

Differential blood cell counts were performed by SWAPS technical staff using standard human pathology laboratory protocols based on flow cytometry. The method is broadly similar to that described in detail in section 3.8, above, with the following variations.

White cell count (WCC) and percentage of lymphocytes were determined by performing a full blood count using an Abbott Cell-Dyn 3500 instrument.
Lymphocyte subsets were analysed on a Becton Dickinson FACScan instrument using SimulSET software on a HP340 computing system (Hewlett Packard). Lymphocyte subset proportions were expressed both as a percentage of total lymphocytes and as absolute counts calculated from the WCC and % lymphocytes determined by full blood count.

3.13 Record of infections - Army recruit study

The general health of the subjects was evaluated by keeping a running tally of the nature, frequency and duration of infectious episodes. A tally sheet for recording the relevant information was inserted into the medical record of each soldier. Whenever a relevant entry was made in the medical record, it was also entered on the tally sheet. At the end of the study period the tally sheets, identified only by an ID code, were removed by Kapooka Medical Centre staff and sent to me. I had no access to the actual medical records.

3.14 Data analysis and statistics

3.14.1 Confinement stress study

Repeated measures were made on each sheep, making it possible to use statistically powerful within-subject comparisons for data analysis (Dhabar et al., 1995). For comparisons of changes in cortisol levels, repeated measures analysis of variance was used to test for overall significance of changes observed over time. In addition, differences between timepoints were analysed using the paired t test, as a test for significant differences between means; differences between group A and group B cortisol values at each time point were tested for significance using the Student's t
test for dependent measures.

For the behavioural data, one-way analysis of variance was used to test for differences between the three treatment groups (penned sheep in weeks 1 and 2; group A, control confined sheep; group B, startle confined sheep). The Student's t-test was used as a test of significant differences between mean values.

3.14.2 Isolation stress study

There were five separate trials with the same four treatments in a 2x2 factorial combination:

1. Confined in isolation for 19 days (3 week isolated),
2. Confined in isolation for 12 days (2 week isolated),
3. Confined in groups for 19 days (3 week grouped),
4. Confined in groups for 12 days (2 week grouped).

Each treatment group comprised four sheep, resulting in 20 sheep per group over the five replicates.

For statistical analysis the data from the five trials were combined to provide appropriate error terms for significance testing. Looking first at the analysis of inter-sheep distance, or spread, the following procedures were followed.

With the effects of treatments and trials removed, the successive residuals for animals over days 1-4 (called period 1) and 9-12 (called period 2) were found to be significantly correlated; correlations within periods were uniform and averaged 0.41 and 0.72 for periods 1 and 2, respectively. The daily error terms (the trial x treatment "interactions") were also satisfactorily uniform over days within periods, so separate split-plot-in-time analyses were performed within periods with treatments as the
main plots and days as sub-plots. Although the main plot error for period 2 was significantly greater (p<0.05) than that for period 1, the sub-plot errors were almost identical, so an error term pooled from both periods could be used to test sub-plot effects and interactions. Within each period orthogonal polynomial contrasts (linear, quadratic, cubic) were used to assess trends over time. Period effects and the period x treatment interactions were assessed using the sum of the trial x period and trial x treatment x period terms as error.

For the analysis of motor activity (distance travelled) and approach distance, the measures analysed were: (i) the mean and linear trend for days 1-4; (ii) the mean and linear trend for days 9-12; and (iii) the mean of days 1-4 plus 9-12 and the difference between these means. The trial x treatment term on 12 degrees of freedom was used as experimental error.

For the physiological measures, analyses of variance were performed on each measure at day 2 and at day 12 and also for the difference between day 12 and day 2 (the change during the sampling period). The trial x treatment "interaction" term on 9 degrees of freedom was used as the experimental error. The logarithmic (base 10) transformation was applied to the cortisol data.

3.14.3 Army recruit study

Repeated measures were made on each recruit. Hence, within-measure significant differences between baseline and later time points, and between time points, were tested with the paired t test. Within-measure between-group comparisons at particular time points were examined using the Student’s t test. The degree of association between different immune and psychological measures was tested with Spearman rank-order correlations, one for each combination at each sampling point.
CHAPTER 4

PHYSIOLOGICAL AND BEHAVIOURAL RESPONSES OF SHEEP TO CONFINEMENT STRESS

4.1 Introduction

This pilot study is a preliminary investigation designed to establish a suitable animal experimental model. It should permit the exploration of adaptation, or homeostatic responses, following a putative stressful change applied to sheep.

The sheep is "a defenceless, vigilant, tight flocking, visual, wool covered ruminant" (Kilgour, 1976) whose main protection from predators is flocking and flight. The initial movement of a few individuals, usually older sheep, results in the movement of the whole flock. Moreover, a Merino, a member of the most highly gregarious of sheep breeds, tends to respond only as a member of a flock (Lynch, Hinch and Adams, 1992).

As well as providing defence from predation, flocking behaviour is important for the social transmission of information among sheep, especially in connection with food selection (Chapple, Wodzicka-Tomaszewska and Lynch, 1987; Hinch et al., 1987; Key and MacIver, 1980; Thorhallsdottir, Provenza and Balph, 1990). The social structure within a flock is maintained by visual, auditory, olfactory and tactile
mechanisms (Arnold, 1985; Grubb, 1974; Kendrick and Baldwin, 1987; Lynch, Hinch and Adams, 1992; Shillito-Walser, 1978; Shillito-Walser, Walters and Ellison, 1982). “Flocking is so important to the animal that it is not surprising that separation from the group causes distress” (Ryder, 1983). Separation, and the anxiety that it produces, provide a major stimulus to the hypothalamus-pituitary-adrenal axis in sheep that then leads to high plasma levels of cortisol (Lynch, Hinch and Adams, 1992). In addition, visual separation (isolation) can lead to: an increase in heart rate (Baldock, Sibly and Penning, 1988); unpredictable fearful behaviour or abnormal stereotypic behaviour (Lynch, Hinch and Adams, 1992); or generalised psychological stress (Parrott et al., 1987).

Thus, this investigation was undertaken to monitor sheep as they were being subjected to three notionally increasing levels of stress: confinement in an indoor pen of a small group taken from a relatively undisturbed flock; being individually penned within sight and hearing of other sheep (termed “contact separation” in this study); then, subjection to a visually and aurally startling stimulus. Twenty sheep spent two weeks in a group in an indoor pen. Group A (10 of those sheep) then spent four weeks (weeks 3 to 6) in individual adjoining pens in one animal house. Group B (the remaining 10 sheep) were similarly housed in another building where they were also subjected to a daily startle stimulus during weeks 5 and 6 (sections 3.3 and 3.4). Plasma cortisol (section 3.2) and “naturalistic” behaviour were monitored. Grouping behaviours into active and non-active enabled comparisons with earlier work (Fordham et al., 1991; Marsden and Wood-Gush, 1986); head orientation and head angle were also considered as they are recognised agonistic postures in sheep (Stolba et al., 1990).
4.2 Results

4.2.1 Cortisol levels

Cortisol data were available only from 21 days spanning the 40 days of the study (Table 4.1, Figure 4.1), as an attempt was being made to strike a balance between frequency of sampling and unnecessary disturbance of the sheep. There were no significant differences in cortisol levels between groups A and B on 17 occasions. The significant differences on days 15, 16, 18 and 36 did not conform to any consistent pattern; however, the 20 sheep kept together in a pen for 14 days had been dispersed into individual pens on day 15. Subsequent analyses were based on a single combined group.

Using the day 1 cortisol value as a baseline, the reduction in cortisol over time first became significant on day 4 (p < 0.01) and persisted until day 40. No significant changes in cortisol were found following separation of the penned group on day 15 (with reference to a day 12 baseline) and following the initiation of the startle procedure on day 29 (with reference to a day 26 baseline).

4.2.2 Behavioural data

Behavioural comparisons were made between the initial penned group of 20 sheep (weeks 1 and 2) and the two groups of separated sheep in weeks 5 and 6. Group A was the control group and group B was subjected to the startle procedure. Results were analysed with respect to general level of activity, head orientation and head angle (Table 4.2).
Table 4.1

Cortisol levels (mean ± se) in nmol.L\(^{-1}\), with daily sample sizes (n), in 20 sheep sampled on 21 of 40 days. The combined data represent groups A and B treated as one group. Sheep were penned in a group in weeks 1 and 2 and separated from each other in weeks 3 to 6. Group B were subjected to a startle stimulus in weeks 5 and 6.

<table>
<thead>
<tr>
<th>Day</th>
<th>Group A</th>
<th>Group B</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>129.6 ± 4.18 (10)</td>
<td>109.3 ± 3.66 (10)</td>
<td>119.5 ± 2.03 (20)</td>
</tr>
<tr>
<td>2</td>
<td>136.2 ± 5.18 (10)</td>
<td>132.1 ± 4.13 (10)</td>
<td>134.2 ± 2.35 (20)</td>
</tr>
<tr>
<td>3</td>
<td>87.6 ± 2.97 (10)</td>
<td>73.5 ± 3.08 (10)</td>
<td>80.6 ± 1.56 (20)</td>
</tr>
<tr>
<td>4</td>
<td>55.0 ± 5.46 (10)</td>
<td>80.8 ± 3.02 (10)</td>
<td>67.9 ± 2.30 (20)</td>
</tr>
<tr>
<td>5</td>
<td>86.7 ± 3.63 (10)</td>
<td>96.1 ± 4.54 (10)</td>
<td>91.4 ± 2.07 (20)</td>
</tr>
<tr>
<td>8</td>
<td>48.5 ± 2.89 (10)</td>
<td>75.4 ± 4.21 (10)</td>
<td>62.0 ± 1.93 (20)</td>
</tr>
<tr>
<td>12</td>
<td>47.4 ± 5.16 (5)</td>
<td>44.6 ± 4.52 (5)</td>
<td>46.0 ± 2.43 (10)</td>
</tr>
<tr>
<td>15</td>
<td>30.0 ± 5.50 (5)</td>
<td>65.4 ± 6.02 (5)</td>
<td>47.7 ± 3.38 (10)</td>
</tr>
<tr>
<td>16</td>
<td>67.2 ± 3.54 (10)</td>
<td>38.2 ± 2.07 (9)</td>
<td>53.5 ± 1.69 (19)</td>
</tr>
<tr>
<td>17</td>
<td>34.8 ± 1.67 (10)</td>
<td>49.4 ± 2.70 (9)</td>
<td>41.7 ± 1.15 (19)</td>
</tr>
<tr>
<td>18</td>
<td>43.8 ± 2.42 (10)</td>
<td>80.4 ± 2.74 (10)</td>
<td>62.1 ± 1.58 (20)</td>
</tr>
<tr>
<td>19</td>
<td>38.1 ± 2.26 (9)</td>
<td>51.2 ± 3.64 (10)</td>
<td>45.0 ± 1.61 (19)</td>
</tr>
<tr>
<td>22</td>
<td>53.4 ± 2.14 (9)</td>
<td>70.9 ± 2.84 (10)</td>
<td>62.6 ± 1.37 (19)</td>
</tr>
<tr>
<td>26</td>
<td>47.2 ± 4.70 (5)</td>
<td>60.8 ± 14.0 (5)</td>
<td>54.0 ± 5.27 (10)</td>
</tr>
<tr>
<td>29</td>
<td>37.8 ± 3.02 (9)</td>
<td>53.6 ± 3.08 (10)</td>
<td>46.1 ± 1.59 (19)</td>
</tr>
<tr>
<td>30</td>
<td>48.9 ± 2.48 (9)</td>
<td>39.5 ± 1.27 (10)</td>
<td>43.9 ± 0.97 (19)</td>
</tr>
<tr>
<td>31</td>
<td>48.4 ± 2.07 (9)</td>
<td>40.0 ± 1.05 (8)</td>
<td>44.5 ± 0.90 (17)</td>
</tr>
<tr>
<td>32</td>
<td>51.9 ± 2.27 (10)</td>
<td>57.7 ± 3.84 (10)</td>
<td>54.8 ± 1.59 (20)</td>
</tr>
<tr>
<td>33</td>
<td>47.6 ± 2.15 (10)</td>
<td>64.8 ± 4.82 (10)</td>
<td>56.2 ± 1.92 (20)</td>
</tr>
<tr>
<td>36</td>
<td>62.0 ± 3.39 (10)</td>
<td>36.8 ± 1.56 (10)</td>
<td>49.4 ± 1.46 (20)</td>
</tr>
<tr>
<td>40</td>
<td>67.3 ± 5.13 (9)</td>
<td>53.7 ± 3.69 (10)</td>
<td>60.2 ± 2.22 (19)</td>
</tr>
</tbody>
</table>
Figure 4.1 Mean cortisol levels (nmol.L\(^{-1}\)) in 20 sheep over 40 days. Groups A and B are shown separately (Figure 4.1a), then in a combined group (Figure 4.1b).
Table 4.2

