The Effect of Major Surgery on Systemic and Splanchnic Immune Function: Examining the Two-hit and Gut hypotheses of the Systemic Inflammatory Response Syndrome

An Experimental and Clinical Study

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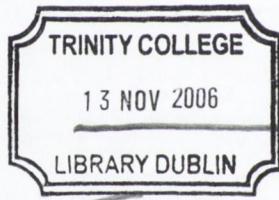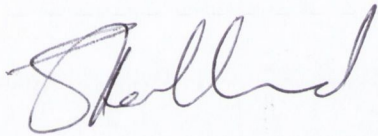
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Summary

The systemic inflammatory response syndrome (SIRS), sepsis and multiple organ dysfunction syndrome (MODS) are recognized sequelae of complex major surgery and underlie significant morbidity and mortality. The systemic immunoinflammation underlying SIRS and MODS in the presence or absence of infection is not fully understood, but two of the current hypotheses are the 'two-hit' and gut hypotheses. The 'two-hit' hypothesis proposes that if the host immune system has been primed by an initial insult, the immune response to any subsequent or second insult will be greatly amplified. The gut hypothesis incriminates bacteria and endotoxins derived from the gastrointestinal tract as triggers, which initiate, perpetuate or exacerbate a systemic inflammatory response resulting in the development of SIRS.

In this work, a cohort of patients undergoing curative surgery for upper gastrointestinal malignancy was studied, some of who received neoadjuvant chemoradiotherapy. The acute-phase response was assessed by measurement of C-reactive protein levels. T-cell and monocyte function were assessed by measuring expression of activation markers on these cells *in vivo*, while neutrophil function was assessed by measurement of intranuclear NF-kappa B *in vivo*. Intraoperative measurement of splanchnic blood flow was performed by means of gastric tonometry, while pre- and postoperative gut function was estimated by measurement of intestinal permeability.

The studies found that neo-adjuvant chemoradiotherapy resulted in phenotypic priming and activation of the CD4+ T-cell subpopulation, which in theory heightens the consequences of a "second-hit" in the postoperative period and increases the risk of perioperative sepsis or systemic inflammatory response syndrome. The research also demonstrated that a reduced intraoperative splanchnic blood flow is significantly associated with increased post-operative complications, increased intestinal permeability and significant elevation in systemic acute phase markers. Alteration in monocyte activation markers both intraoperatively and in the postoperative period was also evident as a consequence of this hypoperfusion.

Publications to date

Published Article:

- Intraaoperative splanchnic hypoperfusion, increased intestinal permeability, downregulation of monocyte class II major histocompatibility complex expression, exaggerated acute phase response, and sepsis.

  Jane Holland, Michael Carey, Niall Hughes, Karl Sweeney, Patrick J Byrne, Martin Healy, Narayanasamy Ravi, John V Reynolds.

  Am J Surg 2005; 190: 393 – 400

Published Abstracts:

- Association of intraoperative splanchnic hypoperfusion with heightened acute phase response, increased intestinal permeability and downregulation of monocyte function.

  J C Holland, K J Sweeney, N Hughes, M Carey, M Healy, A R McGill, P J Byrne, J V Reynolds.

  Br J Surg 2003; 90: p631

- Multimodal Therapy results in systemic T-cell activation and heightened T-cell response to surgery.

  J C Holland, K J Sweeney, P J Byrne, J V Reynolds.

  Ir J Med Science 2002; 171 (3) (Suppl 2): p 42.

Presentations to Learned Societies

- Association of intraoperative splanchnic hypoperfusion with heightened acute phase response, increased intestinal permeability and downregulation of monocyte function.

  J C Holland, K J Sweeney, N Hughes, M Carey, M Healy, A R McGill, P J Byrne, J V Reynolds.

  RAMI Registrar's Prize Meeting (Surgical Section), RCSI, 15/1/2003

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  SARS, Leeds, 10/1/2003

- Multimodal therapy results in systemic T-cell activation and heightened T-cell response to surgery.

  J C Holland, K J Sweeney, P J Byrne, J V Reynolds.

  Association of Upper GI Surgeons, Manchester, September 2002

- Suppression of Monocyte HLA-DR expression following major upper gastrointestinal surgery is associated with reduced peroperative splanchnic perfusion.

  J C Holland, J M W O'Riordan, K J Sweeney, P J Byrne, N Hughes, M Carey, J V Reynolds.

  Irish Society of Gastroenterology, Westport, June 2002

- Neo-adjuvant chemoradiotherapy primes the immune system and alters subsequent immune response to surgery for upper gastrointestinal carcinoma.

  J C Holland, J M W O'Riordan, K J Sweeney, P J Byrne, J V Reynolds.

  Irish Society of Gastroenterology, Westport, June 2002

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  J C Holland, K J Sweeney, P J Byrne, J V Reynolds.

  Sir Peter Freyer Symposium, Galway, 2002

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Abbreviations

ABG	Arterial blood gas
Ag(s)	Antigens
APC	Antigen presenting cell
ARDS	Adult respiratory distress syndrome
CAM	Cell adhesion molecule
CARS	Compensatory anti-inflammatory response syndrome
CSF	Colony stimulating factor
CTLA-4 Ig	Cytolytic T lymphocyte associated molecule-4
CRP	C-reactive protein
DCIS	Disseminated intravascular coagulation
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
FasL	Fas Ligand
FITC	Fluourescin isothiocyanate
GALT	Gut-associated lymphoid tissue
G-CSF	Granulocyte colony stimulating factor
HDU	High dependency unit
HLA	Human leucocyte antigens
HPLC	High Performance Liquid Chromatography
ICU	Intensive care unit
Ig	Immunoglobulin
IFN-γ	Gamma interferon
IL	Interleukin
IL-2 R	IL-2 receptor (CD25)
IMA	Inferior mesenteric artery
LMER	Lactulose: mannitol excretion ratio (dual sugar test)
LPB	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
M cell	Microfold cell
MHC	Major histocompatibility complex
MODS	Multiple organ dysfunction syndrome
NF kappa B	Nuclear factor kappa B

NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PEG	Polyethylene glycols
PGE_2	Prostaglandin E_2
pHi	(Gastrointestinal) intramucosal pH
PI	Propidium Iodide
PMN	Polymorphonuclear leucocyte
SAA	Serum amyloid A
SIRS	Systemic inflammatory response syndrome
SMA	Superior mesenteric artery
TCR	T-cell receptor
TGF β	Transforming growth factor β
Tc	Cytotoxic T cell
Th	T helper cell
TNF-α	Tumour necrosis factor alpha
TNF-β	Tumour necrosis factor beta
Ub	Ubiquitin

CHAPTER 1: INTRODUCTION

1.1 ABSTRACT OF THESIS

The systemic inflammatory response syndrome (SIRS), sepsis and multiple organ dysfunction syndrome (MODS) are recognized sequelae of complex major surgery and underlie significant morbidity and mortality. The systemic immunoinflammation underlying SIRS and MODS in the presence or absence of infection is not fully understood, but two of the current hypotheses are the 'two-hit' and gut hypotheses. The 'two-hit' hypothesis proposes that if the host immune system has been primed by an initial insult, the immune response to any subsequent or second insult will be greatly amplified. The gut hypothesis incriminates bacteria and endotoxins derived from the gastrointestinal tract as triggers, which initiate, perpetuate or exacerbate a systemic inflammatory response resulting in the development of SIRS.

In this work, a cohort of patients undergoing curative surgery for upper gastrointestinal malignancy was studied, some of who received neoadjuvant chemoradiotherapy. The acute-phase response was assessed by measurement of C-reactive protein levels. T-cell and monocyte function were assessed by measuring expression of activation markers on these cells *in vivo*, while neutrophil function was assessed by measurement of intranuclear NF-kappa B *in vivo*. Intraoperative measurement of splanchnic blood flow was performed by means of gastric tonometry, while pre- and postoperative gut function was estimated by measurement of intestinal permeability.

The studies found that neo-adjuvant chemoradiotherapy resulted in phenotypic priming and activation of the CD4+ T-cell subpopulation, which in theory heightens the consequences of a "second-hit" in the postoperative period and increases the risk of perioperative sepsis or systemic inflammatory response syndrome. The research also demonstrated that a reduced intraoperative splanchnic blood flow is significantly associated with increased post-operative complications, increased intestinal permeability and significant elevation in systemic acute phase markers. Alteration in monocyte activation markers both intraoperatively and in the postoperative period was also evident as a consequence of this hypoperfusion.

1.2   LITERATURE REVIEW

1.2.1   THE IMMUNE SYSTEM

(i)   Introduction

The immune system has evolved in order to protect us from foreign and harmful substances, such as infectious agents and toxins. An essential task of the immune system is to differentiate whether a stimulus, or antigen, is dangerous or harmless, and to mount the appropriate response. The destruction of foreign bodies or toxins is desirable; attacks against the host's own tissues are not.

The human body mounts two different types of immune response. The first of these is the innate, or non-specific, immune system. This system becomes active independently of the invading pathogen and the response occurs to the same extent no matter how many times the infectious agent is encountered (Delves et al, 2000). The specific, or adaptive, immune system can produce highly specific reactions to their respective antigens, which becomes more efficient on repeated exposure to that antigen.

The first of these systems to develop evolutionally was the innate, or non-specific, immune system. It has a limited number of antigen receptors (or recognition sites), which focus on recognizing a small number of structures typically found on the surfaces of infectious microorganisms. These receptors are genetically inherited and passed from one generation to the next (Medzhitov et al, 2000). The innate immune system incorporates both cellular and soluble components, the former including granulocytes, monocytes and natural killer cells. The mechanisms of innate immunity are usually deployed within hours of exposure to a pathogenic agent (Burmester et al, 2003; Delves et al, 2000).

The adaptive immune system may respond to a stimulus with either a humoral or cellular line of defense, dependent on the stimulating antigen and the cytokine milieu. It involves the proliferation of specialised lymphocytes, T cells and B cells (Delves et al, 2000). Clonal expansion of these cells is necessary in order to mount an efficient immune response, but takes a minimum of 3 to 5 days, and may take weeks (Medzhitov et al, 2000). The humoral response is mediated by the B cells, which mature in healthy bone marrow, and results in the generation of an antibody reactive to the stimulating antigen. All antibodies are glycoproteins with similar structures, known collectively as immunoglobulins (Ig). They can be transferred passively to another individual by injection of serum, thus conferring some immunity (vaccination). The cellular response is mediated by T cells. Examples of cellular immune responses include the rejection of a graft by host lymphoid tissue cells, and the reverse, graft versus host disease. The adaptive immune system has a tremendous capacity to recognize almost any antigenic structure, but can fail to distinguish foreign antigens from self antigens, leading to auto-immune disorders. In contrast, the innate immune system deploys a more limited number of receptors with specificity for conserved microbial structures (Medzhitov et al, 2000).

(ii)   Origin of cells of the immune system

All blood components, including blood cells, arise from stem cells within the bone marrow. These cells are among the few body cells that can self-replicate, i.e. divide without differentiating. These stem cells may differentiate into either myeloid or lymphoid progenitor cells. Myeloid progenitor cells may differentiate into the following: megakaryocytes, which break up to form platelets; erythroblasts, which differentiate into red blood cells; myeloblasts, which differentiate into the polymorphonuclear leucocytes (basophils, eosinophils and neutrophils); monoblasts, the precursors to monocytes; and dendritic cells. The most important cells of the immune system are the lymphocytes, which originate from the lymphoid progenitor cells of the bone marrow. Two main types can be identified: T cells, which mature in the thymus and are responsible for the cellular adaptive immune response, and B cells, which produce antibodies (humoral response) (Burmester et al, 2003). The initial stages of development of these lymphocytes do not require the presence of an antigen, but once these cells express a mature antigen receptor, their further survival and differentiation become antigen dependent (Delves et al, 2000). A third type of lymphocyte, the natural killer cell, also originates from the lymphoid precursor cell. These cells are related to T cells, but their exact origin is still undecided.

(iii)   Antigens and superantigens

Molecules recognized by receptors on lymphocytes are generally referred to as antigens and range from small chemical structures to highly complex molecules. Antigens are recognized on the basis of the shape of a region of their structure - this site is named an epitope; a paratope is the corresponding section of the receptor on the immune cell to which they bind. They are able to stimulate an immune response by reacting with the T-cell receptor (TCR) and some with the B-cell receptor (antibody). Although antibodies and T-cell receptors can accurately distinguish between closely related antigens, they may sometimes cross-react with apparently unrelated antigens, either because the two antigens happen to share an identical epitope or because two different epitopes have similar shapes (Delves et al, 2000).