Proportion of time (mean ± sd) spent in various behaviours (defined in the text) for penned sheep in weeks 1 and 2 (P), and groups A (separated, control) and B (separated, startle) in weeks 5 and 6. Sample size is 10 pooled daily observations for all groups. Groups are listed in descending order of magnitude to indicate significant pairwise comparisons. Significance of differences is indicated as follow: ***: p<0.01; **: p<0.1; NS: no significant difference in any pairwise comparison.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Group</th>
<th>P</th>
<th>B</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>P</td>
<td>0.957 ± 0.036</td>
<td>0.762 ± 0.076</td>
<td>0.634 ± 0.105</td>
</tr>
<tr>
<td>Orientation</td>
<td>P</td>
<td>0.862 ± 0.036</td>
<td>0.741 ± 0.079</td>
<td>0.639 ± 0.116</td>
</tr>
<tr>
<td>Angle</td>
<td>P</td>
<td>0.816 ± 0.070</td>
<td>0.819 ± 0.054</td>
<td>0.799 ± 0.074</td>
</tr>
</tbody>
</table>
Lying down, feeding, drinking and standing were grouped together as non-active behaviour. All other activity, including such things as: biting/butting the food bin or the pen; butting, or butting towards, another sheep; climbing up the pen structure, was classified as active behaviour. The proportion of time spent in active behaviour was highest in the penned sheep (0.957), followed by group B (0.762), followed by group A (0.634). All the differences were statistically significant (p < 0.01).

Head orientation refers to the head facing either forward or to the left/right of that sheep. The proportion of time spent with the head facing forward was highest in the penned sheep (0.862), followed by group B (0.741), followed by group A (0.639). All the differences were statistically significant (p < 0.1).

Head angle distinguishes between the head held either horizontally or up/down. The proportion of time spent with the head held horizontally was highest in group B (0.819), followed by the penned sheep (0.816), followed by group A (0.799). None of the differences was statistically significant.

4.3 Discussion

The cortisol levels found in this study suggest that the initial removal from the paddock flock was the major stressor for these 20 sheep. From about day 4 onward, they were effectively recovering or, perhaps more correctly, adapting to their new circumstances. Being placed in contact separation appeared to have little effect on the process of adaptation to confinement; neither did the introduction of a stimulus expected to startle the sheep in group B.
A number of factors may have moderated the impact of, first, individual separation, then the startle. The separated sheep were deprived of physical contact, but were able to see, hear and smell each other. This level of partial confinement was being evaluated for its suitability as a stressor. Nevertheless, the sheep continued to be members of a small group, albeit a more dispersed group. In addition, throughout the duration of the study, the sheep were housed in a large shed in the presence of other sheep, cattle, goats and associated human activity. They were therefore subjected to a variety of unexpected and unusual sounds, sights and smells. It may not be a surprise, then, that the “startle” stimulus may not have been particularly startling, at least in terms of provoking an additional cortisol response.

The methodology used in this study imposed no a priori influence on the type of behaviour to be exhibited by the sheep. Thus the resultant behaviour could be described as free-running or even somewhat naturalistic, so that whatever happened was recorded. Observations were then grouped into three potentially useful categories: active/non-active, head orientation, head angle.

A reduction in time spent in active behaviour was seen in the comparison between the penned sheep in weeks 1 and 2 and group A in weeks 5 and 6. Such a change has been termed withdrawal in earlier work with Merino ewes (Done-Currie, Heckler and Wodzicka-Tomaszewska, 1984), with withdrawal lasting up to three weeks (Fordham et al., 1991). In this study, however, the withdrawal persisted for six weeks, but the extent of withdrawal appeared to have been mitigated by the startle procedure (group B).

Sheep prefer to orient themselves away from each other, rather than head to head which is a threatening posture (Lynch, Hinch and Adams, 1992). The changes in head orientation seen in this study suggested that the sheep were becoming
increasingly threatening towards each other, with the startle procedure moderating
the extent of the change.

The head-up angle is seen in rams exerting dominance, while head-down is a
common form of submission, especially in encounters between rams (Lynch, Hinch
and Adams, 1992). It is not surprising, then, that no significant differences were seen
in head angle in these ewes which had come from a flock of ewes.

Separation of 20 sheep from their flock had a short-term effect on their cortisol
levels and a longer-term effect on behaviour. Both effects, however, are consistent
with the impact of the procedures being imposed on the sheep. Clearly, being
separated from the larger flock to be housed in a shed constituted a significant and
stressful change in environment. Subsequent contact separation, followed by a startle
procedure for half the group, appeared to not add to, or sustain, the level of stress.
Thus, during the six weeks of this study the sheep were being subjected to a chronic
level of confinement stress. The cortisol response is likely to have developed
quickly, perhaps within hours of initial confinement (Cockram et al., 1994), and
recovered more slowly, taking four days to fall to a significant degree. The
persistence of the reduction in active behaviour, the withdrawal phenomenon,
beyond the expected three weeks (through to week 6 in groups A and B) contrasted
with other studies in which a recurrence of active behaviour has been ascribed to
frustration, monotony or a lack of stimulation (Done-Currie, Hecker and Wodzicka-
Tomaszewska, 1984; Fordham et al., 1991). This difference is most likely due to
different breeds of sheep, with different histories, being used in different studies.
Thus, in the absence of a secondary breakout of active behaviour, the sheep were
recovering from the physiological response to confinement while, at the same time,
the behavioural response continued to develop. The increase in apparently
threatening head orientation postures is likely to have been an artefact of the group
of sheep being spread further apart in weeks 3 to 6, reducing the usual controls on
agonistic behaviours in a flock.

This study has contributed to the development of a protocol. It has shown that being
taken from a paddock flock into a small group housed in a shed was sufficiently
stressful to be seen in a measureable increase in cortisol level. The cortisol response
appeared virtually immediately, certainly within hours, and had shown a significant
level of recovery by day 4. Behaviour also changed in measureable ways. In the shed
environment in which this study was conducted, neither contact separation nor a
startle procedure was sufficient to alter the course of the response to the initial
confinement. Separation anxiety was not evident.

Naturalistic behaviour may be too “open-ended” to be amenable to rigorous analysis
with respect to physiological parameters. It may be more useful to concentrate on a
particular, appropriate aspect of behaviour. In addition, the ability to identify an
individual sheep when behaviour is being recorded (not possible on all occasions in
this study) would add significantly to the available statistical repertoire, both in
analysis of behaviour and in comparisons between behaviour and other variables.

Contact separation, when individuals continued to see, hear and smell each other, did
not separate a sheep sufficiently to result in separation anxiety. Sensory isolation is
likely to be required in order to induce separation anxiety.

The observations made in this pilot study were used to refine the methodology
described in Chapter 5.
CHAPTER 5

IMMUNOLOGICAL AND BEHAVIOURAL RESPONSES OF SHEEP TO ISOLATION STRESS

5.1 Introduction

To some extent, animals and humans can cope by adapting to stressors within hours or days, but insufficient adaptation can lead to a "pre-pathological state" (Moberg, 1985, 1996) that is characterised by reduced immune competence.

It is known that confinement of sheep in pens and, especially, their isolation from other sheep, are stressful for a time. The immediate effects include changes in plasma cortisol levels, and disrupted behaviour (section 2.5). However, various behaviours and the adrenocortical response to handling exhibit adaptation, returning to normal over two to three weeks. Since, as a generalisation, stress is said to cause immunosuppression (section 2.5.1), this experiment was designed to see if the numbers of various circulating lymphocytes also changed during this period of adaptation.

A pilot study (Chapter 4) has confirmed that, while confinement of sheep in pens can be a major stressor, the immediate adrenocortical response has resolved within four days. It also suggested that isolation from other sheep should be total if it is to be more stressful than confinement in a group. Finally, the observation of naturalistic
behaviour during the period of adaptation provided only a broad indication of behavioural changes, so that a more structured approach to behaviour may provide more useful fine detail. Hence the arena test is being used in this investigation.

The arena test, a motivational-choice open-field test, was originally described by Fell and Shutt (1989). It is a sensitive method that detects changes in the complex behaviour of sheep, changes that have been shown to co-vary with immune parameters. To date, it has been used to study aspects of the sheep’s response to internal parasites (Adams and Fell, 1997; Fell et al, 1991; Gates et al., 1992; Hohenhaus et al., 1998).

In this investigation the sheep are not responding to an antigen, nor are they subject to parasites. Therefore, the peripheral immune system, the compartment of the immune system that generates clonally-driven immune responses to antigens (section 2.6), is expected to be relatively quiescent (Boise and Thompson, 1996). On the other hand, any immunological activity that does arise is likely to reflect the actions of the central immune system. The central immune system is a self-referential, connected network of variable region molecules (immunoglobulins (section 2.3.1), B cell receptors (section 2.3.3.1) and T cell receptors (section 2.3.5.3)), engaged in a stable dynamics that precludes any engagement in antigen-driven immune responses (section 2.6). Rather, outside influences would be expected to perturb the network so that its activity changes to compensate for the perturbation.

Therefore, this investigation is a way of seeing whether a procedure that is known to be stressful and to perturb the behavioural repertoire (Chapter 4) is accompanied by compensation by the immune network, principally the central immune system. The outcome is expected to provide some insight into the relationship between the psychoneural and immune systems.
In this study, Merino ewes were brought in from pasture (section 3.1) and kept indoors either in groups of four per pen or in total isolation from other sheep. Half the sheep were kept for 19 days while the other half were kept for 12 days. Blood sampling and behavioural testing were carried out during the last 12 days of the experiment, beginning on the day that the second group was brought in which was designated as day 1 (section 3.6). The blood samples were used to determine plasma cortisol levels (section 3.2) and lymphocyte counts (section 3.8). Behaviour was recorded in the arena test (section 3.7).

5.2 Results

5.2.1 Lymphocyte sub-types

There were no significant differences between the treatment groups for CD4 or CD8 cells on either day 2 or day 12, although the 2 week grouped sheep had a higher level of CD4 cells than the other groups on day 2 (34.8 compared with 31.0%). The percentage of CD4 cells increased during this period for each group (mean rise from 32.0 to 37.9%), while the level of CD8 cells decreased slightly over the same period in three of the four groups. The mean increase in CD4 cells was significantly greater (p<0.01) for isolated sheep (8.15 ± 0.80) (mean ± se) than for grouped sheep (3.82 ± 0.80) (Figure 5.1).

Similarly, there was a very small, but statistically significant, difference (p<0.05) between the isolated and the grouped sheep in the mean change in CD8 cells between day 2 and day 12. There was a mean decrease of 1.98 ± 0.80 in the percentage of CD8 cells for the sheep held in isolation compared to a mean increase of 1.19 ± 0.80 for those held in small groups (Figure 5.2).
Figure 5.1 The increase (mean ± se) in the level of CD4 cells (CD4%) in sheep after being taken from pasture and kept indoors for 12-19 days either in individual isolation or in pens in groups of four sheep.
Figure 5.2 The change (mean ± se) in the level of CD8 cells (CD8%) in sheep after being taken from pasture and kept indoors for 12-19 days either in individual isolation or in pens in groups of four sheep.
The combined effect of these changes was an increase in the CD4:CD8 ratio in three of the treatment groups during the sampling period. Again there were no significant effects of the treatments apparent on either day 2 or day 12, although the isolated sheep had consistently lower CD4:CD8 values than the grouped sheep on day 2. The increase in the CD4:CD8 ratio was significantly greater (p<0.01) for isolated sheep than for grouped sheep (0.97 ± 0.16 compared with 0.16 ± 0.16) (Figure 5.3), and also significantly greater (p<0.05) for 2 week sheep than for 3 week sheep (0.87 ± 0.16 compared with 0.26 ± 0.16) (Figure 5.4). The 3 week grouped sheep were unusually high at day 2 and their CD4:CD8 ratio had actually fallen by day 12.

The percentage of CD5 cells was the same in all groups at day 2, but a significant treatment effect was evident by the end of the sampling period. On day 12 the level of CD5 cells was 52.3 ± 2.0 for the isolated sheep compared with 62.6 ± 2.0 for the grouped sheep (p<0.01). Furthermore, the mean increase from day 2 to day 12 was significantly less (p<0.05) for the isolated sheep (4.8 ± 2.6) than for those kept in groups (14.2 ± 2.6) (Figure 5.5).

Thus, the main treatment effect on lymphocyte sub-types was isolation versus grouping. For CD4 cells and the CD4:CD8 ratio the isolated sheep tended to have lower values at first than the grouped sheep, but clearly rose more quickly during the sampling period. For CD5 cells the levels in the isolated sheep rose more slowly and were clearly lower at the end of the recovery period than for those kept in groups. The extra seven days of treatment (3 week versus 2 week) also affected the CD4:CD8 ratio in that the 3 week grouped sheep were already high at the start of sampling.
Figure 5.3 The increase (mean ± se) in the CD4:CD8 ratio in sheep after being taken from pasture and kept indoors for 12-19 days either in individual isolation or in pens in groups of four sheep.
Figure 5.4 The increase (mean ± se) in the CD4:CD8 ratio in sheep after being taken from pasture and kept indoors for either 12 days (2 week stress) or 19 days (3 week stress) in individual isolation and in pens in groups of four sheep.
Figure 5.5 The increase (mean ± se) in the level of CD5 cells (CD5%) in sheep after being taken from pasture and kept indoors for 12-19 days either in individual isolation or in pens in groups of four sheep.
5.2.2 Adrenocortical response

Plasma cortisol concentration showed a marked decline in all groups from day 1 to day 3 of the sampling and appeared to have levelled out thereafter (Figure 5.6). Due to the very large variation in plasma cortisol concentration between animals and between samples, which is normal, there were few statistically significant differences.

The 2 week groups had substantially higher cortisol levels than the 3 week groups, particularly on day 1, but also on several of the following days (Figure 5.6). This difference was significant (p<0.05) for the isolated sheep, but not for the grouped sheep. There was also a significant interaction (p<0.05) on day 1 between the type of confinement (isolation versus grouping) and the duration of confinement (2 week versus 3 week).

5.2.3 Arena behaviour

For approach distance no significant effects were detected over days 1-4. For days 9-12 the interaction between type of confinement (isolation versus grouping) and the duration of confinement (2 week versus 3 week) was significant (p<0.05), but neither of the main effects was significant and no linear trend was detected. The mean approach distance was 10.54 m for 3 week isolated sheep, 9.73 m for 2 week isolated sheep, 9.32 m for 3 week grouped sheep and 10.35 m for 2 week grouped sheep (se = 0.56 m in each case). No significant effects of interaction were detected for the overall mean, nor for the difference between means.
Figure 5.6  Plasma cortisol concentration (mean ± se) in sheep after being taken from pasture and kept indoors for either 12 days (2 week) or 19 days (3 week) either in individual isolation or in pens in groups of four sheep.
For distance travelled during days 1-4 there was a significant effect of duration of confinement (p<0.05), the 3 week isolated sheep having much higher motor activity than the other three groups (Table 5.1). However, the effect of isolation was not significant during this period. During days 9-12 the effect of isolation approached significance (0.05<p<0.10), the grouped sheep having travelled less than half the distance of the isolated sheep (Table 5.1). At this stage there was no longer any effect of the 3 week versus 2 week treatment. For the overall mean the effect of isolation was significant (p<0.05), but the effect of confinement duration was not. For the difference between periods neither main effect was significant and there were no significant linear trends.

During the sampling period the inter/sheep distance, or spread, increased by a small, but significant amount. The mean spread in period 2 was 1.438 ± 0.045 m compared with 1.212 ± 0.045 m in period 1 (p<0.05). In period 1 there was a significant interaction (p<0.05) between type of confinement (isolation versus grouping) and duration of confinement (2 week versus 3 week). The mean spread for 3 week isolated sheep (1.576 ± 0.084 m) was significantly greater than the means for the other three groups (1.094 m for 2 week isolated, 1.115 m for 3 week grouped and 1.063 m for 2 week grouped (se = 0.084 m)).

The linear component of the trend over days 1-4 was significant (p<0.05) and positive for the 3 week groups (regression coefficient 0.098 ± 0.042 units per day), but no significant trend was present for the 2 week groups. No curvature terms were significant.
The distance travelled by sheep in an arena test (mean ± se) after they had been taken from pasture and kept indoors for either 12 days (2 week) or 19 days (3 week) either in individual isolation or in pens in groups of four sheep.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean distance travelled during the test (m)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Days 1 - 4</td>
</tr>
<tr>
<td>Isolated 3 week</td>
<td>20.8 ± 5.6</td>
</tr>
<tr>
<td>Isolated 2 week</td>
<td>4.8 ± 5.6</td>
</tr>
<tr>
<td>Grouped 3 week</td>
<td>9.2 ± 5.6</td>
</tr>
<tr>
<td>Grouped 2 week</td>
<td>3.6 ± 5.6</td>
</tr>
</tbody>
</table>
In period 2 no significant treatment effects were detected, although the isolated versus group effect just failed significance at the 5% level. The means were 1.598 m for isolated sheep compared with 1.279 m for grouped sheep (se = 0.108 m; actual difference 0.319; 1 sd value 0.331).

Thus, the main effects were that the inter-sheep distance was significantly greater for 3 week isolated sheep than for the others and it tended to increase over the period, but less so in that group. Motor activity in the test was significantly greater for isolated sheep than for grouped sheep.

5.3 Discussion

The physiological effects described here are consistent with the imposition of a severe stressor on the sheep and their subsequent adaptation to the stressful situation. These sheep had been running at pasture in a large flock and had no previous experience of confinement indoors. From previous studies there is good reason to believe that the individual isolation would be a more severe stressor than confinement in groups (Balock, Sibly and Penning, 1988; Lynch, Hinch and Adams, 1992; Parrott et al., 1987; Chapter 4). However, the effect in this experiment would be chronic in nature, allowing for adaptation to occur (section 2.5.1), even though isolation leads to separation anxiety in sheep (Lynch, Hinch and Adams, 1992). This adaptation makes it very difficult to measure the level of stress at any particular time. This type of acute immune response, followed by chronic adaptation, has been observed previously, not only in sheep (Cockram et al., 1994) but also in rabbits (Bensoussan, 1991).
Judging from the cortisol data, there was no indication that isolated confinement was any more stressful than group confinement. However, plasma cortisol concentration is not a good indicator of chronic stress (Baldwin and Stephens, 1973; Cockram et al., 1994; Dalin et al., 1993; Fell and Shutt, 1989; Mitchell, Hattingh and Ganhao, 1988; Shutt et al., 1988b), being a measure of the acute adrenal response around the time of blood sampling - the sheep’s reaction to handling. The cortisol data showed that this response declined rapidly over successive sampling days in all groups, presumably due to habituation by the sheep to the sampling procedure (section 2.5.1).

At the start of sampling, however, the adrenocortical response was greater for those sheep that had only just been brought into confinement (one hour earlier) compared to those that had already experienced one week of confinement prior to the first sampling. Whether they were kept singly or in groups, the handling at sampling was less stressful for sheep that had already experienced a period of confinement and the interaction with humans associated with it (daily feeding and cleaning). This implies that the stress associated with the procedure of taking the sheep into confinement was at its maximum on the first day of the treatment and would decline thereafter. It is impossible to establish this with great certainty because of the problem of stress measurement mentioned above, but it is a reasonable assumption from these findings. The daily human interaction while in confinement may also explain the persistence of the behavioural withdrawal for six weeks reported in Chapter 4. The effect may have been to prevent frustration or monotony, the supposed triggers for resumption of active behaviour (Fordham et al., 1991).

In this situation, the imposition of the stress upon the sheep’s immune system is likely to have been maximal at the start of the treatment, although the time course of any effects is largely unknown. There was an overall rise in the levels of CD4 and
CD5 cells and the CD4:CD8 ratio during the sampling period that was not large, but was reasonably consistent. This kind of change can be interpreted as indicating a recovery of immune competence following an adverse reaction to the introduction of a stressor. The likelihood that this is the recovery period becomes more evident in comparisons with other studies, especially those that have documented an acute response to a stressor, during the time before recovery. The immediate response to brief stress has been shown to include: constant levels of total T lymphocytes (CD5 cells in the sheep); constant or decreased levels of CD4 cells; an increase in CD8 cells; and a decrease in the CD4:CD8 ratio (Bachen et al., 1992; Brosschot et al., 1992; Naliboff et al., 1991). Furthermore, taking the cortisol results into account suggests a process of recovery from immunosuppression, rather than one of immune enhancement. Indeed, in healthy subjects, such as these sheep, an increase in circulating lymphocyte numbers may indicate the reversal of a departure from immunological health (Booth, Petrie and Pennebaker, 1997; Petrie et al., 1995). To put it another way, these results are suggestive of a period of immune compensation following a perturbation.

At the end of the trial the only significant difference between treatment groups was in CD5 cells, where the isolation treatment resulted in lower numbers of CD5 lymphocytes and the extent of their recovery had been retarded by this treatment. CD5 cells represent the whole population of T cells (Dhabhar et al., 1995), together with the B-1 cells, a relatively small subset of the population of B cells (section 2.3.3.2), so this suggests that the immune system had been more severely affected in an adverse manner by the isolation than by the group confinement (Copping er et al., 1991; Minton and Blecha, 1990). The CD4 cells and the CD4:CD8 ratio were not significantly affected by the treatments either at the beginning or the end of the sampling period.
The isolation treatment did tend to produce lower levels of CD4 cells and the CD4:CD8 ratio at the start of sampling than the grouping treatment, but it clearly increased the extent of the recovery in these cell populations during this period. The CD4 cells, consisting of TH1 inflammatory T cells and TH2 helper T cells (section 2.3.5.2), are an important element in the overall competence of the immune system, both subsets having been shown to be active in mice under individual/group housing stress (Karp, Cohen and Moynihan, 1994). Thus, the stressful effect of adapting to isolation was initially more severe than the stress of adapting to group confinement, yet recovery was greater. It is conceivable that the individual sheep-human interaction experienced by the isolated sheep was more conducive to successful physiological adaptation (for CD4 cells) than the sheep-human interaction for sheep grouped in pens, although no mechanism is known for this effect. Perhaps the intensity of the interaction is different in the different situations. It is also possible that the initially more severe stressor (isolation) generated a greater perturbation that, in turn, resulted in a correspondingly greater level of compensation.