The first stage of an immune response to any antigen involves modification of the antigen by antigen presenting cells (APC), such as dendritic cells and monocytes. Unlike the subsequent restricted binding of antigen to lymphocytes this is not an antigen specific process. Antigen is processed by these cells and then carried and 'presented' to lymphocytes. T-cells cannot recognize antigen without such processing and since activation of T-cells is essential for most immune responses, antigen processing is crucial. The efficiency of T-cell activation is enhanced by secretion of cytokines by APC's previously activated by processing antigens (Delves et al, 2000).

Superantigens are mainly bacterial or viral products that induce nonselective T-cell activation. The response of T-cells to superantigen is not clonal; a large number of different T-cells may be activated and an excessive cellular response is triggered that can lead to lethal toxic shock (Arad et al, 2000; Marrack et al, 1990). Included in this family are the staphylococcal enterotoxins A to E (among which SEB is the most prominent), toxic shock syndrome toxin 1 (TSST-1) and the streptococcal pyrogenic exotoxins SPEA and SPEC (Bohach et al, 1990; Marrack et al, 1990). Bypassing the APC-dependent presentation of conventional antigens, superantigens bind directly to most major histocompatibility (MHC) class II molecules and can stimulate 30 – 40% of all T-cells to divide and produce cytokines. Toxicity results from massive induction of cytokines derived from T-helper-type-1 (Th1) cells, which include interleukin (IL)-2, gamma interferon (IFN-γ) and tumor necrosis factor (TNF)-β (Uchiyama et al, 1989).

(iv)   Major histocompatibility complex antigens

The major histocompatibility complex (MHC) antigens were discovered in humans in the 1950's and consist of cell-surface glycoproteins that exhibit highly extensive genetic polymorphism with multiple alleles at each locus. Human MHC antigens are also called human leucocyte antigens (HLA). The HLA genes that are involved in the immune response fall into two classes, one and two, which are structurally and functionally different (the classes denote the historical order of discovery) (Klein et al, 2000).

Class I molecules are expressed by most nucleated cells, although the level of expression varies depending on the tissue. Each HLA class I molecule consists of a heavy chain (α) and a light chain (β). The α chain is a transmembrane (TM) protein, which is subdivided into three domains (α_1, α_2 and α_3). The α chains form non-covalent bonds with the extracellular β microglobulin. The three main types of human class I molecules are HLA-A, B and C. In contrast, expression of class II molecules is restricted to a subgroup of immune cells that includes B-cells, activated T-cells, macrophages, dendritic cells and thymic epithelial cells. In the presence of interferon-γ, however, other types of cells can express class II molecules (i.e. pancreas and gut). Each class II molecule consists of 2 chains, an α and a β chain. Both of these are transmembrane proteins and are both subdivided into two parts (α_1, α_2, β_1, β_2). The α_2 and β_2 chains are highly preserved, whereas the α_1 and β_1 are highly polymorphic. In humans there are three groups of class II antigens: the loci are known as HLA-DP, HLA-DQ and HLA-DR (Burmester et al, 2003; Delves et al, 2000; Klein et al, 2000).
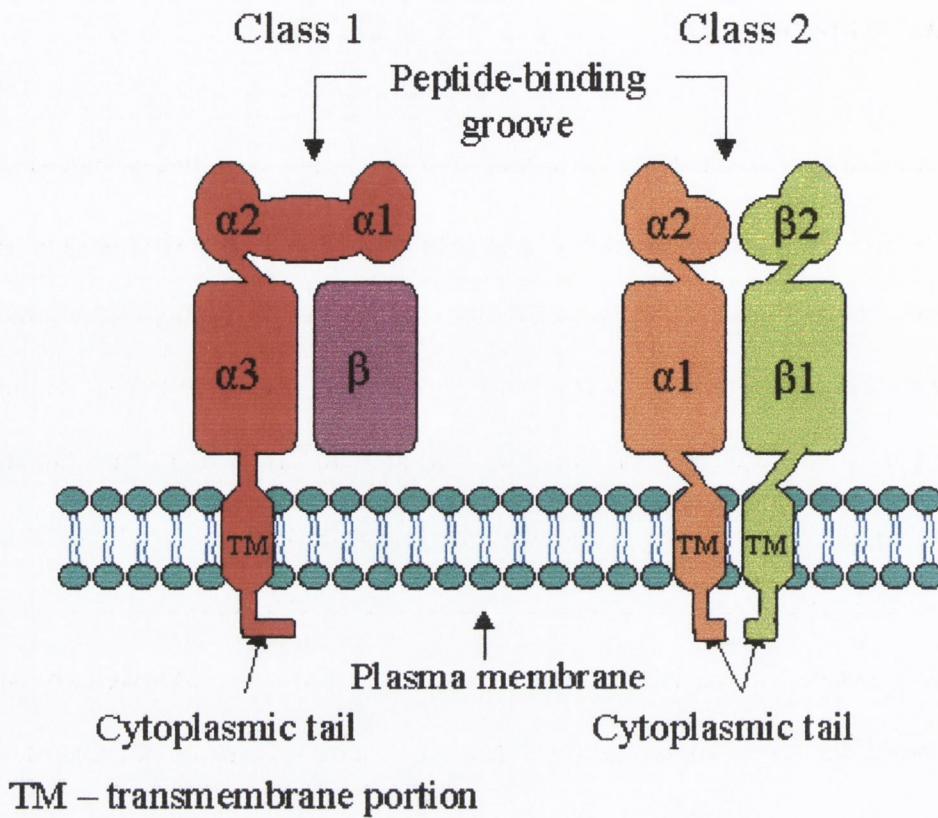
Figure 1.1.   MHC class I and II molecules

The function of both class I and II molecules is the presentation of short, pathogen derived peptides, to T-cells, a process that initiates the adaptive immune response. T-cells characteristically possess T-cell receptors (TCR) that recognize processed antigen presented by MHC molecules. Endogenous antigens (i.e. within cells infected by viruses or bacteria, or mutated cancer proteins) are processed by the endoplasmic reticulum and presented by MHC class-1 bearing cells exclusively to T-cells expressing the CD8 receptor (cytotoxic T-cells). Exogenous antigens are processed by the lysosomal (endosomal) route and presented by MHC class II antigens to T-cells expressing CD4 receptors (helper T-cells) (Delves et al, 2000; Klein et al, 2000).

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(v)   Adhesion molecules

In order for cells of the immune system to be effective in mounting a defense, they must be able to leave the circulating blood system and migrate into the tissues. As they circulate within the blood stream, leucocytes are subject to extreme physical conditions – the blood exerts a shear stress of approximately 50 dyn per square centimeter within blood vessels, quickly dislodging cells that touch the vessel wall. Therefore, the recruitment of leucocytes to within the tissues is regulated, and assisted, in part by cell adhesion molecules (CAMs), which participate in the initial attachment, adhesion and subsequent extravasation. Not only are these molecules anchors to assist in leucocyte extravasation, they may vary from tissue to tissue, and so can function as tissue-specific recognition molecules. CAMs are divided into four main families: selectins, integrins, cadherins and the immunoglobulin superfamily (Tedder et al, 1995; von Andrian et al, 2000). Selectin function is restricted to the vascular system, whereas the integrins, cadherins and immunoglobulin superfamily are involved in a wide variety of cell-cell interactions (Tedder et al, 1995). Although proper antigen presentation is required for T-cell recognition and activation, additional signals derived from accessory or co-stimulatory receptors are also required. Many distinct T-cell surface molecules appear capable of delivering these second signals, including concurrent binding of cell adhesion molecules on the cell surface (Brunmark et al, 1997; de Fougerolles et al, 1994; Springer, 1990; Ybarrondo et al, 1994).

The selectin family is composed of three glycoproteins designated by the prefixes E (endothelial), L (leucocyte) and P (platelet) to denote the cells on which they were first described (Ley et al, 2004). They are multifunctional adhesion molecules whose primary function is to mediate the initial interaction between circulating leucocytes and the cells of the endothelium for leucocyte migration, specifically the tethering, rolling and slow rolling steps. This rolling begins within minutes of tissue injury, reaching a peak after approximately 20 to 40 minutes (Ehrhardt et al, 2004; Schlossman et al, 1995; Tedder et al, 1994).
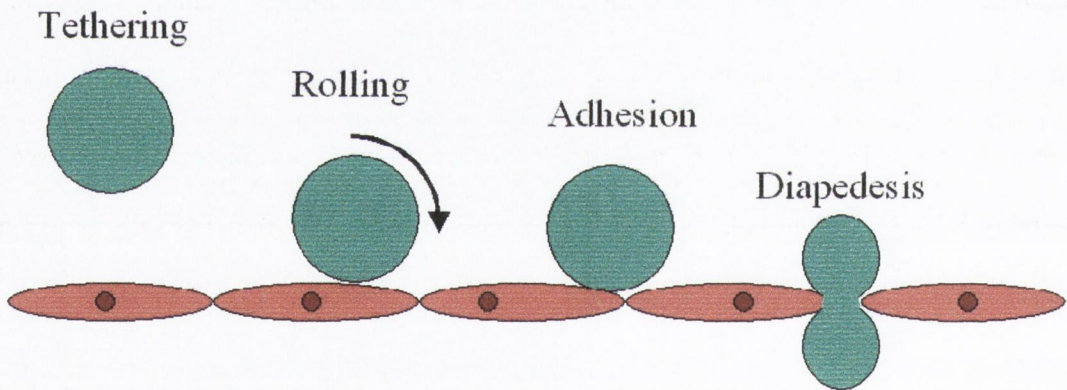
The selectins mediate the initial interactions between circulating leucocytes and the cells of the endothelium for leucocyte migration, specifically the tethering, rolling and slow rolling steps. Subsequently, integrins and members of the immunoglobulin superfamily interact to stop rolling and mediate firm adhesion, which precedes PECAM-1 dependant diapedesis.

Figure 1.2.   Leucocyte migration

L-selectin (CD62-L) expression is limited to haemopoetic cells, with most classes of leucocytes expressing L-selectin at some stage of development, including naïve T-cells and various subpopulations of mature T-cells. L-selectin is shed by proteolytic cleavage from the surfaces of neutrophils and lymphocytes during inflammation. It is shed rapidly following major injury, resulting in the generation of soluble L-selectin (sCD62-L) which then circulates in plasma. Leucocyte expression of CD62-L and plasma concentrations of sCD62-L have been studied to determine whether they are predictive of subsequent clinical outcome (Carlos et al, 1994; Kerner et al, 1999; Tedder et al, 1994).

The integrins are transmembrane cell surface proteins that bind to cytoskeletal proteins and communicate extracellular signals. Each integrin contains a noncovalently bonded α and β subunit. They are subdivided into 5 main families according to the β chain subunit, with the β1 and β2 integrins playing a key role in leucocyte-endothelial interaction, mediating firm adhesion. These integrins are not constitutively active, but must be activated by chemokines present at sites of inflammation (Brown et al, 2003; Springer, 1994; Weber, 2003).

Cadherins are calcium dependent cell adhesion molecules with responsibility for both establishing molecular links between adjacent epithelial cells and a number of subsequent cell-cell interactions. The regulated formation of these cell-cell and cell-matrix junctions is crucial for normal embryogenesis and in maintenance of normal tissue architecture in the adult (Wheelock et al, 2003; Zbar et al, 2004).

The immunoglobulin superfamily of cell adhesion molecules consists of molecules that share a common immunoglobulin-like structure. Members of this family include the antigen-specific receptors of T and B lymphocytes, specific examples being CD4, CD8, CD2, lymphocyte function antigen-3 (LFA-3 or CD58) and intracellular adhesion molecules (ICAM-1 to –3) (Springer, 1990).

(vi)   Cytokines

Cytokines are low molecular-weight proteins involved in mediating cellular and physiological responses, particularly (but not exclusively) within the immune system. Cytokines have in the past often been divided into groups according to their source (lymphokines and monokines), but this characterization can be problematic as many cytokines may be produced by a variety of cell types. These cytokines are induced by specific stimuli (i.e. bacterial products) and are responsible for the generation, stimulation and differentiation of multiple cell types within the immune system. In addition, they control other cytokines that may enhance or inhibit protein synthesis or other cellular biological processes. With regard to the immune system, cytokines that act as mediators between the cells of this system are known as interleukins (IL) while those that induce chemotaxis are referred to as chemokines. Other types of cytokine include interferons (IFN), lymphotoxins, colony-stimulating factors (CSF), tumour-necrosis factor (TNF) and other miscellaneous cytokines (Curfs et al, 1997). The major cytokines are described in table 1.1, along with their main functions.