The only effect of the duration of the treatment (3 week versus 2 week) was on the recovery of the CD4:CD8 ratio in that the ratio for the 3 week grouped sheep was already high at the start of sampling; either their recovery had already begun or they had never fallen to a low level. This cannot be determined from the current data, although the former alternative seems more likely, given the stressful effect of confinement on sheep.

Behavioural adaptation may have already taken place during this period, although the results are far from clear. The increase during the testing period in inter-sheep distance, or spread, may have indicated a declining level of fear in the sheep. Under normal circumstances sheep tend to flock more closely when they are fearful (Lynch, Hinch and Adams, 1992). On the other hand, it could have indicated impairment of
the natural flocking tendency of these Merinos, the most highly gregarious sheep breed (Lynch, Hinch and Adams, 1992), given that confinement in groups of 25 animals for several weeks can reduce flocking behaviour (Fell et al., 1991). The 3 week isolated sheep had significantly greater spread than other groups from the start of testing so it is possible that their natural flocking ability had been impaired by the treatment. Their excessive motor activity during the early tests (see below) may have contributed to this.

The isolation treatment resulted in increased motor activity by the sheep in the arena test which has been interpreted as indicating a higher level of agitation or fear in animals in an open-field test (Fell, 1992; Fell et al., 1991). This provides further evidence that the isolation treatment was a more severe stressor than group confinement. The 3 week isolated sheep had much the highest motor activity in the first period of testing, a period that began after one week spent in relatively undisturbed isolation. Thus, the combination of greater spread and greater motor activity characterised the 3 week isolated sheep predominantly. In effect, the sheep held in groups and those isolated for a shorter period exhibited less behavioural disruption, that is, a greater degree of behavioural adaptation.

In conclusion, physiological and behavioural adaptation over the period were characterised by a decline in the adrenocortical response, a resumption of the normal pattern of flocking behaviour and a reduction in motor activity during the test. There was an accompanying recovery in T lymphocyte numbers in the direction of immunological health. This provides further evidence of close links among behaviour, the neuroendocrine system and the immune system.

In this investigation a non-antigenic challenge (confinement/isolation stress) has been applied and concomitant changes in complex behaviour patterns, cortisol secretion and numbers of various types of lymphocytes have been described. Further
work is necessary to establish a causal relationship among the psychoneural system, the hypothalamo-pituitary-adrenal axis and the immune system, but these findings point to the possibility of some correspondence between critical features of each of these systems.

Nevertheless, this study has provided tentative evidence that is suggestive of deterioration followed by recovery in both behaviour and circulating lymphocytes in response to a substantial stressor. Assuming that behaviour is an element of psychoneural operation, and that circulating lymphocytes reflect immune activity, these findings support the idea that the immune system and the psychoneural system are not operating independently, but are interconnected in some way, as has been suggested in many previous reports.
CHAPTER 6

PSYCHOLOGICAL STATUS, IMMUNE FUNCTION AND HEALTH IN ARMY RECRUITS

6.1 Introduction

The responses to stress have been shown to be diverse and seemingly contradictory (section 2.5.1), and include endocrine, psychoneural and immune elements. Army recruit training, the first three months of a soldier's career, is undoubtedly a stressful time. Nevertheless, prospective soldiers are selected for recruit training with the expectation that they will succeed. The majority do so, and continue their Army careers, apparently healthy and well-adjusted.

In the study reported here, recruit training extended over 10-12 weeks, providing an opportunity for a comprehensive investigation of these issues. As was the case with the sheep isolation study (Chapter 5), the stressor was non-antigenic. In addition, the study examined the possibility that soldiers who did not complete recruit training could be characterised in terms of the parameters being measured.

Of the 55 male Army recruits that participated in this study, those that did not finish recruit training became the study group, allocated a posteriori. Sampling times were at induction into training (#0) and at the end of each of the three stages of training (#1, #2, #3). Blood and saliva were collected, and psychological state characteristics
were measured (section 3.11), on all four occasions. A psychological trait characteristic was measured at #1 and #3 (section 3.10). Blood was used to count lymphocytes (section 3.12) and cortisol was measured in saliva (section 3.11). A record of infections was also maintained (section 3.13).

6.2 Results

By the end of the period of recruit training six soldiers (11% of the participants) had not completed the course and became the study group (section 3.10). The remaining 49, who had succeeded, became the control group. However, a Student’s t test comparison between the two groups showed no significant differences for any of the parameters on any occasion (Table 6.1). Therefore, the results were combined so that the remainder of the analysis was performed on the data from a single group of 55 soldiers. Thus, it was not possible to distinguish between a “study group” and the other soldiers.

At the conclusion of recruit training, soldiers were posted to various Army units throughout the nation. Due to a misunderstanding between the researcher and the medical staff at Kapooka, records of infection were not retrieved before the soldiers’ personal medical records had been sent on to their new postings. As a result, only 19 records of infection could be traced and recovered. Of those, five (26%) contained evidence of infection. Again, a Student’s t test comparison between the two groups (no infection, or infection) showed no significant differences for any of the parameters on any occasion (Table 6.2).

The different sample sizes for different parameters on different occasions were a reflection of varying levels of compliance by the subjects, as well as a loss of some stored saliva samples when a freezer broke down.
Table 6.1

Mean values (± standard error), with sample size in parentheses, of the parameters measured at the first three sampling points during Army recruit training. The values for the recruits who completed training are followed by those for the recruits who did not. None of the pairwise Student's t test comparisons was significant. Abbreviations and units are defined in the text.

<table>
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<th>Parameters</th>
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<th>Sampling points</th>
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<td>#0</td>
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<td>#2</td>
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<td>8.62 ± 0.90 (6)</td>
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<td>915.0 ± 61.7 (6)</td>
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<td>565.0 ± 33.2 (6)</td>
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Table 6.2

Mean values (± standard error), with sample size in parentheses, of the parameters measured at the four sampling points during Army recruit training. The values for the recruits whose records of infection indicated no infection are followed by those whose records indicate infection. None of the pairwise Student’s t test comparisons was significant. Abbreviations and units are defined in the text.

<table>
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<th>Parameters</th>
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<td>6.80 ± 1.11 (5)</td>
<td>7.32 ± 2.03 (4)</td>
</tr>
<tr>
<td>LCB</td>
<td>25.5 ± 0.8 (14)</td>
</tr>
<tr>
<td>P(TMD)</td>
<td>67.63 ± 2.61 (14)</td>
</tr>
<tr>
<td>63.44 ± 4.60 (5)</td>
<td>64.52 ± 6.80 (4)</td>
</tr>
</tbody>
</table>
6.2.1 Adrenocortical response

Beginning with a baseline (#0) value of 18.74 nmol.L\(^{-1}\), salivary cortisol dropped to a nonsignificant extent at #1 (Table 6.3). By #2, it had risen significantly, compared both with #0 (p<0.01) and with #1 (p<0.001). Although the level had risen further by #3, there appeared to be no significant difference from #0, despite the values at #2 and #3 also showing no significant difference. Perhaps the statistical disparity was an artefact of comparing a sample of nine data points with nine of 51 at #0 and nine of 45 at #2. Nevertheless, the trend was for the initial level of cortisol to remain essentially unchanged during the first period of training, then to rise significantly over the remainder of the time (Figure 6.1).

6.2.2 Lymphocyte responses

White blood cell (WBC) numbers rose significantly (p<0.05) at #1, from an initial value of 8.42 x 10\(^3\) cells.mm\(^{-3}\) (Table 6.3). They then fell significantly at #2 (p<0.001) and rose slightly but remained significantly lower at #3 (p<0.05).

The level of CD16 lymphocytes (NK cells) began at 298.0 cells.mm\(^{-3}\) (Table 6.3). This was followed by a sustained significant reduction throughout recruit training (p<0.001 on all three occasions). The slight decrease at #2 and slight increase at #3 were not significantly different from the value at #1 and each other, indicating minor fluctuations around a level that was significantly lower than baseline.
Figure 6.1 Percentage changes in cortisol (Figure 6.1a) and lymphocyte (Figure 6.1b) measures for the four sampling points during Army recruit training. Abbreviations are defined in the text.
Table 6.3

Adrenocortical and lymphocyte measures (mean ± standard error), with sample sizes (n), for the four sampling points during Army recruit training. The levels of significance of paired t test comparisons are shown for comparisons with the baseline value (column A) and comparisons between adjacent values (column B). Significance of differences is indicated as follows (NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.001). Abbreviations and units are defined in the text.

<table>
<thead>
<tr>
<th>Adrenocortical measure</th>
<th>Comparisons A</th>
<th>Comparisons B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortisol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>18.74 ± 0.19 (51)</td>
<td>NS</td>
</tr>
<tr>
<td>#1</td>
<td>14.86 ± 0.42 (36)</td>
<td>**</td>
</tr>
<tr>
<td>#2</td>
<td>30.58 ± 0.50 (45)</td>
<td>NS</td>
</tr>
<tr>
<td>#3</td>
<td>31.89 ± 2.52 (9)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Lymphocyte measures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WBC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>8.42 ± 0.05 (55)</td>
<td>*</td>
</tr>
<tr>
<td>#1</td>
<td>9.40 ± 0.05 (53)</td>
<td>***</td>
</tr>
<tr>
<td>#2</td>
<td>6.86 ± 0.03 (50)</td>
<td>***</td>
</tr>
<tr>
<td>#3</td>
<td>7.29 ± 0.03 (45)</td>
<td>*</td>
</tr>
<tr>
<td><strong>CD16</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>298.0 ± 2.7 (55)</td>
<td>***</td>
</tr>
<tr>
<td>#1</td>
<td>214.2 ± 2.1 (53)</td>
<td>***</td>
</tr>
<tr>
<td>#2</td>
<td>209.4 ± 2.1 (50)</td>
<td>***</td>
</tr>
<tr>
<td>#3</td>
<td>236.2 ± 2.1 (45)</td>
<td>***</td>
</tr>
<tr>
<td><strong>CD19</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>333.1 ± 2.2 (55)</td>
<td>**</td>
</tr>
<tr>
<td>#1</td>
<td>386.6 ± 2.7 (53)</td>
<td>NS</td>
</tr>
<tr>
<td>#2</td>
<td>306.2 ± 2.0 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>#3</td>
<td>378.0 ± 2.7 (45)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CD3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>1287.5 ± 9.2 (55)</td>
<td>NS</td>
</tr>
<tr>
<td>#1</td>
<td>1414.0 ± 6.8 (53)</td>
<td>NS</td>
</tr>
<tr>
<td>#2</td>
<td>1251.1 ± 5.1 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>#3</td>
<td>1464.7 ± 7.6 (45)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CD4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>701.1 ± 5.0 (55)</td>
<td>**</td>
</tr>
<tr>
<td>#1</td>
<td>815.5 ± 4.1 (53)</td>
<td>NS</td>
</tr>
<tr>
<td>#2</td>
<td>685.6 ± 2.9 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>#3</td>
<td>806.2 ± 4.2 (45)</td>
<td>***</td>
</tr>
<tr>
<td><strong>CD8</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>526.9 ± 4.8 (55)</td>
<td>NS</td>
</tr>
<tr>
<td>#1</td>
<td>554.9 ± 3.8 (53)</td>
<td>NS</td>
</tr>
<tr>
<td>#2</td>
<td>498.0 ± 3.9 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>#3</td>
<td>582.2 ± 5.4 (45)</td>
<td>*</td>
</tr>
<tr>
<td><strong>CD4:CD8</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#0</td>
<td>1.49 ± 0.01 (55)</td>
<td>*</td>
</tr>
<tr>
<td>#1</td>
<td>1.61 ± 0.01 (53)</td>
<td>NS</td>
</tr>
<tr>
<td>#2</td>
<td>1.53 ± 0.01 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>#3</td>
<td>1.56 ± 0.01 (45)</td>
<td>NS</td>
</tr>
</tbody>
</table>
CD19 lymphocytes (B cells) began at a level of 333.1 cells\(\text{mm}^{-3}\), rose by a significant amount \(p<0.01\) at #1, then returned to values not significantly different from #0 at #2 and #3 (Table 6.3). However, the value at #2, significantly lower than that at #1 \(p<0.001\), was followed by a significant increase at #3 \(p<0.001\). Nevertheless, #2 and #3 fluctuated about the baseline, but to statistically non significant degrees. In effect, the increase at #1 was a spike above a level that otherwise persisted throughout training.