Table 1.1   Selected cytokines and their functions

Cytokine	Source	Major Functions
Interleukin-1 (IL)	Monocytes & many others	'Endogenous pyrogen' – fever; production of acute-phase proteins, activates T,B & NK cells
Interleukin-2 (IL-2)	CD4+ T-cells	T-cell growth, differentiation and activation; B-cell growth; induces IFN-γ & TNF-β production, leading to monocyte, neutrophil & NK cell activation
Interleukin-3 (IL-3)	T- & NK cells Mast cells	Growth factor for haemopoetic cells
Interleukin-4 (IL-4)	CD4+ T-cells Mast cells	B-cell growth and stimulation, promotes IgE & IgG synthesis; growth factor for T_H2 CD4+ cells
Interleukin-5 (IL-5)	CD4+ T-cells Mast cells	B-cell growth & differentiation; Eosinophil differentiation
Interleukin-6 (IL-6)	T & B-cells & many others	B-cell stimulation & Ig production; T-cell activation & IL-2 production; production of acute phase proteins; haemopoetic cell growth
Interleukin-7 (IL-7)	Bone marrow & thymic stromal cells	Proliferation of lymphoid progenitor cells
Interleukin-8 (IL-8)	T-cells, monocytes endothelial cells	Chemotaxis and activation of leucocytes
Interleukin-9 (IL-9)	T-cells	T-cell & mast cell growth & proliferation
Interleukin-10 (IL-10)	T_H2 cells, macrophages	Inhibition of cytokine production of T_H1 cells & macrophages; B-cell stimulation
Interleukin-12 (IL-12)	B-cells & macrophages	Activates NK cells; promotes generation of T_H1 cells
Interferon-α IFN-α	Lymphocytes, monocytes	Regulates class I MHC expression; induces viral resistance
Interferon-γ IFN-γ	T & NK cells	Activation of macrophages B-cells, T-cells, NK cells; Enhances MHC class II expression on APC's
TNF α	Monocytes	Cell proliferation & apoptosis. Enhances cytolytic activity of NK cells
TNF β	Lymphocytes	Growth & differentiation of numerous cells

(vii) NF-kappa B

Transcription factors are DNA-binding proteins that regulate gene expression. Nuclear factor kappa B (NF-κB) is a transcription factor first identified in 1986, which is involved in the regulation of genes encoding a variety of pro-inflammatory mediators such as cytokine receptors, cell adhesion molecules and acute phase proteins. NF kappa B is also required for the maximal transcription of many cytokines, including TNF-α, IL-1, IL-6 and IL-8, which are thought to be important in the generation of acute inflammatory responses. This transcription factor is therefore important with respect to control of the inflammatory response (Blackwell et al 1997; Sen et al, 1986).

NF-κB is a heterodimer, consisting of two DNA binding subunits. These subunits include p50 (NF kappa B1), p52 (NF kappa B2), p65 (RelA), RelB and c-Rel. Also included in this family are two precursor subunits; p105 is the precursor to the p50 subunit and p100 the precursor of p52. The most prevalent activated form consists of a combination of a p50 or 52 subunit and p65 (RelA) (Tak et al, 2001). In inactivated cells, NF-κB is usually found in an inactive form within the cytoplasm. This may consist either of the p50/p65 subunits complexed with an inhibitory unit known as IκB, or as the precursor p105/p65 (Baeuerle et al, 1994; Blackwell et al, 1997).

Activation is the process by which NF-κB is disassociated from IκB, or the p105 subunit is converted to the active p50, and then translocated to the nucleus. This complex process occurs rapidly (within minutes of extra-cellular stimulation) and involves phosphorylation, proteolysis and redox changes (Chen et al, 1999; Tak et al, 2001).
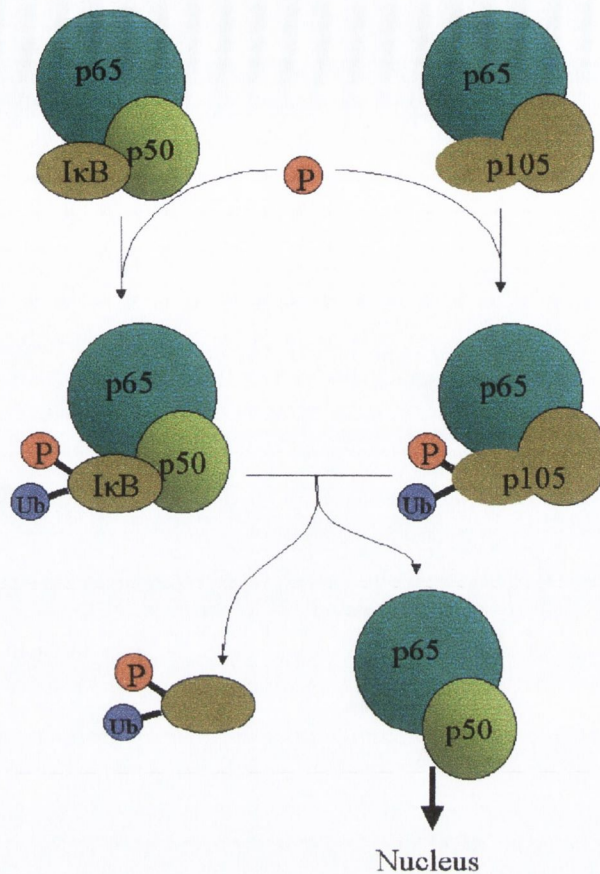
NF kappa B activation: Phosphorylation is the initial step,
then addition of Ubiquitin (Ub) to the inhibitory unit (p105 or
IκB). The Inhibitory subunit is then removed by a proteosome.
The active NF kappa B then translocates to the Cell nucleus.

Figure 1.3.   Activation of NF-κB

NF-κB is activated in response to numerous stimuli including bacterial endotoxins, viral proteins, oxidative or shear stresses, ionizing radiation, UV light, hypoxia and cytokines such as IL-1β and TNF-α (Baeuerle et al, 1994; Blackwell et al, 1997; Bowie et al, 1999). Because it is such an integral part of the immune response, this activation is a tightly controlled event. Feedback control occurs both intracellularly and extracellularly. Positive feedback may occur in order to amplify inflammatory signals; NF-κB enhances the transcription of TNF-α and IL-1β, which in turn activate NF-κB. Negative feedback

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may occur either intracellularly or extracellularly. NF-κB activation leads to transcriptional upregulation of the IκB and p105 genes, resulting in increased production of these inhibitory subunits. In addition to this intracellular mechanism, inflammatory stimuli such as TNF-α and IL-1β can stimulate the production of counter-regulatory cytokines, such as IL-10, which suppresses further inflammatory cytokine release, and blocks further NF-κB activation (Baeuerle et al, 1996; Blackwell et al, 1997).

NF-κB has a number of functions, including control of cellular apoptosis and embryonic development, but it is its role in immune functions that has relevance here. It is an important participant in cytokine regulation (for example IL-6 and IL-8), by affecting transcription of many of the genes involved in their generation. As mentioned in the previous paragraph, while TNF-α and IL-1β are upregulated by NF-κB, they themselves are two of the main activators of NF-κB, undermining its importance in regulating the acute phase response. With regard to the cells of the immune system, it is becoming likely that NF-κB plays a role in activating B cell function and in maintaining the activated state; in pre-B cell lines NF-κB is only present after stimulation, whereas in mature antibody-producing B cells, it is an active nuclear protein. The activation of T cells via their receptors induces nuclear transcription factors, including NF-κB. As a transcription factor of both growth factor and growth factor receptor, NF-κB is also involved in T-cell proliferation. Nuclear expression of NF-κB in neutrophils has been repeatedly demonstrated following cell stimulation, and it is likely that it plays an important role in inducible gene expression in neutrophils. (Aronica et al, 1999; Baeuerle et al, 1994 & 1996; Blackwell et al, 1997; Chen et al, 1999; Lenardo et al, 1989; McDonald, 2004).

(viii) C-reactive protein

The molecules collectively referred to as acute phase proteins are one of the three main soluble components of the innate immune system. An acute-phase protein has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25 percent during inflammatory disorders (Gabay et al, 1999). C-reactive protein (CRP) was the first acute phase protein to be described, as early as 1930, and is an exquisitely sensitive systemic marker of inflammation and tissue damage (Pepys et al, 1983; Tillet et al, 1930). Other acute phase proteins include serum amyloid A protein, proteinase inhibitors and coagulation proteins (Delves et al, 2000).

CRP is a member of an old and stable family of plasma proteins, the pentraxin family, the other main member of which is serum amyloid A. The pentraxin family is named for its electron microscopic appearance from the Greek *penta* (five) *ragos* (berries), and the human CRP molecule is composed of five identical polypeptide subunits. While there is some slight variation of physiological properties of CRP between species, the composition of sequence, subunit organization and protein fold is well conserved. The failure thus far to detect any polymorphism or deficient states in man, implies that CRP has a very important physiological role. The functions described to date include activation of the classical complement pathway for opsonophagocytosis of microorganisms or necrotic host cells (Ablij et al, 2002; Pepys et al, 2003; Thompson et al, 1999).

The normal plasma CRP concentration in healthy adults is <0.8 mg/l, the 90th centile is 3.0 mg/l and the 99th centile is 10mg/l (Shine et al, 1981). It is produced by hepatocytes, and its production is regulated by IL-6. Synthesis occurs rapidly following stimulus – serum concentrations rise after 6 hours and can peak at 48 hours; the half-life is approximately 19 hours, plasma concentrations will therefore fall rapidly after cessation of the stimulus (Vigushin et al, 1993). In most, though not all, diseases, the circulating value of CRP reflects ongoing inflammation and/or tissue damage much more accurately than do other laboratory parameters of the acute-phase response, such as the erythrocyte sedimentation rate (ESR). Importantly, there is no diurnal or post-prandial variation, and while liver failure impairs production, no other pathologies and few drugs affect CRP values, unless they also affect the underlying cause of the CRP elevation. CRP is therefore useful as a screening tool for organic pathology, as a marker to monitor disease progression or response to treatment. Table 1.2 lists the main pathologies that give rise to a plasma CRP response. There is excellent correlation between circulating CRP concentration with the severity, extent and progression of many of these different pathologies, and the prognostic significance of these associations are felt to be consistent with CRP not just being a marker of disease, but also perhaps contributing to pathogenesis (Pepys et al, 2003).

Table 1.2   CRP responses in disease (Pepys et al, 2003)

Major CRP acute phase response

Infections	Bacterial
	Systemic/Severe fungal
	Mycobacterial, viral
Allergic complications of Infection	Rheumatic fever
	Erythema nodosum
Inflammatory disease	Rheumatoid arthritis
	Juvenile chronic arthritis
	Ankylosing spondylitis
	Psoriatic arthritis
	Systemic vasculitis
	Polymyalgia rheumatica
	Reiter disease
	Crohn's disease
	Familial Mediterranean fever
Necrosis	Myocardial infarction
	Tumour embolization
	Acute pancreatitis
Trauma	Surgery
	Burns
	Fractures
Malignancy	Lymphoma
	Carcinoma
	Sarcoma

Modest or absent CRP response

Systemic lupus erythematosus
Scleroderma
Dermatomyositis
Ulcerative colitis
Leukaemia
Graft-versus-host disease

1.2.2 THE INNATE IMMUNE SYSTEM

(i) Introduction

The innate immune system consists of all the immune defenses that lack immunologic memory. This system developed earlier in evolution than did the adaptive immune system. The main distinction between these two systems is the mechanism used for immune recognition; by contrast with the adaptive system, innate immune recognition is mediated by germ-line-encoded receptors, i.e. they are passed from generation to generation. This system mobilizes immediately following infection, unlike the adaptive system, which may take 3 to 5 days to mount a response. In addition, activation of the adaptive immune response is controlled by the innate immune system; it responds to a pathogen only after it has first been recognized by the innate immune system. The cellular components of this innate system are phagocytic cells (neutrophils, monocytes and macrophages), cells that release inflammatory mediators (basophils, mast cells and eosinophils) and natural killer (NK) cells, which represent an interface between the specific and non-specific immune systems. Soluble or molecular components include complement, acute-phase proteins and cytokines (Delves et al, 2000; Fearon et al, 1996; Medzhitov et al, 2000; Minchinton et al, 2004).

(ii)   Monocytes

The mononuclear phagocyte system includes the blood monocytes and macrophages of various kinds in extravascular tissues. They are an integral part of the innate immune system functioning as efficient phagocytic killers. In addition to this, they are also important accessory cells for the generation of specific adaptive immune responses, presenting peptides to T cells for antigen recognition and subsequent T cell activation (Burmester et al, 2003; Medzhitov et al, 2000; Minchinton et al, 2004).

CD14 is a myeloid differentiation antigen found primarily on the surfaces of monocytes and macrophages, but can be expressed at low levels on the surface of polymorphonuclear leucocytes (PMN). It is a receptor for complexes of lipopolysaccharide (LPS) and the serum protein LPS-binding protein (LPB). Monocytes and macrophages binding LPS/LPB via their CD14 surface receptor become activated and release cytokines such as TNF-α (Knapp et al, 1999; Schlossman et al, 1995).

The structure and primary function of class II MHC molecules has been discussed in detail in the previous section. With regard to monocyte function and activation, expression of the class II antigen HLA-DR appears to be critical for recognition of foreign antigens. T helper cells will only recognize a foreign antigen if it is presented in conjunction with monocyte/macrophage surface expression of HLA-DR (Burmester et al, 2003; Cheadle et al, 1991; Ditschkowski et al, 1999; Medzhitov et al, 2000; Wakefield et al, 1993; Welsh et al, 1996).

(iii)   Neutrophils

Neutrophils are part of the rapid response of the innate immune system and represent approximately 60% of all circulating leucocytes. They rapidly extravasate from the blood stream, migrating towards an inflammatory focus (chemotaxis), and are usually the first leucocytes to arrive. When they approach a pathogen, they adhere to it and then commence phagocytosis, subsequently killing the invader with intracellular reagents. Neutrophils have receptors for antibody and complement, so that coating of foreign antigens with antibody or complement enhances phagocytosis.   Neutrophil numbers and function are crucial to defence against severe bacterial and fungal infections, clearing invaders from the circulation (Delves et al, 2000; McDonald, 2004; Minchinton et al, 2004; Wakefield et al, 1995).

The presence of NF-κB in neutrophils was first confirmed in 1994, following stimulation with granulocyte colony stimulating factor (G-CSF). Since then, it has been confirmed that activation of neutrophils leads to rapid loss of the inhibitory protein IκB-α and subsequent accumulation of intranuclear NF-κB (Foulds et al, 2001; McDonald, 2004).

1.2.3   THE ADAPTIVE IMMUNE SYSTEM

(i)   Introduction

The T cells of the adaptive immune system comprise mainly of lymphocytes expressing the αβ T cell receptor (TCR) that recognize antigenic peptides presented by major histocompatibility complex (MHC) molecules. These cells can be divided into two major sub-populations based on whether their TCR recognize antigenic peptides presented by MHC class I or MHC class II molecules. Endogenous antigens are presented by MHC class-1 bearing cells exclusively to T-cells expressing the CD8 receptor (cytotoxic T-cells). Exogenous antigens are presented by MHC class II antigens to T-cells expressing CD4 receptors (helper T-cells) (Delves et al, 2000; Klein et al, 2000; Singer et al, 2004).

Naïve T-cells migrate preferentially from the blood to lymph nodes and other secondary lymph node tissues, a process referred to as homing. In inflamed tissues, antigen presenting cells (APCs) are mobilised to carry antigen to lymph nodes, where they stimulate antigen-specific T-cells via the TCR. This encounter with antigen results in the proliferation of T-cell clones, which differentiate into effector cells and then home to the sites of inflammation. Although most effector cells are short lived, dying after the antigen is cleared, a few antigen-experienced memory cells remain for long-term protection. There is therefore an increase in the number of specific helper T-cells available at re-exposure to antigen, i.e. an expanded clone. The immune response on the second and subsequent exposures is quicker and more vigorous (von Andrian et al, 2000).

(ii)   T-cell surface markers

T-cells possess cell-surface glycoproteins which act as markers of differentiation and activation. Subsets of T-cells can therefore be recognized by monoclonal antibodies.

The T-cell receptor is associated with the CD3 complex of molecules, which transmit activation signals into the cell when the TCR bonds antigen. The TCR belongs to the immunoglobulin superfamily, and its role is well accepted for recognition of antigens which are expressed in association with MHC-antigens. The CD3 antigen is present on up to 85% of normal human peripheral blood lymphocytes and thymocytes. When TCR binds to an antigen-APC complex, it causes phosphorylation of the CD3 complex and this initiates downstream signaling, involving a number of biochemical pathways to the transcriptional activation of genes involved in cellular proliferation and differentiation (Beverley et al, 1981; Klein et al; 2000; Knapp et al, 1989).

The CD4 antigen is present on T-helper/inducer cell populations (i.e. CD3+CD4+), which comprise up to 50% of peripheral blood lymphocytes and 80% of thymocytes. It binds to HLA class II molecules during the interaction of CD4+ T-cells with APCs or with target cells and also serves as the primary receptor for the human immunodeficiency virus. In addition, the CD4 antigen is also present in low density on the cell surfaces of monocytes, tissue macrophages and granulocytes (Knapp et al, 1989; Mason et al, 2002).

Thymocytes and approximately 30% of peripheral T-cells express the CD8 membrane-bound glycoprotein, functionally corresponding to the T-cytotoxic/suppressor cell populations (i.e. CD3+CD8+). It is responsible for binding to class I MHC molecules. In addition to being present on the surface of cytotoxic T-cells, this antigen is also weakly characteristic of natural killer (NK) cells (Knapp et al, 1989; McMichael et al, 1987).

The CD25 molecule is a cell surface glycoprotein that forms the α chain of the interleukin 2 receptor complex. It was originally thought only to be expressed on activated T-cells, but is now known to be a non-lineage specific activation marker, which is also present on activated B-cells and monocytes/macrophages. It is a relatively early activation marker, appearing after 1 to 3 days, with studies showing expression on up to 80% of cells 48 hours after stimulation induction (Burmester et al, 2003; Knapp et al, 1989; Mason et al, 2002; Nelson et al, 1998; Schlossman et al, 1995).

The CD69 molecule (also known as the activation inducer molecule, early activation antigen or Leu-23) is a cell surface protein transiently expressed on T-cells through signals via TCR cross-linking and is involved in TCR/CD3 signalling. It is the earliest marker of T-cell activation, detectable on T-cells within 30 minutes of stimulation and usually reaches maximal clonal expression within 24 hours of initial antigen presentation. It is also the earliest inducible cell surface glycoprotein to appear upon in vitro activation of NK and B cells. With regard to its function, the CD69 antigen is thought to be a regulatory protein (or a part thereof), involved in T- and B-cell activation pathways. (Craston et al, 1997; Knapp et al, 1989; López-Cabrera et al, 1993; Mason et al, 2002; Schlossman et al, 1995; Simms et al. 1996)

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The CD71 molecule was originally identified as the transferrin receptor in 1981. It is widely distributed over many different cell types and organs that require transferrin for iron-dependant growth. The correlation between expression of CD71 and cell proliferation is well established; a rapid increase in expression is observed after stimulation of lymphocytes with mitogens. Thus, CD71 is a prototype activation marker associated with increased proliferative activity in most tissues, regardless of lineage. As with CD69, CD71 is usually upregulated on the cell membrane within a few hours of activation (Burmester et al, 2003; Knapp et al, 1989; Mason et al, 2002; Ponka et al, 1999; Schlossman et al, 1995).

(iii)   Helper T-cell responses

Once antigen presentation occurs, T-cells are induced to change from a naïve phenotype (von Andrian et al, 2000). Multiple phenotypes have been described, including Th1, Th2, Thp, Th0 and Th3. The abbreviations Th1 (T helper cell type 1) and Th2 (T helper cell type 2) refer to CD4+ T-cell subsets that provide help to cells of both the innate and adaptive immune systems. Helper T-cells may be either effector or memory T-cells. Thp refers to T cells which are antigen-naïve, or precursor cells; these cells have traditionally been described as IL-2 producers, but they can be highly diverse in cytokine production on initial activation (i.e. IL-2, IL-4, TNF-α, IL-13, IFNγ). Upon exposure, the naïve cell may then undergo differentiation to an uncommitted cell named Th0. The exact nature of this cell type is still not clear, but it appears to be a Th1/Th2 precursor cell, which secretes mainly IL-4 and IFNγ (Mosmann et al, 1996; Nakamura et al, 1997).

Antigen exposure of a naïve or undifferentiated T-cell results in the creation of either a Th1 or Th2 helper, with polarization occurring within 48 hours after stimulation. These cells may be either memory or effector cells. If they are actively secreting cytokines they can be considered either Th1 or Th2 primary effector cells. If they are "resting" but polarized (i.e. committed to a Th type), they could be considered Th1 or Th2 memory cells, which retain functional plasticity and, when reactivated, form Th1 and Th2 effector cells (Ahmed et al, 1996; Burkett et al, 2004; Dutton et al, 1998; Nakamura et al, 1997).

The Th phenotypes are characterized by the cytokines they produce; these patterns were first described in mouse, then human, Th1 and Th2 cells. In general, Th1 cells are responsible for generating strong cellular immunity against intracellular pathogens; human Th1 cells secrete IFNγ, IL-2 and TNF-β. In contrast, Th2 cells promote the development of humoral responses that direct effector functions using specific antibodies against extracellular pathogens; these cells characteristically secrete IL-4, IL-5, IL-10 and IL-13. Another Th cell type with a unique cytokine secretion profile is the Th3 cell, which appears to be a CD4+ immune regulatory T cell that secretes the immunosuppressive cytokine Transforming growth factor (TGF) β (Delves et al, 2000; Ferrick et al, 1995; Jiang et al, 2004; Mosmann et al, 1996; O'Garra et al, 2004; Stetson et al, 2004).
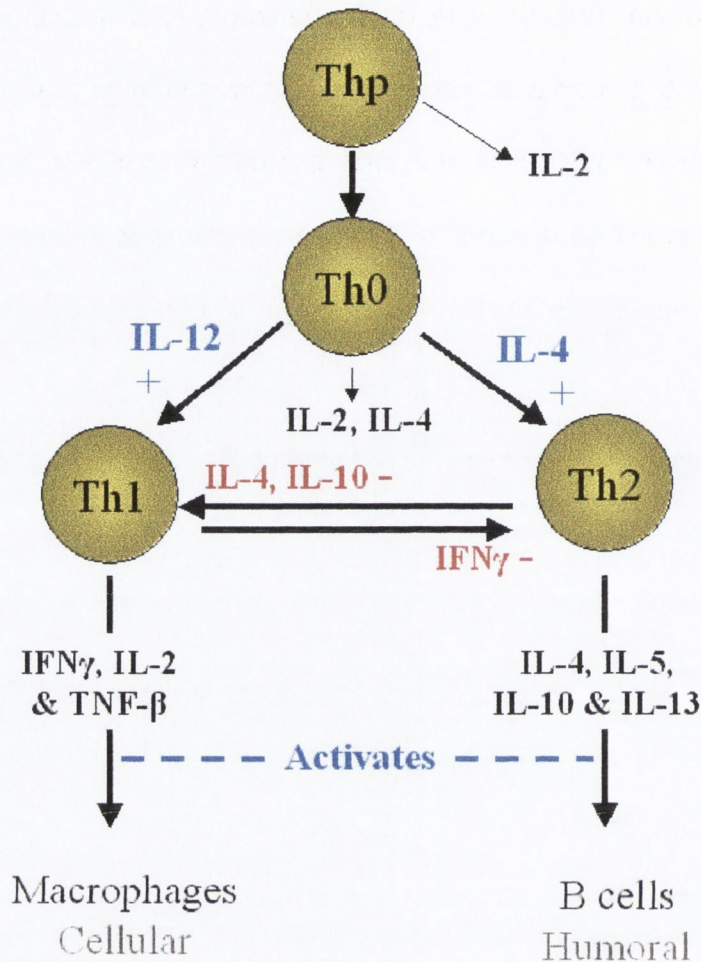
Figure 1.4   T-helper cell subsets

It is still unclear how naïve T cells select which cytokine profile to exhibit, but there is evidence to suggest that exposure to certain cytokines is an important influence. Both Th1- and Th2-specific cytokines can promote growth or differentiation of their own respective T cell subset, but can also inhibit the development and function of the opposing subset. Th1 derived IFNγ can inhibit the development of Th2 cells, whereas IL-4 can inhibit Th1 development (while enhancing growth of Th2 cells). IL-12, which is principally produced by monocytes and macrophages, drives differentiation of naïve T cells towards a Th1 phenotype, and the presence of IL-12 appears to be an essential requirement for Th1 development – this process can be enhanced by the presence of IFNα

and IFNβ (but not IFNγ). It is still unclear whether increased IL-4 production or decreased IL-12 production is the fundamental abnormality involved in the shift toward Th2-cytokine production seen after injury. Once one Th phenotype is dominant, a positive feedback mechanism maintains the cytokinetic response (Delves et al, 2000; Mosmann et al, 1996; O'Garra et al, 2004; O'Sullivan et al, 1995).