Variations in CD3 cell (T cell) numbers (Table 6.3) were not significantly different from #0 until #3, when they rose significantly \(p<0.01\) to 1464.7 cells\(\text{mm}^{-3}\). However, the decrease at #2 was significantly lower than the increase at #1 \(p<0.001\), and was followed by a significant increase at #3 \(p<0.001\). A similar pattern was evident with CD8 cells, but with a lesser degree of significance \(p<0.05\) in the final increase, and non significant fluctuations at #1 and #2.

Beginning with 701.1 cells\(\text{mm}^{-3}\), CD4 lymphocyte levels rose significantly \(p<0.01\) at #1, returned to values not significantly different from baseline at #2, then again rose significantly \(p<0.001\) at #3 (Table 6.3). The decrease in values from #1 to #2 was significant \(p<0.001\), however, as was the increase from #2 to #3 \(p<0.001\). The combined effect of levels of CD4 and CD8 lymphocytes, the CD4:CD8 ratio, remained greater than 1.0, with the value at #1 being significantly higher \(p<0.05\) than at other times.

Finally, throughout training the mean value of CD3 lymphocytes (the entire T cell population) always exceeded the sum of CD4 and CD8 T cell mean values. The difference was likely to have been due to the presence of \(\gamma\delta\) T cells, although they had not been specifically labelled or counted.
In summary, CD16 lymphocyte (NK cell) numbers dropped significantly over time, with WBC following the same trend but with a spike at #1. CD19 lymphocyte (B cell) numbers remained essentially unchanged but with a spike at #1. While both total (CD3 lymphocyte) and CD8 T cells only increased significantly at the end of training (#3), CD4 T cells fluctuated up and back throughout the time. Thus, the CD4:CD8 ratio rose significantly above baseline on the occasion (#1) when CD4 numbers were significantly higher and CD8 values were not. While the levels of significance may not have followed a consistent trend, a pattern did emerge among all the lymphocytes, except NK cells (Figure 6.1). The baseline values were followed by rises, falls and rises.

6.2.3 Psychological indicators

The scores for both the Beck Anxiety Inventory (BAI) and the Beck Depression Inventory (BDI) decreased progressively over time (Table 6.4), to a significant extent (p<0.001) at #2 and #3.

Locus of Control of Behaviour (LCB) was measured twice (#1 and #3), registering a significant drop (p<0.05) on the second occasion (Table 6.4).

Profile of Mood States (POMS) was measured as a Total Mood Disturbance score, P(TMD), subdivided into six identifiable mood states (Table 6.4). They were: anger/hostility - P(ANG); confusion/bewilderment - P(CON); depression/dejection - P(DEP); fatigue/inertia - P(FAT); tension/anxiety - P(TEN); and, vigour/activity - P(VIG) (McNair, Lorr and Droppleman, 1992). The overall score showed a continuing significant decrease over time (p<0.05 at #1 and p<0.001 at #2 and #3).
Table 6.4
Mean values of psychological measures for the four sampling points during Army recruit training. Baseline values (#0) include mean ± standard error, with sample size in parentheses. Values from the end of each component of training (#1, #2, #3) include the level of significance of a paired t test comparison with #0 (NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.001). Abbreviations are defined in the text.

<table>
<thead>
<tr>
<th>Psychological measures</th>
<th>#0</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAI</td>
<td>6.18 ± 0.11 (55)</td>
<td>5.83 ± 0.12 (54) NS</td>
<td>3.08 ± 0.10 (50) ***</td>
<td>2.91 ± 0.15 (34) ***</td>
</tr>
<tr>
<td>BDI</td>
<td>4.95 ± 0.11 (55)</td>
<td>4.26 ± 0.11 (54) NS</td>
<td>1.98 ± 0.06 (50) ***</td>
<td>1.15 ± 0.07 (34) ***</td>
</tr>
<tr>
<td>LCB</td>
<td>21.8 ± 0.19 (54)</td>
<td>17.8 ± 0.29 (35) *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(TMD)</td>
<td>58.82 ± 0.53 (55)</td>
<td>53.81 ± 0.48 (54) *</td>
<td>39.00 ± 0.39 (50) ***</td>
<td>40.65 ± 0.55 (34) ***</td>
</tr>
<tr>
<td>P(ANG)</td>
<td>6.49 ± 0.15 (55)</td>
<td>8.22 ± 0.14 (54) NS</td>
<td>3.80 ± 0.09 (50) **</td>
<td>4.65 ± 0.13 (34) NS</td>
</tr>
<tr>
<td>P(CON)</td>
<td>8.22 ± 0.10 (55)</td>
<td>5.83 ± 0.09 (54) ***</td>
<td>4.00 ± 0.06 (50) ***</td>
<td>3.03 ± 0.09 (34) ***</td>
</tr>
<tr>
<td>P(DEP)</td>
<td>8.55 ± 0.20 (55)</td>
<td>7.43 ± 0.17 (54) NS</td>
<td>3.10 ± 0.10 (50) ***</td>
<td>3.29 ± 0.17 (34) *</td>
</tr>
<tr>
<td>P(FAT)</td>
<td>7.58 ± 0.09 (55)</td>
<td>8.09 ± 0.10 (54) NS</td>
<td>5.74 ± 0.10 (50) *</td>
<td>6.00 ± 0.13 (34) NS</td>
</tr>
<tr>
<td>P(TEN)</td>
<td>11.45 ± 0.12 (55)</td>
<td>8.37 ± 0.11 (54) ***</td>
<td>5.98 ± 0.09 (50) ***</td>
<td>6.12 ± 0.15 (34) ***</td>
</tr>
<tr>
<td>P(VIG)</td>
<td>16.53 ± 0.10 (55)</td>
<td>15.87 ± 0.11 (54) NS</td>
<td>16.38 ± 0.13 (50) NS</td>
<td>17.59 ± 0.16 (34) NS</td>
</tr>
</tbody>
</table>
This pattern was repeated, with minor variations, in P(CON), P(DEP) and P(TEN). P(ANG) and P(FAT) shared a different pattern of scores; both measures registered a significant drop (p<0.01 and p<0.05, respectively) but only at #2. There were no significant differences in the level of P(VIG) throughout training.

In general terms, then, the soldiers experienced a significant decrease in their confusion/bewilderment, depression/dejection, and tension/anxiety. These improvements were reflected in BAI and BDI scores, and in the relevant POMS scales. The reduction in LCB score also suggested that a degree of personality maturation had taken place (Andrews, Page and Neilson, 1993). At the same time, they sustained their vigour/activity throughout training. However, anger/hostility and fatigue/inertia tended to rise, fall and rise (Figure 6.2).

6.2.4 Lymphocyte/psychological interactions

The degree of association between different lymphocyte and psychological measures was tested with Spearman rank-order correlations, one for each combination at each sampling point (Tables 6.5 to 6.11), in the manner of Lee et al. (1995). The significant correlations were then assembled in Table 6.12. However, given the large number of comparisons undertaken, a probability level of p<0.01 was selected to determine significance and inclusion in Table 6.12. Incidentally, it would be a mistake to think of Tables 6.5 to 6.11 as multiple statistical comparisons. Rather, they are an attempt to spread out what would have otherwise been one large table of correlations of 10 psychoneural measures x seven immune measures x four occasions.
Figure 6.2 Percentage changes in psychological measures for the four sampling points during Army recruit training. Abbreviations are defined in the text.
Spearman rank-order correlations (with sample sizes in parentheses) between psychological measures and white blood cell count for the four sampling points during Army recruit training. Level of significance is also indicated (NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.001). Abbreviations are defined in the text.

<table>
<thead>
<tr>
<th>Psychological measures</th>
<th>Sampling points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#0</td>
</tr>
<tr>
<td>BAI</td>
<td>-0.23 (55) NS</td>
</tr>
<tr>
<td>BDI</td>
<td>-0.04 (55) NS</td>
</tr>
<tr>
<td>LCB</td>
<td></td>
</tr>
<tr>
<td>P(TMD)</td>
<td>-0.12 (55) NS</td>
</tr>
<tr>
<td>P(ANG)</td>
<td>-0.15 (55) NS</td>
</tr>
<tr>
<td>P(CON)</td>
<td>-0.10 (55) NS</td>
</tr>
<tr>
<td>P(DEP)</td>
<td>-0.02 (50) NS</td>
</tr>
<tr>
<td>P(FAT)</td>
<td>-0.01 (55) NS</td>
</tr>
<tr>
<td>P(TEN)</td>
<td>-0.28 (55) *</td>
</tr>
<tr>
<td>P(VIG)</td>
<td>0.08 (55) NS</td>
</tr>
</tbody>
</table>
Table 6.6

Spearman rank-order correlations (with sample sizes in parentheses) between psychological measures and CD16 lymphocytes for the four sampling points during Army recruit training. Level of significance is also indicated (NS: not significant; *: \( p < 0.05 \); **: \( p < 0.01 \); ***: \( p < 0.001 \)). Abbreviations are defined in the text.

<table>
<thead>
<tr>
<th>Psychological measures</th>
<th>Sampling points</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#0</td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
</tr>
<tr>
<td><strong>BAI</strong></td>
<td>-0.16 (55) NS</td>
<td>-0.26 (53) NS</td>
<td>-0.15 (50) NS</td>
<td>-0.03 (33) NS</td>
</tr>
<tr>
<td><strong>BDI</strong></td>
<td>-0.19 (55) NS</td>
<td>-0.20 (53) NS</td>
<td>-0.03 (50) NS</td>
<td>-0.40 (33) *</td>
</tr>
<tr>
<td><strong>LCB</strong></td>
<td></td>
<td>0.16 (53) NS</td>
<td></td>
<td>0.13 (33) NS</td>
</tr>
<tr>
<td><strong>P(TMD)</strong></td>
<td>-0.13 (55) NS</td>
<td>-0.41 (53) **</td>
<td>-0.02 (50) NS</td>
<td>-0.18 (34) NS</td>
</tr>
<tr>
<td><strong>P(ANG)</strong></td>
<td>-0.13 (55) NS</td>
<td>-0.30 (53) *</td>
<td>0.10 (50) NS</td>
<td>-0.24 (32) NS</td>
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<tr>
<td><strong>P(CON)</strong></td>
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<td>-0.38 (53) **</td>
<td>-0.09 (50) NS</td>
<td>-0.26 (33) NS</td>
</tr>
<tr>
<td><strong>P(DEP)</strong></td>
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<td>-0.35 (53) **</td>
<td>-0.26 (49) NS</td>
<td>-0.18 (33) NS</td>
</tr>
<tr>
<td><strong>P(FAT)</strong></td>
<td>-0.11 (55) NS</td>
<td>-0.20 (53) NS</td>
<td>-0.19 (50) NS</td>
<td>-0.01 (32) NS</td>
</tr>
<tr>
<td><strong>P(TEN)</strong></td>
<td>-0.12 (55) NS</td>
<td>-0.30 (53) *</td>
<td>0.11 (50) NS</td>
<td>-0.21 (33) NS</td>
</tr>
<tr>
<td><strong>P(VIG)</strong></td>
<td>0.26 (55) *</td>
<td>0.13 (53) NS</td>
<td>0.14 (50) NS</td>
<td>0.21 (33) NS</td>
</tr>
</tbody>
</table>
Spearman rank-order correlations (with sample sizes in parentheses) between psychological measures and CD19 lymphocytes for the four sampling points during Army recruit training. Level of significance is also indicated (NS: not significant; *: p<0.05; **: p<0.01; *** p<0.001). Abbreviations are defined in the text.