(iv) Cytotoxic T-cell responses

The elimination of virally infected cells falls to the cytotoxic T cell, or CD8+ T cell. The infected cell marks itself by displaying peptides derived from intracellular viral protein on its surface. These viral peptides are bound to the peptide-binding regions of class 1 MHC molecules; this combination probably directly activates CD8+ T cells and provides target cells for virally induced T-cell cytotoxicity and a possible mechanism for autoimmune damage. Once the cytotoxic T-cell binds to this complex, it may destroy the cell by one of two mechanisms. They may insert perforins into the target cell membrane, which produce pores, allowing granzymes to be passed from the T cell to within the target cell; these enzymes then induce apoptosis of the cell. The FasL/Fas pathway involves membrane receptor expression of FasL on the target cell, to which the T cell then binds. This process will also activate caspases within the target cell and ultimately induce apoptosis. In addition to killing cells directly, it is now recognized that T cells also produce a number of cytokines, including TNF-α and lymphotoxin. IFNγ, another product of CD8+ T cells, reinforces antiviral defenses by rendering adjacent cells resistant to infection (Delves et al, 2004; Henkart et al, 2004; Mosmann et al, 1996).

Parallel to the Th1 vs Th2 cytokine secretion patterns of CD4+ cells, CD8+ cells follow a tendency to become differentiated along similar lines. Both Tc1 and Tc2 cells have potent cytotoxic activity along the perforin-dependant granule exocytosis pathway and the FasL/Fas pathway. However, Tc1 cells secrete predominantly IL-2 and IFNγ, while Tc2 cells secrete predominantly IL-4, IL-5 and IL10. IL-12 and IFNγ encourage the differentiation of precursors into Tc1 cells, whereas IL-4 induces the generation of Tc2 cells. After commitment of CD8+ cells to either the Tc1 or Tc2 phenotype, neither subset can be converted to the other cytokine-secreting pattern. Tc1 vs Tc2 differentiation has not been shown to play a crucial role in in vivo immune responses, but is an area of growing interest (Henkart et al, 2004; Mosmann et al, 1996).
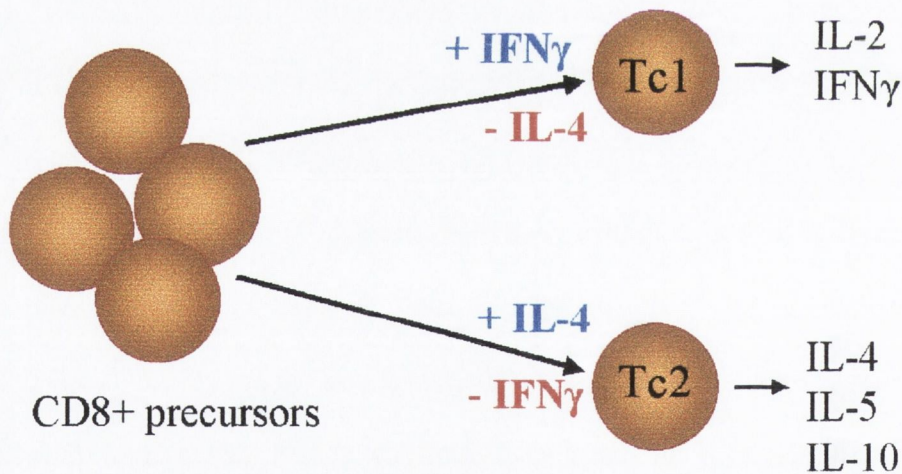
Figure 1.5   Cytotoxic T cell subsets

1.2.4   GUT BARRIER FUNCTION

(i)   Introduction

The intestinal barrier is a complex entity, allowing selective absorption of nutrients and other macromolecules from the gut lumen, whilst excluding pathogenic organisms or their products, which may be harmful to the host. As will be discussed in detail in a further section, failure of the gut barrier to contain endogenous bacteria and endotoxins within the gastrointestinal tract is central to the gut hypothesis of multiple organ failure (Deitch, 1992; Reynolds, 1996).

(ii)   Nature of the Gut Barrier

The healthy gastrointestinal tract is thought to contain as many as 10^{12} total bacteria, 10^9 of these being potentially pathogenic gram-negative bacteria, as well as lethal amounts of endogenous endotoxin. Under normal conditions, the gut acts as a selective route of entry. It allows easy passage of some molecules through the epithelium, primarily absorption of nutrients, but remains impervious to bacteria and endotoxin, confining them within the bowel lumen. The epithelial cells of the intestine are a heterozygous population, including enterocytes, goblet cells, endocrine cells, paneth cells, microfold (M) cells, tuft and cup cells. The enterocyte is the most common cell and is responsible for the majority of the absorption of nutrients and other substances from the intestine. The barrier function of the gut can be likened to that of other epithelial surfaces of the body, such as the skin and respiratory tract. Just as these other epithelial barriers may be breached, so may the gut barrier. There are four main components to this physiological barrier: the balance of the normal microbial flora; immune defenses; mechanical factors; and the gut-liver axis.

Disruption or alteration of any of these components may lead to a failure of the gut barrier, increased intestinal permeability and translocation of bacteria or endotoxin (Balimane et al, 2000; Doig et al, 1998; Fink, 1991; Swank et al, 1996).

(ii)   (a)   Intestinal microflora:

The first component of the gut barrier is the ecological balance of the normal gut microflora. The normal acidic stomach is an inhospitable environment and as such is usually sterile. The normal duodenum and jejunum are also relatively sterile, due to lack of organisms entering from the stomach above, the antibacterial effect of bile and pancreatic juice and the flow effect of large volumes of intestinal secretions. The concentration of bacteria within the bowel lumen increases towards the terminal ileum, with the most complex combination of endogenous flora found within the large colon. Overall, the anaerobic bacteria within the bowel outnumber the gram-negative bacteria by about 100 to 1000-fold and occupy the space closest to the intestinal epithelial cells, thereby reducing the potential for enterocyte adherence or attachment by potentially pathogenic bacteria. This has been termed colonization resistance and alteration of this balance can allow infection of the gut with organisms such as clostridium difficile and also increases the likelihood of bacterial translocation. One common way in which the normal microflora may be disrupted is by the administration of broad-spectrum antibiotics, to which these obligate anaerobic bacteria are more sensitive than their pathogenic cousins (Marston et al, 1989; O'Boyle et al, 1998; Swank et al, 1996).

(ii)   (b)   *Mechanical defenses*

The gut has a variety of mechanical defenses that maintain the functional integrity of the gut barrier. As stated above, the upper gastrointestinal tract is relatively sterile, due to intrinsic gastric acidity and the mild anti-microbial activity of biliary and pancreatic secretions. The mucous layer overlying the luminal epithelium comprises primarily of a high-molecular-weight glycoprotein secreted by goblet cells. In addition, it contains secretory immunoglobulin A (sIgA), which is secreted by submucosal plasma cells. This layer provides an optimum environment for the protective obligate anaerobes within the gut, while preventing adherence of pathogenic bacteria and toxins to enterocytes, thus helping to maintain colonization resistance. Intestinal peristalsis prevents stasis, further reducing adherence and also facilitating the removal of damaged cells, which are rapidly replaced by the continually renewing epithelial layer. The cellular barrier of the small intestine is composed of simple columnar epithelial cells (enterocytes), interspersed with specialized cells such as goblet cells, endocrine cells and lymphocytes. These enterocytes are joined by desmosomes and tight junctions and develop from stem cells within the mucosal crypts, migrating from there to the villous tips. At the tip, the cell undergoes apoptosis and is either shed within the bowel lumen or phagocytosed by surrounding cells. This continuous renewal reduces the likelihood of pathogenic adherence to damaged cells. Enterocytes rely primarily on glutamine as an energy-yielding substrate, and depletion of this amino acid may result in decreased proliferation of this layer, to the detriment of gut barrier function (Balimane et al, 2000; Marston et al, 1989; Swank et al, 1996).

(ii)   (c)   Immune defenses:

In addition to the colonization resistance and protection conferred by healthy endogenous flora, the gut has a number of additional immunological defense systems. The gut-associated lymphoid tissue (GALT) is the largest in the body, comprising of intraepithelial and lamina propria lymphocytes, lymphoid follicles, Peyer's patches and mesenteric lymph nodes. Exposed as it is to a substantial antigen load, it must continually maintain a delicate balance between active immunity, tolerance and suppression of immune responses.

The mucous layer overlying the luminal epithelium comprises primarily of a high-molecular-weight glycoprotein secreted by goblet cells. Within the submucosa of the bowel are found a large concentration of plasma cells, approximately 80% of which secrete the secretory immunoglobulin A (sIgA) found within the mucous layer of the intestinal lumen. This layer provides an optimum environment for the protective obligate anaerobes within the gut, while preventing adherence of pathogenic bacteria and toxins to enterocytes, thus helping to maintain colonization resistance. Additional numbers of T-cells and macrophages are also to be seen within the lamina propria. The significance of these is as yet unclear, but initial clinical studies suggest increased activation levels of T-cells, macrophages and the enterocytes themselves, in conditions such as cholestatic jaundice and malnutrition, where an increase in intestinal permeability is known to occur (Reynolds et al, 1995; Swank et al, 1996; Welsh et al, 1998).
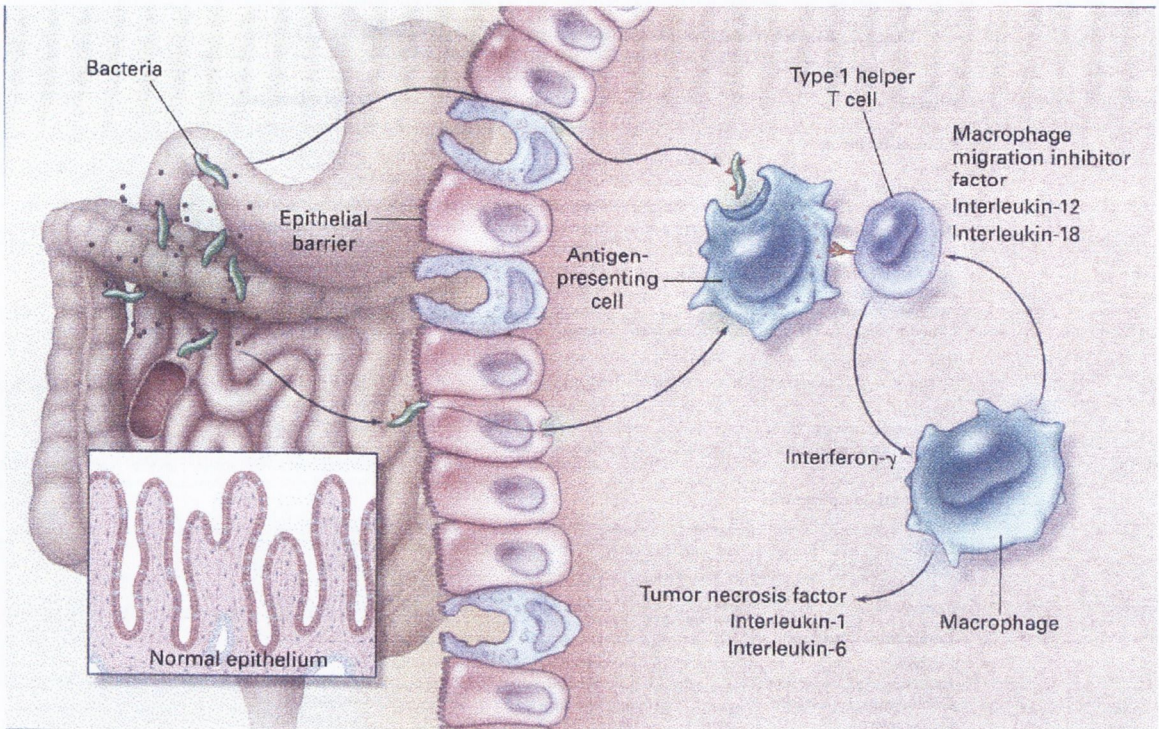
Figure 1.6   Priming of gut immune and epithelial cells

(ii)   (d)   The gut-liver axis

The final defense of the gut barrier is the gut-liver axis. Biliary salts appear to be responsible for binding intraluminal endotoxin, thus hindering its translocation. In experimental models, administration of bile salts or restoration of internal flow has been shown to reduce endotoxaemia and decrease bacterial translocation. Additional studies have shown that restoration of flow also increases the responsiveness of Kupffer cells, intrahepatic macrophages that are responsible for clearance of endotoxin from the portal circulation (Reynolds et al, 1995; Swank et al, 1996).