<table>
<thead>
<tr>
<th>Psychological measures</th>
<th>Sampling points</th>
<th>#0</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>#0</td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
</tr>
<tr>
<td>BAI</td>
<td>-0.11 (55)</td>
<td>-0.23 (53)</td>
<td>-0.20 (50)</td>
<td>-0.21 (33)</td>
<td>NS</td>
</tr>
<tr>
<td>BDI</td>
<td>-0.25 (55)</td>
<td>-0.16 (53)</td>
<td>-0.09 (50)</td>
<td>-0.25 (33)</td>
<td>NS</td>
</tr>
<tr>
<td>LCB</td>
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<td></td>
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<td>-0.01 (33)</td>
</tr>
<tr>
<td>P(TMD)</td>
<td>-0.18 (55)</td>
<td>-0.26 (53)</td>
<td>-0.04 (50)</td>
<td>-0.02 (34)</td>
<td>NS</td>
</tr>
<tr>
<td>P(ANG)</td>
<td>0.04 (55)</td>
<td>-0.22 (53)</td>
<td>0.15 (50)</td>
<td>0.08 (32)</td>
<td>NS</td>
</tr>
<tr>
<td>P(CON)</td>
<td>-0.24 (55)</td>
<td>-0.39 (53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(DEP)</td>
<td>-0.13 (50)</td>
<td>-0.26 (53)</td>
<td>0.08 (49)</td>
<td>-0.01 (33)</td>
<td>NS</td>
</tr>
<tr>
<td>P(FAT)</td>
<td>-0.27 (55)</td>
<td>-0.16 (53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(TEN)</td>
<td>-0.31 (55)</td>
<td>-0.27 (53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(VIG)</td>
<td>0.27 (55)</td>
<td>0.25 (53)</td>
<td>0.29 (50)</td>
<td>0.14 (33)</td>
<td>NS</td>
</tr>
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</table>
Table 6.8

Spearman rank-order correlations (with sample sizes in parentheses) between psychological measures and CD3 lymphocytes for the four sampling points during Army recruit training. Level of significance is also indicated (NS: not significant; *: p<0.05; **: p<0.01; *** p<0.001). Abbreviations are defined in the text.

<table>
<thead>
<tr>
<th>Psychological measures</th>
<th>Sampling points</th>
<th>#0</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAI</td>
<td></td>
<td>-0.26 (55)</td>
<td>-0.18 (52)</td>
<td>-0.43 (50)</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>BDI</td>
<td></td>
<td>-0.18 (55)</td>
<td>-0.13 (52)</td>
<td>-0.28 (50)</td>
<td>-0.16 (33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>LCB</td>
<td></td>
<td></td>
<td>-0.12 (52)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(TMD)</td>
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<td>-0.17 (52)</td>
<td>-0.02 (50)</td>
<td>-0.23 (34)</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>P(ANG)</td>
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<td>-0.18 (50)</td>
<td>-0.06 (32)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P(CON)</td>
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<td>-0.25 (52)</td>
<td>-0.29 (50)</td>
<td>-0.20 (33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>P(DEP)</td>
<td></td>
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<td>-0.16 (52)</td>
<td>-0.11 (49)</td>
<td>-0.19 (33)</td>
</tr>
<tr>
<td></td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P(FAT)</td>
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<td>-0.19 (50)</td>
<td>-0.46 (32)</td>
</tr>
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<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>P(TEN)</td>
<td></td>
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<td>-0.22 (52)</td>
<td>-0.21 (50)</td>
<td>-0.34 (33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td>P(VIG)</td>
<td></td>
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<td>0.14 (52)</td>
<td>0.22 (50)</td>
<td>0.12 (33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*</td>
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</table>
Table 6.9

Spearman rank-order correlations (with sample sizes in parentheses) between psychological measures and CD4 lymphocytes for the four sampling points during Army recruit training. Level of significance is also indicated (NS: not significant; *: p<0.05; **: p<0.01; *** p<0.001). Abbreviations are defined in the text.

<table>
<thead>
<tr>
<th>Psychological measures</th>
<th>Sampling points</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#0</td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
</tr>
<tr>
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<td>-0.12 (53) NS</td>
<td>-0.37 (50) **</td>
<td>-0.29 (33) NS</td>
</tr>
<tr>
<td>BDI</td>
<td>-0.24 (55) NS</td>
<td>-0.13 (53) NS</td>
<td>-0.43 (50) **</td>
<td>-0.29 (33) NS</td>
</tr>
<tr>
<td>LCB</td>
<td></td>
<td>-0.13 (53) NS</td>
<td></td>
<td>-0.11 (33) NS</td>
</tr>
<tr>
<td>P(TMD)</td>
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<td>-0.12 (53) NS</td>
<td>-0.21 (50) NS</td>
<td>-0.16 (34) NS</td>
</tr>
<tr>
<td>P(ANG)</td>
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<td>-0.12 (53) NS</td>
<td>-0.33 (50) *</td>
<td>-0.11 (32) NS</td>
</tr>
<tr>
<td>P(CON)</td>
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<td>-0.19 (53) NS</td>
<td>-0.45 (50) ***</td>
<td>-0.07 (33) NS</td>
</tr>
<tr>
<td>P(DEP)</td>
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<td>-0.17 (49) NS</td>
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<td>-0.26 (32) NS</td>
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<tr>
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<td>-0.24 (50) NS</td>
<td>-0.05 (33) NS</td>
</tr>
<tr>
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<td>0.17 (50) NS</td>
<td>0.05 (33) NS</td>
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</table>
Table 6.10

Spearman rank-order correlations (with sample sizes in parentheses) between psychological measures and CD8 lymphocytes for the four sampling points during Army recruit training. Level of significance is also indicated (NS: not significant; *: p<0.05; **: p<0.01; *** p<0.001). Abbreviations are defined in the text.

<table>
<thead>
<tr>
<th>Psychological measures</th>
<th>Sampling points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#0</td>
</tr>
<tr>
<td>BAI</td>
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</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>BDI</td>
<td>-0.15 (55)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>LCB</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>P(TMD)</td>
<td>-0.18 (55)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>P(ANG)</td>
<td>-0.26 (55)</td>
</tr>
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<td></td>
<td>*</td>
</tr>
<tr>
<td>P(CON)</td>
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</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>P(DEP)</td>
<td>-0.11 (50)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>P(FAT)</td>
<td>-0.21 (55)</td>
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<td>P(TEN)</td>
<td>-0.31 (55)</td>
</tr>
<tr>
<td></td>
<td>*</td>
</tr>
<tr>
<td>P(VIG)</td>
<td>0.10 (55)</td>
</tr>
<tr>
<td></td>
<td>*</td>
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</table>
Table 6.11

Spearman rank-order correlations (with sample sizes in parentheses) between psychological measures and CD4: CD8 lymphocyte ratios for the four sampling points during Army recruit training. Level of significance is also indicated (NS: not significant; *: p<0.05; **: p<0.01; *** p<0.001). Abbreviations are defined in the text.

<table>
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<tr>
<th>Psychological measures</th>
<th>Sampling points</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td></td>
<td>#0</td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
</tr>
<tr>
<td>BAI</td>
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<td>0.19 (53)</td>
<td>-0.05 (50)</td>
<td>-0.06 (33)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BDI</td>
<td>0.10 (55)</td>
<td>0.05 (53)</td>
<td>-0.18 (50)</td>
<td>0.09 (33)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LCB</td>
<td></td>
<td>0.10 (53)</td>
<td></td>
<td>-0.15 (33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P(TMD)</td>
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<td>0.15 (53)</td>
<td>-0.34 (50)</td>
<td>0.09 (34)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>P(ANG)</td>
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<td>-0.25 (50)</td>
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</tr>
<tr>
<td></td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P(CON)</td>
<td>0.10 (55)</td>
<td>0.14 (53)</td>
<td>-0.25 (50)</td>
<td>0.09 (33)</td>
</tr>
<tr>
<td></td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P(DEP)</td>
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<td>0.10 (53)</td>
<td>-0.17 (49)</td>
<td>-0.01 (33)</td>
</tr>
<tr>
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<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>P(FAT)</td>
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<tr>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P(TEN)</td>
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<td>-0.14 (50)</td>
<td>-0.35 (33)</td>
</tr>
<tr>
<td></td>
<td>**</td>
<td>*</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>P(VIG)</td>
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<td>-0.02 (50)</td>
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</tr>
<tr>
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Table 6.12

Significant Spearman rank-order correlations between psychological measures and lymphocyte measures across the four sampling points (#0, #1, #2, #3) during Army recruit training. Entries include correlation coefficient, sample size in parentheses, and the level of significance (**: p<0.01; ***: p<0.001). Abbreviations are defined in the text.

<table>
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<tr>
<th>Psychological measures</th>
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<th>Correlation coefficients</th>
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</tr>
<tr>
<td></td>
<td>CD4, #2</td>
<td>-0.37 (50) **</td>
</tr>
<tr>
<td>BDI</td>
<td>WBC, #3</td>
<td>-0.45 (33) **</td>
</tr>
<tr>
<td></td>
<td>CD4, #2</td>
<td>-0.43 (50) **</td>
</tr>
<tr>
<td>P(TMD)</td>
<td>CD16, #1</td>
<td>-0.41 (53) **</td>
</tr>
<tr>
<td>P(CON)</td>
<td>CD16, #1</td>
<td>-0.38 (53) **</td>
</tr>
<tr>
<td></td>
<td>CD19, #1</td>
<td>-0.39 (53) **</td>
</tr>
<tr>
<td></td>
<td>CD4, #2</td>
<td>-0.45 (50) ***</td>
</tr>
<tr>
<td>P(DEP)</td>
<td>CD16, #1</td>
<td>-0.35 (53) **</td>
</tr>
<tr>
<td>P(FAT)</td>
<td>CD3, #3</td>
<td>-0.46 (32) **</td>
</tr>
<tr>
<td>P(TEN)</td>
<td>WBC, #1</td>
<td>-0.36 (53) **</td>
</tr>
<tr>
<td></td>
<td>CD4, CD8, #0</td>
<td>0.40 (55) **</td>
</tr>
</tbody>
</table>
The vast majority of the 265 correlations (95%) were statistically not significant. No statistically significant correlations were found with LCB, P(ANG), P(VIG) and CD8 cells. None of the psychological measures correlated significantly with any lymphocyte measures on more than one occasion. However, CD16 cells correlated significantly with P(TMD), P(CON) and P(DEP) at time #1, while CD4 cells correlated significantly with BAI, BDI and P(CON) at time #2. Thus, none of the significant correlations was consistent over time.

6.3 Discussion

A comparison between successful recruits and those who did not complete recruit training had been expected to identify possible features that might predict success or failure. That expectation was not realised, however, as there were no significant differences between the two groups for any of the parameters being measured in this study (Table 6.1). Statistical comparisons between the so called study group and the remaining soldiers were not particularly robust, however, because of the small number in the study group. A larger total sample size, yielding a greater number of subjects who fail to finish recruit training, would have been required to adequately test this difference. Nevertheless, the failed soldiers were characterised, in a statistically non significant fashion, by lower cortisol levels and higher total white blood cell, CD3 cell and CD4 cell numbers. The decision was made, therefore, to perform subsequent analyses on the combined group which had become the study group. In effect, this study became more like that of Lee and colleagues (Lee et al., 1992, 1995) who examined the psychological, immunological and health aspects of US Air Force basic cadet training. Rather than comparing a study group with a control group, they followed the time course of their measures, using as their baseline the initial samples collected from cadets upon arrival at the training
academy. Since their sampling timetable was similar to that adopted in this study, their analytical procedures informed the present analysis.