(iii)   Intestinal Permeability

Measurement of intestinal permeability for macromolecules is thought to reflect the functional integrity of the gut barrier and can be measured by a variety of probe molecules. Early studies of permeability, or passive transport, have shown that there are at least two permeation pathways in the small intestine mucosa. The first pathway is the paracellular pathway, where molecules diffuse passively via the tight junctions or zonulae occludens between the enterocytes. This is the path taken by macromolecules such as lactulose and cellubiose. The other diffuse pathway is transcellular, where molecules are transported through the cell membrane of enterocytes. This pathway admits smaller molecules such as urea, mannitol and rhamnose. In addition to these two passive mechanisms, molecules may be transported across the cell by specific carriers or by receptor-mediated endocytosis (Balimane et al, 2000; Bjarnson et al, 1995).

Intestinal permeability may be measured in vivo by the use of orally administered, non-metabolisable, test molecules that are absorbed via the intestine and excreted unchanged into the urine, where they may be measured. The ideal marker should be water soluble, biochemically inert, cross the intestinal barrier by passive diffusion and be palatable for the patient to ingest. Additionally, they should not ordinarily be present in urine and their measurement should be sensitive, accurate and easy (Bjarnson et al, 1995).

Gut Lumen
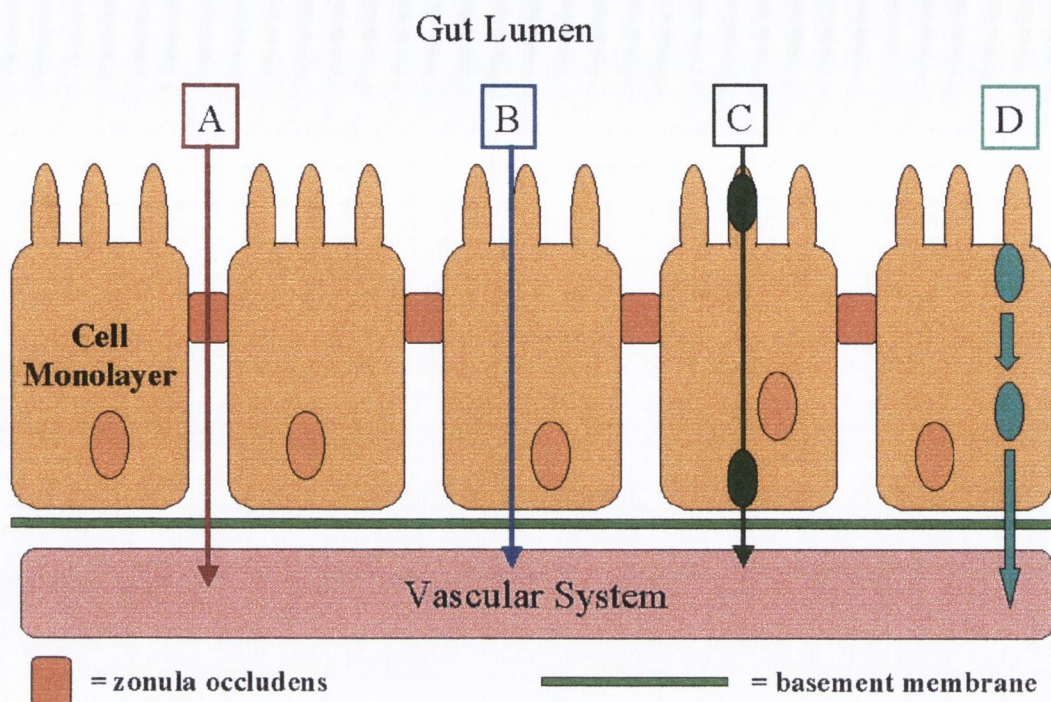
A – Paracellular pathway
B – Transcellular, passive
    diffusion

C – Transcellular, carrier
    mediated
D – Transcellular, endocytosis

Figure 1.7   The parallel pathways of intestinal absorption

Absorption of these test substances can be affected by a number of factors other than the mucosal integrity itself. These can be divided into pre- and post-absorption factors. Preabsorption factors include rate of gastric emptying, dilution of the test substance by intrinsic gastrointestinal secretions, bacterial degradation and intestinal transit time. Post-absorption factors include systemic distribution, endogenous production, urinary excretion and completeness of urine collection. These problems are overcome by differential permeability testing, whereby two markers are administered which are equally affected by the pre- and post-absorption factors, the only difference between the two being the route of permeation across the intestinal mucosa. Therefore, the urinary excretion ratio

is considered to be a parameter for intestinal permeability per se. The diagnostic accuracy of this test may be increased by using a combination of a large molecule, which is absorbed paracellularly, and a small molecule, which is absorbed transcellularly (Juby et al, 1985; van Nieuwenhoven et al, 1999).

Differential sugar absorption tests, or dual sugar tests, are among the best-documented tests, having been studied in a wide variety of gastrointestinal diseases. Mannitol is the most commonly used monosaccharide, but may be naturally present in human urine. Obtaining a pre-test or 'blank' urine specimen for measurement of endogenous mannitol levels may ameliorate the potential for inaccuracy of the test. Alternatively, another monosaccharide such as rhamnose may be employed. Lactulose and cellubiose are the best-documented disaccharides, but cellubiose may be hydrolysed to a small degree by small intestine lactase. The test solution is often administered with an osmotic 'filler' such as glucose, which renders the solution hyperosmolar. This increases the absorption of the disaccharide and has been shown to increase the diagnostic discrimination of the test. The advantages of these tests are that they are cheap, sensitive, simple to perform and the variations in hepatic and renal function have little effect on the disaccharide/monosaccharide recovery ratio. The test solution is also generally palatable to patients, ensuring compliance and complete ingestion (Akinbami et al, 1989; Bjarnson et al, 1995; Juby et al, 1985; Laker et al, 1977; Lunn et al, 1989; Northrop et al, 1990; Uil et al, 2000).

One variation of the dual sugar test described above measures the serum concentration of the sugar probes, rather than the urinary concentrations. While this may reduce sampling error due to inability to collect complete urine samples (i.e. in children and neonates), the method employs High Performance Liquid Chromatography (HPLC) analysis of the serum samples, a more time-consuming and technically demanding method than the enzymatic one used for analysis of urine (Cox et al, 1997; Fleming et al, 1996).

A variety of other probes for measurement of intestinal permeability are available. Polyethylene glycols (PEG) are usually used as solvents and food additives, in suppositories and in bowel preparations for large bowel investigations (i.e. Klean-prep), but can also be used to assess intestinal permeability. The most commonly used PEG in human studies is PEG-400. ^{51}CR-labelled ethylenediaminetetraacetic acid (^{51}Cr-EDTA) and ^{99}Tc-diethylenetriaminopentaacetate (^{99}Tc-DTPA) share many physical characteristics with oligosaccharides with the advantage of ease of measurement. However, in addition to the disadvantages of single marker tests, their use of radioactivity renders them less suitable for serial measurements, or use in children (Bjarnson et al, 1995).

1.2.5   SPLANCHNIC BLOOD FLOW

(i)   Introduction

Under normal conditions, the human gastrointestinal tract contains 30% of the total body blood volume and receives approximately 25% of cardiac output from the heart. This large vascular capacity and the extensive length of the mucosa make ischaemic injury and cellular hypoxia a constant hazard. During cardiogenic or hypovolaemic shock, it is thought that blood is preferentially diverted away from the gut to the "vital" organs, such as the brain and heart – one author has proposed that as little as a 15% reduction in circulating blood flow may result in a 40% reduction in splanchnic blood volume (Mythen et al, 1994; Price et al, 1966). Once the intestinal mucosa is damaged, the gut barrier is breached, allowing translocation of bacteria and endotoxin from the bowel lumen, which may result in activation of the systemic immune system (Deitch, 1992; Saadia, 1995; Swank et al, 1996; Taylor, 1998; Vatner, 1974).

(ii)   Anatomy

The three main visceral arteries of the gastrointestinal tract correspond to the embryological foregut, midgut and hindgut. Of these, the largest is the midgut, which includes the small intestine, where approximately 90% of all absorption in the gastrointestinal tract occurs (Balimane et al, 2000; Marston et al, 1989).

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Aorta and branches   Coeliac trunk
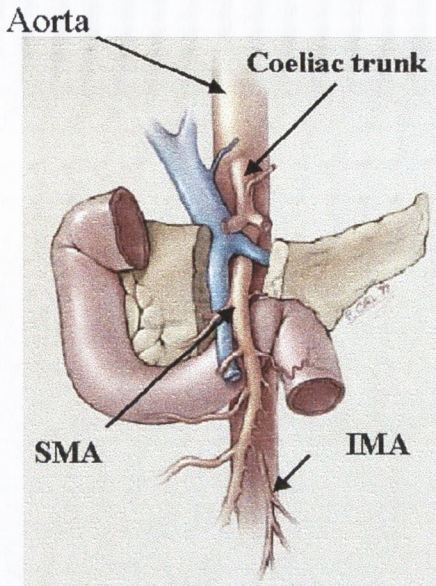
IMA – inferior mesenteric artery
SMA – superior mesenteric artery
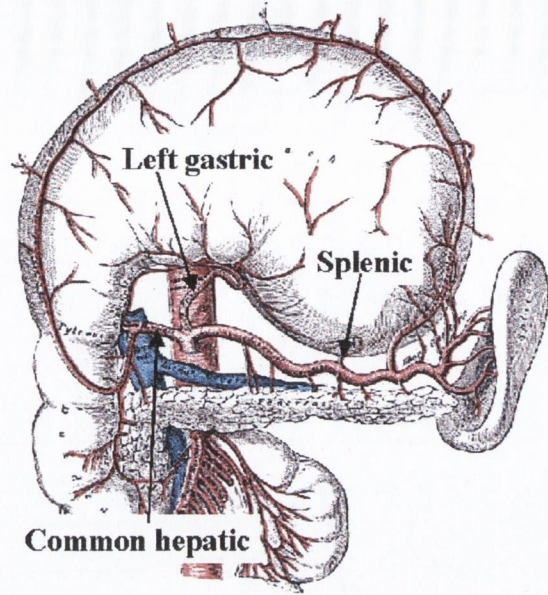
Figure 1.8   The aorta and coeliac trunk (Williams et al, 1995)