The loss of records of infection meant that links between infection and the chosen parameters could also not be established. It should be emphasised, however, that, due to the paucity of data, no conclusions could be drawn from those data (Table 6.2). In the U.S. Air Force Academy study, risk of infection did not correlate with the observed reduction in *in vitro* immune responsiveness (Lee et al., 1992), but could be predicted by self-report measures of hostility, of response to environmental stressors, and of well-being (Lee et al., 1995). Furthermore, the use of a symptom checklist (Lee et al., 1992) may have led to the identification of a larger number of soldiers with infection.

The sustained, significant increase in the level of salivary cortisol (Table 6.3) seen over the three months of recruit training was indicative of continuing activation of the HPA axis (section 2.5.1). Such HPA activation has usually been assumed to result from the experience of an event that had been perceived as stressful, and to result in immune suppression (refer to section 2.5.1 for a detailed discussion). Thus, the perception of the stressor would have an impact on events. Furthermore, the nature of the stressor would also be expected to influence the way it was perceived.

Although it would be easy to assume that a stressful experience lasting some three months was a chronic stressor, the assumption deserves some examination, especially in terms of the adrenocortical response. Cortisol levels are usually thought of as a measure of the immediate adrenal response to acute stress (section 5.3). In humans, the increase in cortisol can take 30 min to develop after exposure to the stressor (Cacioppo et al., 1995; Kuhn, 1989), and would be expected to return to baseline within hours or days. The cortisol levels in the recruits, however, continued to rise over the three months of training, in a manner suggestive of the effects of
strenuous exercise (Nehlsen-Cannarella et al., 1997; Sharp and Parry-Billings, 1992; Smith et al., 1992; Weidemann et al., 1993) and despite the potential for habituation to the sampling protocol (Chapter 5). Perhaps this stressor, which was clearly not acute, was not chronic either (Breivik et al., 1996; Van Raaij et al., 1996), especially since the adaptation by sheep to a chronic stressor (Chapters 4 and 5) was not seen among the recruits. Indeed, a hint of adaptation to a chronic stressor, seen in the drop in cortisol at #1, was emphatically dispelled by subsequent measures. Thus, as soon as the recruits had adapted to an acute stressor (had learned something new) they were subjected to another. Perhaps it would be more accurately characterised as repetitive (Weiss et al., 1996) or prolonged stress (Arnett et al., 1987; Brenner, 1979; McKinnon et al., 1989).

The perception of the stressor may be as important as its characteristics. Coping style and the perception of a stressor can modulate the impact of the stressor on both immune and endocrine responses (Olff et al., 1995). On the one hand, the greater the perception of a stressor, the greater is the immune depression (Ravindran et al., 1996; Vedhara and Nott, 1996). On the other hand, a psychological reaction that is appropriate to the realistic degree of a stressor is least disruptive of immune function (Solomon et al., 1997). For example, the positive emotional engagement seen in air traffic controllers means that the rise in cortisol during a period of work is accompanied by a rise in salivary IgA (Zeier, Brauchli and Joller-Jemelka, 1996).

In the present study, immune function was monitored by counting lymphocytes. The cells that are involved in the adaptive immune response (section 2.3.7.2), and that participate in the network activity of the central immune system (section 2.6), namely the T and B cells, exhibited strikingly similar patterns of change over time (Table 6.3; Figure 6.1). Although the changes were not always statistically significant, they had all risen above baseline at #1, the only time when cortisol fell below baseline. In addition, they all appeared to fluctuate about the baseline over the
three months of recruit training. Thus, although the overall pattern of changes may not have been statistically compelling, it is possible to speculate about the possibilities.

The decrease in cortisol and increase in T and B cells at #1 suggest that, had recruit training continued with more of the same, or ceased at that time, the soldiers would have continued to recover from what was, by then, a chronic stressor. In fact, at that point the stressful regime of stage 1 of training was replaced by the new stressor of stage 2.

The relatively minor changes in CD19 lymphocyte (B cell) numbers measured in the present study are consistent with other studies, to the extent that reported results have been contradictory. It should also be borne in mind that only B cell responses to acute stress have been investigated by others to date. In earlier work it seemed that acute mental stress had no effect on B cell numbers (Bachen et al., 1992; Brosschot et al., 1992; Naliboff et al., 1991). Subsequently, when the chronic stress history of subjects had been taken into account, acute stress either had no effect on B cell numbers (Benschop et al., 1994), or it produced an increase in low chronic stress subjects and a slight decrease in high chronic stress subjects (Brosschot et al., 1994). Obviously, B cell behaviour in the absence of antigen is not well understood.

The confusion and contradictions surrounding B cell responses to stressors has also been seen in investigations of T cells, most of which also examined acute stress. A regime of moderate exercise, a situation vaguely similar to recruit training (the most similar to be found), resulted in an increase in total (CD3) T cells (Sharp and Parry-Billings, 1992; Smith et al., 1992; Weidemann et al., 1993). In the present study CD3 and CD8 T cells only increased significantly at the end of training, while CD4 T cell numbers fluctuated. Nevertheless, CD4 T cells always exceeded CD8 T cells, yielding a CD4:CD8 ratio always greater than 1.0. The overall effect in the present
study, while it may not represent an active cell-mediated response, suggests an absence of the immune suppression expected from the high levels of cortisol.

In the absence of any obvious antigenic challenge, the behaviour of the T and B cells observed in the present study is unlikely to have been clonally driven. Although it may be difficult to determine which is more correct, there are at least two possible explanations for the pattern of changes in circulating lymphocyte numbers. It may represent no more than the usual fluctuations in unstimulated populations of these cells. Alternatively, it may be indicative of compensatory network activity by the central immune system.

A similar pattern of values fluctuating about the baseline, without an overall increase or decrease, was also evident in some measures of mood state. The scores for P(FAT), reflecting a mood of weariness, inertia and low energy level, and P(ANG), a measure of a mood of anger and antipathy towards others (McNair, Lorr and Droppleman, 1992), rose, fell and rose during training (Table 6.4; Figure 6.2). The only measure of positive mood (P(VIG); McNair, Lorr and Droppleman, 1992) also showed non significant movements about the baseline. Maintaining these levels of a mix of negative and positive mood states appeared to have been an appropriate coping strategy since the other measures of negative mood states showed significant improvements over time (Table 6.4; Figure 6.2). Furthermore, it would not be difficult to imagine that P(FAT), P(ANG) and P(VIG) represent mood states associated with challenging physical activity.

P(TEN), which measures heightened musculoskeletal tension (McNair, Lorr and Droppleman, 1992) and correlates with stress symptoms (Kubitz, Peavey and Moore, 1986), has previously been shown to decrease significantly over a period of eight weeks of Army basic training (Kowal, Patton and Vogel, 1978). Such a pattern was obtained in the present study in both P(TEN) and BAI, which is a comparable
measure of anxiety and tension (Beck and Steer, 1993a). The same situation applied to P(DEP) and BDI, measures of depression and dejection (Beck and Steer, 1993b; Kowal, Patton and Vogel, 1978; McNair, Lorr and Droppleman, 1992). P(CON), a measure of confusion and bewilderment, also showed substantial reduction during Army basic training, both in earlier work (Kowal, Patton and Vogel, 1978; McNair, Lorr and Droppleman, 1992), and in the present study. Finally, P(TMD), the single global estimate of affective state (McNair, Lorr and Droppleman, 1992), showed a significant reduction in overall mood disturbance. Thus, the repetitive, prolonged stress of Army recruit training appeared to have been perceived by the participating soldiers as a positive experience, perhaps akin to the positive emotional engagement experienced by air traffic controllers on the job (Zeier, Brauchli and Joller-Jemelka, 1996).

The likely significance of the moderating effect exerted by perception of stressor on the stressor was seen in the CD16 lymphocyte (NK cell) response in the present study. Despite the finding that NK cell numbers correlate positively with cortisol levels in chronic stress (Kronfol et al., 1997), NK cell levels among the recruits fell as cortisol levels rose (Table 6.2; Figure 6.1). Indeed, in the absence of antigen, NK cell numbers have been shown to be more likely to increase, although transiently, as part of the acute fight or flight response (Benschop, Rodriguez-Feuerhahn and Schedlowski, 1996). Thus, a positive perception of a stressor appears to exert major influence on the elements of the immune response mediated by NK cells.

The positive perception of the stress of recruit training seen in the present study has meant that the behaviour of the immune system is unlikely to have been indicative of an immune response to stress. Thus, although a soldier may not wish to express it in these terms, the psychosocial environment of recruit camp may have facilitated a style of bonding and socialising that was conducive to a type of emotional disclosure, a behaviour that has been reported to buffer the effects of stressors on
immune function (Booth, Petrie and Pennebaker, 1997).

A comparison between this study and that of U.S. Air Force Academy cadets undergoing Basic Cadet Training (Lee et al., 1992, 1995), tends to highlight the significance of these findings. For some undefined, perhaps indefinable, reason the Air Force Academy cadets responded psychologically to the stress of Basic Cadet Training in a comparatively negative way. Their sense of well-being decreased, locus of control became more external, and there were increases in perceived stress, response to stress, sleep problems and feelings of hostility. Not surprisingly, their immune status deteriorated, with a significant drop in T cell proliferation. Nevertheless, the cadets that had experienced viral infections were indistinguishable from healthy cadets in terms of the immune parameters being measured, but could be separated on psychological grounds. Thus, the difference in the perception of the stressor between the two studies correlated with different immunological responses. The link between the psychoneuroimmunology and infection has been demonstrated in an indirect fashion by Lee and co-workers (1992, 1995), but has not been examined adequately in the present study due to lack of statistical robustness in the record of infection data.

Rank-order correlations between psychological and immunological measures, calculated in similar ways, revealed a common pattern in both studies (Lee et al., 1995). The correlations were predominantly both negative and statistically non-significant. This seems to suggest either that psychological and immunological measures might not really correlate or that any statistically significant correlation might be at the level of whole system interaction between the psychoneural system and the immune system. Perhaps the components of each system are more closely associated with other elements of their own system than with those of the other - this possibility is consistent with both explanations. Furthermore, the likelihood that other immune measures might have been more appropriate is remote, since the Air
Force Academy study (Lee et al., 1995) was indeed based on different immune measures.

The present study has also suggested that the recruitment and training procedures currently employed by the Australian Army may be adequate. The Army may have to accept that not all recruits will succeed (11% of the study cohort failed), since the majority finish training more mature psychologically and more robust immunologically.

In this study, the non-antigenic challenge of recruit training was accompanied by both psychoneural improvement and the maintenance of positive immune status, despite a sustained elevation of cortisol levels. Thus, although the evidence is not as compelling as for the sheep, it could be interpreted as being not inconsistent with the idea that the immune system and the psychoneural system are not operating independently, but are interconnected in some way.
CHAPTER 7

GENERAL DISCUSSION

7.1 Summary of results

The aim of this investigation was to contribute to an understanding of the homeostatic aspect of immune function whereby the immune system acts collaboratively with the psychoneural and endocrine systems to maintain the functional integrity of the organism as a whole, rather than merely to defend the organism against invaders. The exploration of this issue involved three related experiments.