The coeliac trunk is the artery to the foregut, which includes the stomach, spleen, pancreas, liver and part of the duodenum (to the level of the ampulla of Vater). It arises from the aorta at the level of the twelfth thoracic vertebra, after it passes behind the median arcuate ligament to enter the abdominal cavity, then almost immediately divides into its three branches, the left gastric, the splenic and the common hepatic arteries. The splenic artery passes behind the pancreas, supplying it and the spleen, then the left side of the stomach via its short gastric and left gastro-epiploic branches. The left gastric artery supplies the upper part of the lesser curvature of the stomach, with the remainder of the stomach supplied by the right gastro-epiploic branch of the common hepatic artery, which also supplies the liver and the commencement of the duodenum (Marston et al, 1989; Williams et al, 1995).
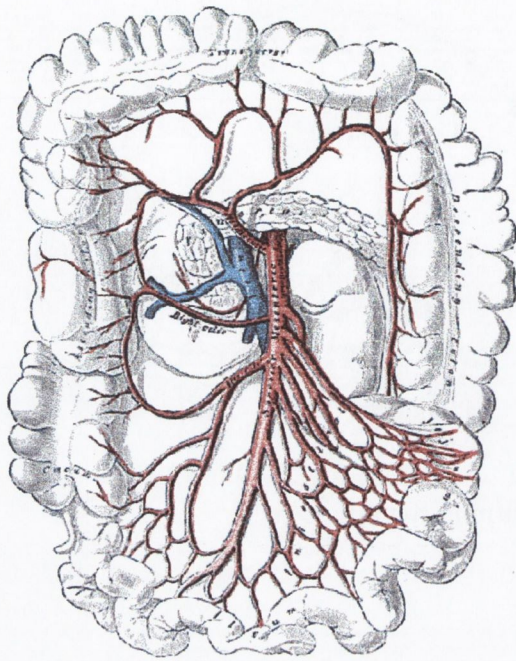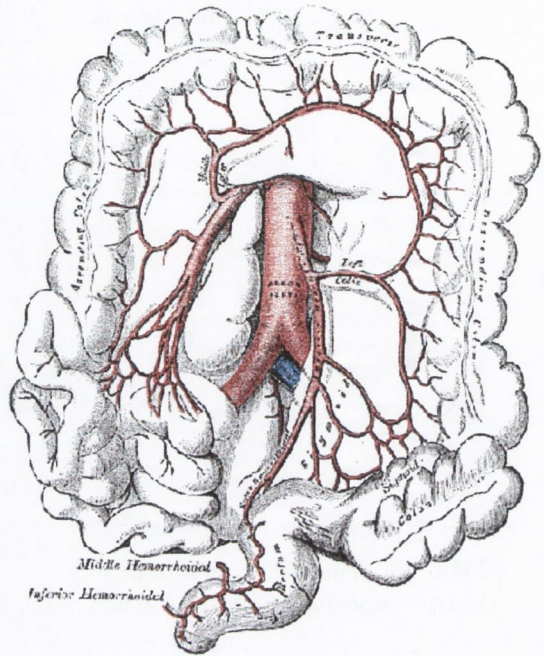
Superior mesenteric artery   Inferior mesenteric artery

Figure 1.9   Superior and inferior mesenteric arteries (Williams et al, 1995)

The superior mesenteric artery is the artery to the midgut, leaving the aorta at the level of the first lumbar vertebra. It is clinically the most important vessel of the gastrointestinal tract, supplying it from the duodenum (distal to the ampulla of Vater) to the splenic flexure of the large bowel, via its jejunal, ileal, ileocolic, right colic and middle colic branches. The inferior mesenteric artery supplies the hindgut and leaves the aorta at the level of the third lumbar vertebra, just to the left of the midline. It supplies the colon from the level of the splenic flexure to the upper anal canal (Marston et al, 1989; Williams et al, 1995).
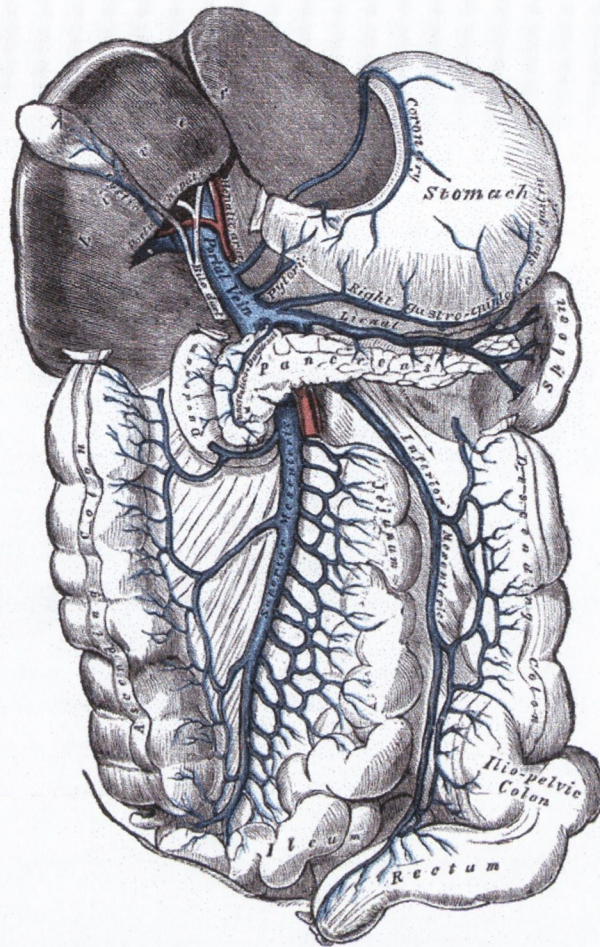
Figure 1.10   The portal system of veins (Williams et al, 1995)

The portal system includes all the veins that drain the blood from the abdominal

part of gastrointestinal tract (with the exception of the lower part of the rectum) and from

the spleen, pancreas, and gall-bladder. From these viscera the blood is conveyed to the

liver by the portal vein. It is about 8 cm. in length, and is formed at the level of the second

lumbar vertebra by the junction of the superior mesenteric and splenic veins, behind the

pancreas. These veins are notable in that they do not contain valves so that flow may occur

in either direction and changes in portal blood pressure are communicated directly to the

intestinal wall (Marston et al, 1989; Williams et al, 1995).

(iii) Physiology

The majority of the total blood flow to the gut (up to 90%) is directed to the mucosa and submucosa. After penetrating the bowel wall, the small arterial branches then course obliquely through the muscularis mucosa to reach the submucosa, where they then anastomose freely with each other. Blood supply to individual villi is provided by a branched arteriole, which arises from a perpendicular feeder vessel in the submucosa. This arteriole is approximately $20\mu m$ in diameter, and loses its muscular coat as it ascends the villus. An extensive capillary network is then present between this arteriole and the venous circulation of the villus. A "counter-current" of blood flow is thus created, which assists in the absorption of nutrients from the gut lumen (Marston et al, 1989; Williams et al, 1995).
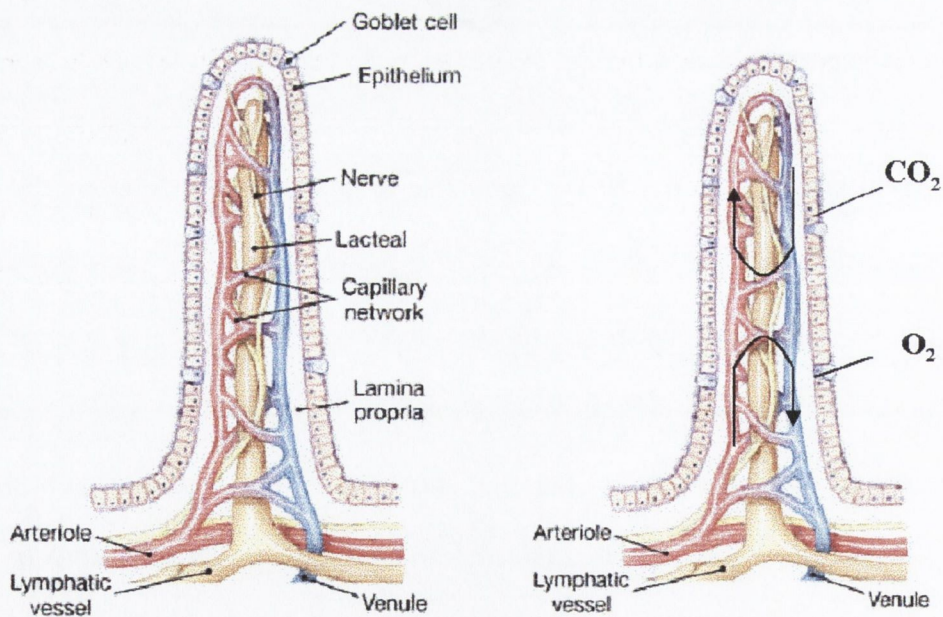
Figure 1.11   Villous anatomy and blood supply

A consequence of the villous counter-current, is that the mucosa of the bowel wall is vulnerable to ischaemia. As oxygen diffuses from the arteriole to adjacent venules, the oxygen tension in arteriolar blood decreases, resulting in a lower oxygen tension at the villous tip than in arterial blood. Also, as oxygen diffuses from the arteriole, carbon dioxide subsequently diffuses from the venule into the arteriole, resulting in accumulation of CO_2 at the villous tip, in addition to the aforementioned reduced oxygen tension. This makes the villi susceptible to tissue hypoxia if the mucosal blood flow is compromised (Taylor, 1998; Marston et al, 1989).

Ischaemia of the gut may occur from low flow, interference in arterial inflow and, less commonly, from an obstruction to venous outflow. Low flow may occur in conditions such as cardiogenic or hypovolaemic shock. As stated above, decrease in mucosal perfusion results in tissue hypoxia as oxygen is shunted away from the villous tip by the "counter-current" exchange. This hypoxia is accompanied by anaerobic metabolism and a reduction in intramucosal pH level. The damage caused by this is enhanced by the presence of bile salts, digestive enzymes, bacteria and their toxins within the gut lumen. The extent of the necrosis is dependant on the degree and duration of the ischaemia, but can develop within minutes of the onset of hypoxia, beginning at the tip and then extending proximally. This damage to the mucosa disrupts the physiological gut barrier, allowing invasion by intraluminal bacteria, and absorption of bacterial endotoxins and the products of necrotic tissue into the portal circulation, resulting in activation of the systemic immune system (Fiddian-Green, 1988; Fink, 1991; Groeneveld et al, 1994; Kolkmann et al, 2000; Taylor, 1998).

(iv)   Measurement

Splanchnic blood flow can be determined non-invasively by means of a method called tonometry. Tonometry specifically refers to the measurement of the partial pressure of a gas, while gastrointestinal tonometry refers to the use of a device to measure the partial pressure of carbon dioxide (PCO_2) of the gastrointestinal mucosa. This was initially performed by injecting saline into a hollow viscus, such as the bladder, and then measuring the PCO_2 of the luminal fluid. The PCO_2 of the intraluminal fluid and the PCO_2 of the mucosa are assumed to be in equilibrium, thus giving an indirect measurement of mucosal oxygenation. In the 1980's, interest in this technique increased after it was proposed that tonometry could be used to estimate gastrointestinal mucosal pH (pHi). This was performed by measuring the gastrointestinal intramucosal CO_2 concentration and the arterial bicarbonate level, which was assumed to be similar to the mucosal bicarbonate level, and then applying these results to the Henderson Hasselbach equation to calculate the pHi (Bass et al, 1985; Clark et al, 1992; Dawson et al, 1965; Fiddian-Green et al, 1982; Taylor, 1998).

$$pHi = 6.1 \; + \log \frac{[\text{arterial HCO}_3]}{PrCO_2 \times 0.03 \times k}$$

Figure 1.12   The Henderson Hasselbach Equation

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Grum et al facilitated the measurement of intraluminal PCO_2 by fashioning a tonometer – a modified nasogastric tube with a silicone balloon incorporated into its tip, and a sampling line along its length. The silicone balloon of the tonometer was permeable to carbon dioxide and the concentration of the saline within this balloon equilibrated with the gastrointestinal mucosal carbon dioxide concentration over a period of time (approximately 30 to 40 minutes). The saline was then aspirated via the sampling line and the PCO_2 measured. Modern tonometers use the same principle, but have substituted gas within the balloon, resulting in a reduced period of time (10 minutes) for equilibration of the PCO_2 concentration inside the balloon to that of the mucosa. Experimental studies in animals have shown that pHi decreases in parallel with decreasing blood flow, tissue PO_2 and oxygen consumption (Antonsson et al, 1990; Dawson et al, 1965; Grum et al, 1984; Guzman et al, 1996 & 1998; Kolkmann et al, 2000).
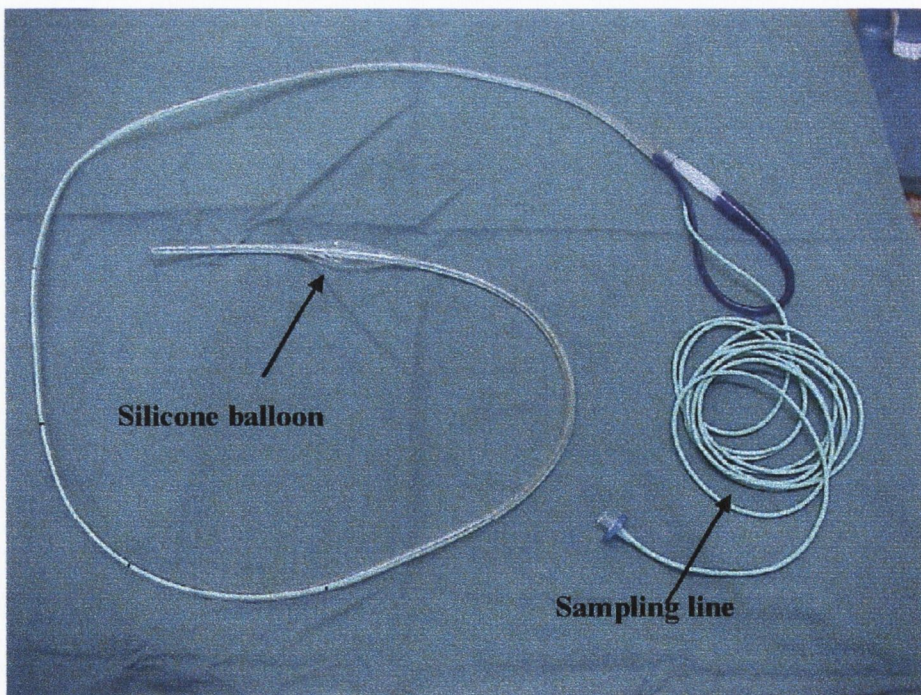
Figure 1.13   Gastrointestinal tonometer