The first sheep experiment was a pilot study. It showed that the sheep appears to be a suitable animal for studies of concomitant changes in immune function, behaviour and cortisol levels. Bringing the sheep from a paddock into indoor confinement proved to be a stressful change. However, it was one to which the sheep were able to adapt over a few days or weeks which seemed to be a suitable time period for such a study. Finally, in order to further refine the study, total isolation of individual sheep and a more formalised measure of behaviour were indicated.

The second sheep experiment followed the time course of changes after sheep were confined, either in groups or individually (a known stressor, based on the pilot study). Measures included an approach-avoidance test for studying behaviour, as
well as enumeration of some immune cells during this period. Several questions were being asked:

(1) are there changes in the numbers of circulating T lymphocytes that accompany changes in cortisol levels and behaviour and, if so, what trend is indicated by these changes (does it suggest a connection between immune and psychoneural systems or not)?

(2) are these changes influenced by the type of stressor (individual versus group confinement) or the duration of the stressor and, if so, does this relationship suggest a connection between immune and psychoneural systems or not?

The decline in cortisol levels over the period suggested that the initial confinement was stressful, as expected. Some changes in behaviour were recorded, notably a resumption of normal flocking behaviour and a reduction in motor activity in the approach-avoidance test. Isolation stress (individual confinement), but not group confinement, had a significant effect on these changes in behaviour. Changes were also seen in numbers of circulating T lymphocytes, with CD4 cell levels increasing and those of CD8 cells decreasing during adaptation to the stressor. Again, isolation stress, but not group confinement, had a significant effect on these changes in T cells. These results can be interpreted as demonstrating recovery (compensation) following an adverse effect of stress on the CIS. Isolation was a more severe stressor than was group confinement. Therefore, considering both the lymphocyte and the behavioural responses, these findings support the idea that the immune system and the psychoneural system are not operating independently, but are interconnected in some way as has been suggested in previous reports.

The third experiment introduced the human psychoneural dimension into the investigation. The Army recruits were stressed, but their expectation of eventual success and the positive reinforcement of completing various stages of the training added quite a different element to the relationship between the psychoneural and
immune systems. In addition, it provided an opportunity to compare the soldiers that failed with those that successfully completed recruit training. The questions being asked were:

(1) are there concomitant changes in cortisol levels, psychological state and numbers of circulating lymphocytes during recruit training and, if so, what trend is indicated by these changes (does it suggest a connection between immune and psychoneural systems or not)?

(2) are these changes (or relationships) influenced by the outcome of recruit training (success or failure) and, if so, does this effect support the idea of a connection between immune and psychoneural systems or not?

These particular measures, with the small number of subjects that failed recruit training, were not able to distinguish any clear differences between the soldiers that succeeded and those that failed. There was a suggestion that lower cortisol levels and higher total white blood cell, CD3 cell and CD4 cell numbers may have characterised the failed subjects. However, with a large standard error, this was not statistically significant. A greater number of subjects would have been required to adequately test this difference. Aggregating the data, there were significant and telling changes in psychological measures during the course of training, but the immune variables measured appeared to fluctuate independently of these changes. Nevertheless, some parallels could be drawn. On the basis of the sheep experiments in this investigation and previous reports, sustained high levels of cortisol would have been expected to correlate with both deteriorating psychological status and immune suppression. In fact, the psychological health of the recruits improved during training while their immune systems remained healthy. Thus, although the evidence is not as compelling as for the sheep, it could be interpreted as being consistent with the idea that the immune system and the psychoneural system are not operating independently, but are interconnected in some way.
7.2 Experimental approach

It is much easier to study the individual parts of a biological system than it is to study the system as a whole, as evidenced by the rarity of publications that report on entire biological systems. However, an understanding of complex unsolved health issues may well require a clearer understanding of how the organism functions as a whole. Homeostasis and autopoiesis are two of the most powerful concepts that contribute to an explanation of the relationships among individual organs, cells and molecules and the function of the body as a whole. These concepts, in turn, rest on the network approach to the study of organisms. This investigation, therefore, has examined interactions among physiological stress, the psychoneural system and the immune system in a way that is consistent with the network paradigm. In other words, the research questions were being asked in the context of network biology. They were concerned with: the non-pathological, physiological state; non-linear, network activities; and, the network of sub-networks that constitutes an autopoietic organism. In addition, the immune system was acknowledged as consisting of a central immune system (CIS - the network) and a peripheral immune system (PIS - the clonally driven defence system), so that defence becomes a subset of overall immune function.

The situations (the experimental conditions) in which the sheep and the recruits found themselves were intended to produce psychoneural responses and to result in activation of the HPA axis. "Separation and the anxiety it causes ......is a potent stimulus of the hypothalamic-hypophyseal-pituitary-adrenal axis in the sheep and leads to high plasma concentrations of hydrocortisone" (Lynch, Hinch and Adams, 1992). The anxiety, and perhaps fear, that arose in the isolated sheep (Chapter 5) was reflected in the acute deterioration and disorganisation of their normal behaviour patterns, followed by recovery. Given that anxiety and fear, and the attendant rise in
cortisol, are also experienced by humans (section 2.5), it is reasonable to make
comparisons between the psychoneural experiences of the sheep and of the recruits.
Measuring the psychoneural status of humans is comparatively straightforward. In a
more-or-less direct comparison with the sheep, anxiety was measured in the recruits,
among an array of standard psychological tests for both state and trait characteristics
(Chapter 6). As was the case with cortisol, the psychoneural measures could have
come from any other PNI study. However, they were being made, and subsequently
analysed, within the context of the network paradigm.

“There is generally a poor correlation between how tense, anxious, or fearful a
person feels and the amount of increase in heart rate, blood pressure, or level of
cortisol in response to a stressful event” (Kagen, 1994). The present investigation
has provided evidence that is indicative of close links among the psychoneural,
neuroendocrine and immune systems. It has confirmed the poor correlation between
psychoneural state (“how a person feels”) and level of cortisol, but has suggested a
stronger correlation between psychoneural and immune states.

The majority of investigations of PNI (section 2.5.4) are consistent with the linearity
that is fundamental to the clonal selection paradigm. Therefore, when they examine
the effects of one system on the operation of another, their analysis could be likened
to solving simultaneous equations. Analysis within the network paradigm, however,
could be said to be attempting to solve a single complex equation in which the
subsystems (sub-networks) become terms in the equation - obviously a much more
difficult task, reflected in the more descriptive tone of network-based PNI studies
(section 2.6.6), including the present investigation. In fact, the bare results of this
investigation of sheep and soldiers can only be described as tentative, at best.
Nevertheless, adopting the logic of the network approach can lead to different
interpretations, so that these results, together with many others that have been
interpreted in other ways consistent with the clonal selection paradigm (section
2.5.4), provide evidence in support of the notion that the immune network (the CIS) appears to be a sub-network of the organismic network.

For example, as limited as they are, the results may suggest an explanation for the observation that the perception of a stressor is important (section 2.5.1). The fact that the correlations between psychoneural measures and immune measures among the recruits were predominantly statistically non significant (Chapter 6) suggests two things. Firstly, there would appear to be a greater degree of connectivity within each of the psychoneural and immune sub-networks than between them. Secondly, there is indeed a low level of connectivity between them. Thus, the perception of a stressor could be said to be able to influence the immune state because of the connectivity between the two relevant sub-networks.

Moving from broad issues to the particular, the robustness of the research design could have been improved in several ways. The decision to allocate soldiers to control or study group a posteriori involved a risk, evident when the two groups emerged as statistically indistinguishable. This situation would not have arisen if a separate control group had been used, perhaps a matched group of young men either in a fitness class or members of a sports team undergoing off-season training.

The third category of VRMs, the immunoglobulins, could also have been measured. Specifically, sIgA has been the focus of several PNI studies in both humans and sheep (sections 2.5.4 and 2.6.6). Changes in its concentration could have provided another surrogate end point for CIS activity, especially as it could be seen as both an enumerative and a functional measure.
7.3 Conclusion

Clonal selection has been characterised as “the central paradigm of immunology” (Paul, 1991). Its usefulness is evident in the way in which it has provided the theoretical basis for the fight against infection, at the same time generating very detailed knowledge about the workings of the immune system (section 2.3). However, “the immune system is at work in health as much as in disease...There is just as much reason to interpret immunity as integrated control within, a control that sustains an appropriate relatedness to the ecological community without (sections 2.4 and 2.5). The immune system is a sophisticated means of preserving biological identity” (Rolston, 1996).

In order to explore, and make sense of, the physiology (biology) of the immune system, it became necessary to move away from the pathology-based clonal selection/defence paradigm into the network paradigm (section 2.6). The adoption of the network paradigm, in turn, imposed an obligation to maintain consistency with the paradigm, to work within the coherent theoretical/intellectual framework of the paradigm. Therefore, logically, the immune network has been seen as a sub-network of the network that is an autopoietic organism. Furthermore, the network and its sub-networks exhibit a number of emergent characteristics, and operate in a self-referential mode whereby external perturbations result in compensatory activity (section 2.7). Hence, the research questions that have been asked have been consistent with the paradigm.

Consequently, a different approach to experimental work in PNI has been adopted. The challenge has been to identify and exploit components of the organism, or the system under observation, that vary in ways that indicate organismic or systemic activity. This indirect approach, necessary if dis-integration is to be avoided, would
be expected to yield progress in very small steps - certainly the case in this investigation. Thus, the sheep study suggested that, in the process of recovering from the adverse effects of stress, the immune and psychoneural systems are not operating independently, but may be interconnected. In a different situation in which success was anticipated, the immune and psychoneural responses of soldiers in recruit training tended to confirm the sheep findings. Hence, it would seem reasonable to propose that the immune and psychoneural systems may behave as sub-networks of the larger organismic network.

Clearly, this proposal requires further testing to resolve the current uncertainty surrounding it. Rather than more of the same, the research design ought to be improved in several ways. Firstly, working only with human subjects would eliminate any questions about the validity of cross-species comparisons. Secondly, a better defined control group (section 7.2) would increase the level of confidence in the findings. Thirdly, non-invasive sampling could lead to both cortisol and sIgA being measured in saliva (section 7.2), while Sense of Coherence, a supposedly culture-neutral scale (section 2.5.1), could become the psychoneural measure. These three improvements would then pave the way for the fourth improvement - the proposal could be tested in a variety of different situations. This would have the dual effect of testing the proposal and of extending its applicability.

For example, the belief that the lasting effects of repeated acupuncture may result from a type of physiological relearning (section 2.5.6) could be examined from the immune network perspective, especially in the light of the non-antigenic nature of the treatment. It could be hypothesised that the stressor that is acupuncture needling is perceived positively by the patient, leading to both psychoneural and immune enhancement.
Given the suggestion that the perception of a stressor may influence psychoneural and immune responses, naturally occurring differences in perception should merit investigation. It could be hypothesised that differences in the perception of a stressor have a cultural as well as an individual basis. Therefore responses to supposedly stressful life-events (for example, bereavement) could be examined in a cross-cultural, comparative study. Such a study could either be an anthropological exercise involving several countries or it could be conducted in a large multicultural city like Sydney.

Perhaps the placebo effect on immune function is mediated by psychoneural responses to the procedure being used (Brown, 1998; Kelkar and Ross, 1994). Perhaps it is a significant factor in the success of a range of therapies (section 2.5.6). Perhaps the physiological (including immune) changes experienced by dissociative identity disorder patients when they switch between personalities (Ross, 1994) is a reflection of the degree of connectedness among the psychoneural, immune and other body systems. Perhaps health could be said to depend on the maintenance of optimal connectedness within and among all the sub-networks of an individual (section 2.6.6).

The evidence presented in this investigation tends to strengthen the network/autopoiesis paradigm. Consequently, it suggests that homeostasis is properly characterised as a property of the whole organism. In autopoietic terms, then, homeostasis could be defined as the maintenance of network stability, a definition that remains faithful to Cannon’s original concept.
CHAPTER 8

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