A number of assumptions underlie the estimation of pHi by the principle of hollow viscus tonometry. As stated before, the main assumptions are that intraluminal PCO_2 parallels intracellular PCO_2 and that the tissue and arterial bicarbonate concentrations are equivalent. In contrast to the saline method, air tonometry eliminates many potential inaccuracies in measurement, such as increased equilibration time, catheter dead space and measurement of PCO_2 concentration of the saline by an arterial blood gas machine. The close approximation of luminal and mucosal PCO_2 may be lost in the stomach when carbon dioxide is produced by buffering of gastric acid, but this may be prevented by inhibition of gastric acid secretion. Luminal PCO_2 may also increase following gastric feeding, again as a result of buffering of the increased acid by the increased bicarbonate output. It is therefore recommended that tonometric readings be performed when the patient is fasting. The tonometer may be used to assess either PCO_2 or calculated pHi, and both measurements have been used to assess outcome in clinical studies. However, while the rise seen in the regional PCO_2 level is mainly attributable to gut hypoperfusion, a number of studies have shown that changes in tonometric PCO_2 may reflect changes in arterial PCO_2. Therefore, the regional PCO_2 may not be an accurate measurement of hypoperfusion, without allowing for arterial PCO_2, either by calculating the PCO_2 gap or pHi (Brinkmann et al, 2001; Gardeback et al, 1995; Guzman et al, 1996; Kolkmann et al, 1994, 1997 & 1998; Ronholm et al, 1999; Taylor, 1998).

1.2.6   THE MODERN PARADIGM OF SIRS/ MODS

(i)   Introduction

Multiple organ dysfunction syndrome (MODS) refers to a condition that has become recognized in the last three decades as a major cause of surgical morbidity and mortality. It is estimated that it is responsible for 50-80% of all surgical intensive care deaths. Treatment for this condition is still supportive and mortality rates have remained unchanged over the last 30 years. Despite the high percentage of ICU deaths attributable to this condition, much of our treatment to date remains supportive (Deitch, 1992; Moore et al, 1996).

MODS is the severe end of the spectrum of illness known as systemic inflammatory response syndrome (SIRS). The term sepsis has been used to describe the clinical syndrome of systemic inflammation resulting from invasive infection. It is now understood that a similar or identical response can arise in response to other stimuli, such as trauma and pancreatitis, in the absence of infection. The American College of Chest Physicians and the Society of Critical Care Medicine have since defined this clinical immune response as systemic inflammatory response syndrome (SIRS), independent of its cause. SIRS therefore is defined as a clinical syndrome whose differential diagnosis includes infection as well as a number of non-infectious causes (Bone et al, 1992; Nathens et al, 1996; Rangel-Frausto et al, 1995).

Table 1.3   Diagnostic criteria for SIRS (Bone et al, 1992)

SIRS – the response is manifested by two or more of the following conditions:

- Temperature > 38°C or <36°C

- Heart rate >90 beats per minute

- Respiratory rate >20 breaths per minute or $PaCO_2$ <32 mmHg

- White blood cell count >12,000mm^3, <4,000mm^3 or >10% immature (band) forms

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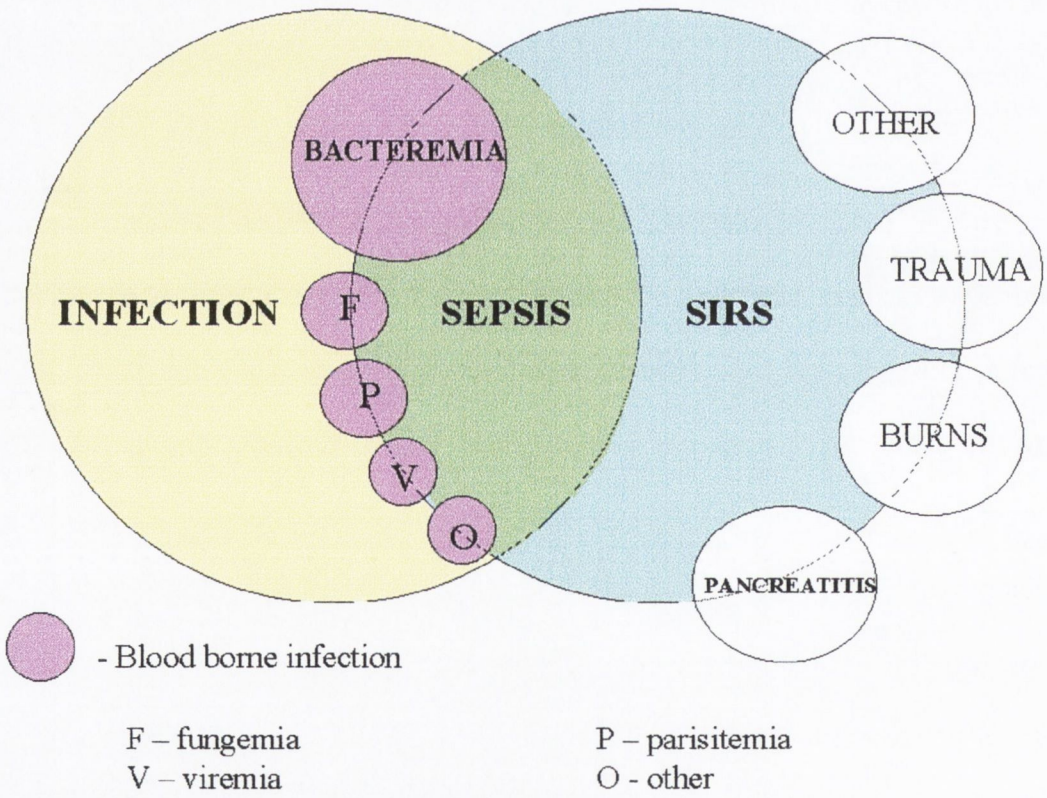
Figure 1.14   The interrelationship between SIRS, sepsis and infection
              (Bone et al, 1992)

The detection of altered organ function in the acutely ill patient is termed multiple organ dysfunction syndrome (MODS) and this is the more severe end of the spectrum of severity of illness in SIRS/sepsis. This may develop as the immediate result of trauma (primary) or as a consequence of the host response (secondary). Secondary MODS therefore usually develops some time after the initial insult to the body. This syndrome was first described by Tilney et al as multiple organ *failure* in a series of patients with ruptured abdominal aortic aneurysms. Since then, it has become recognized as a leading cause of death in trauma and post-operative patients. Although infection/sepsis is a major course of MODS, the syndrome can develop as a result of SIRS in the absence of infection in up to 50% of cases. Despite the high percentage of ICU deaths attributable to this condition, much of our treatment to date remains supportive and research is ongoing to understand the pathophysiology of this condition. Like sepsis and SIRS, it is not clear that MODS is a distinct clinical syndrome, but it does provide a convenient framework for describing morbidity in critical illness. Whatever the initial cause, the progress of MODS is generally uniform, commencing with the lungs and then followed by hepatic, intestinal and renal failure, usually in that order. Coagulopathies and myocardial failure may also occur (Deitch, 1992; Nathens et al, 1996; Tilney et al, 1973).

Table 1.4   Criteria of Organ Dysfunction/failure (Dietch, 1992).

Organ/ System	Dysfunction	Advanced failure
Pulmonary	Hypoxia requiring respirator-assisted ventilation for at least 3 – 5 days	Progressive ARDS requiring PEEP >10cmH$_2$O and FiO$_2$ >0.50
Hepatic	Serum bilirubin $\geq$ 2-3mg/dl or liver function tests $\geq$ twice normal	Clinical jaundice with bilirubin $\geq$ 8-10mg/dl
Renal	Oliguria $\leq$479ml/24hr or rising creatinine ($\geq$2-3mg/dl)	Renal Dialysis
Intestinal	Ileus with intolerance to enteral feeding > 5 days	Stress ulcers requiring transfusion, acalculus cholecystitis
Haematologic	PT and APTT ↑ 25% or platelets < 50-80,000	Disseminated intravascular coagulation
CNS	Confusion, mild disorientation	Progressive coma
Cardiovascular	Decreased ejection fraction or capillary leak syndrome	Hypodynamic response refractory to ionotropic support

As MODS usually develops in organs unrelated to the initial disease, it is hypothesized that MODS is a systemic process, caused by disruption of normal homeostatic mechanisms in the immune system. This can be caused by a number of mechanisms, infection being the most readily recognized. Trauma (mechanical or thermal), pancreatitis or shock can also induce SIRS and MODS. There are a number of interrelated hypotheses as to why SIRS develops, but it is clear that cytokines and other mediators produced by the immune system in response to infection or some other insult cause the inflammatory response seen in SIRS. The two hypotheses examined in this study are the gut hypothesis and the two-hit hypothesis, which is also referred to as cellular priming (Deitch, 1992; Goris, 1996; Rixen et al, 1996; Saadia et al, 1996).

(ii)   The 'Two-Hit' Hypothesis

The two-hit hypothesis, also referred to as cellular priming, states that the host's immune system is primed by an initial insult so that its response is amplified or altered by a subsequent insult ('second-hit'). This may result in either a constructive adaptive response, for example improved tolerance to injury in myocytes, or a destructive maladaptive response (i.e. an exaggerated inflammatory response in neutrophils). In SIRS/MODS, it is the maladaptive response that is of primary interest. In the polytrauma or surgical patient, an episode of hypotension could produce a mild ischaemia-reperfusion injury, resulting in "priming" of resident leucocytes. Any subsequent insult, such as infection, would lead to an amplified or an exaggerated immune response (Deitch, 1992; Meldrum et al, 1997; Sauaia et al, 1996).
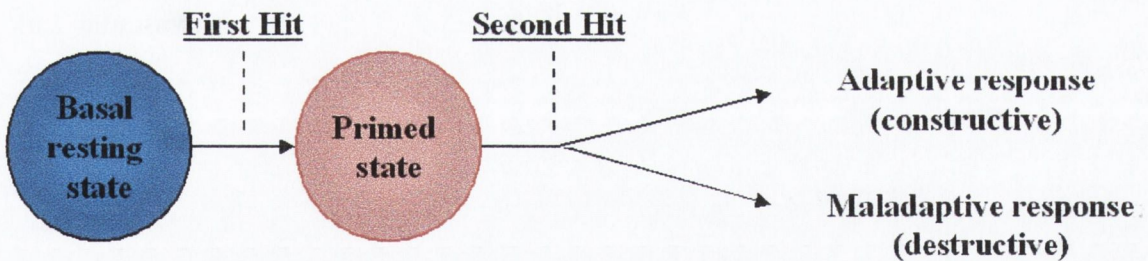
Figure 1.15   The two hit hypothesis (Meldrum et al, 1997)

Following injury or insult to the immune system, the usual response is that T-cell function is altered and neutrophils are stimulated. Suppression of cell-mediated immunity (T-cell response) increases susceptibility to infection and primed neutrophils may cause tissue destruction. It has been demonstrated experimentally that a large number of physiological insults, such as shock, mechanical trauma, or burn injury will prime the host immune system, leaving the host susceptible to infection. In vitro studies have shown that

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neutrophils primed by endotoxin adhere to, but do not damage pulmonary epithelium. The neutrophils detach after 12 hours and cause no injury if stimulated again after this time. If, however, they are subjected to a second stimulus while adherent to the pulmonary epithelium, they cause significant tissue damage. Another study has shown significant differences in pre-operative neutrophil levels of NFκB in patients who develop post-operative MODS as compared to those who do not (Faist et al, 1996; Foulds et al, 2001; Meldrum et al, 1997; Schäffer et al, 1998).
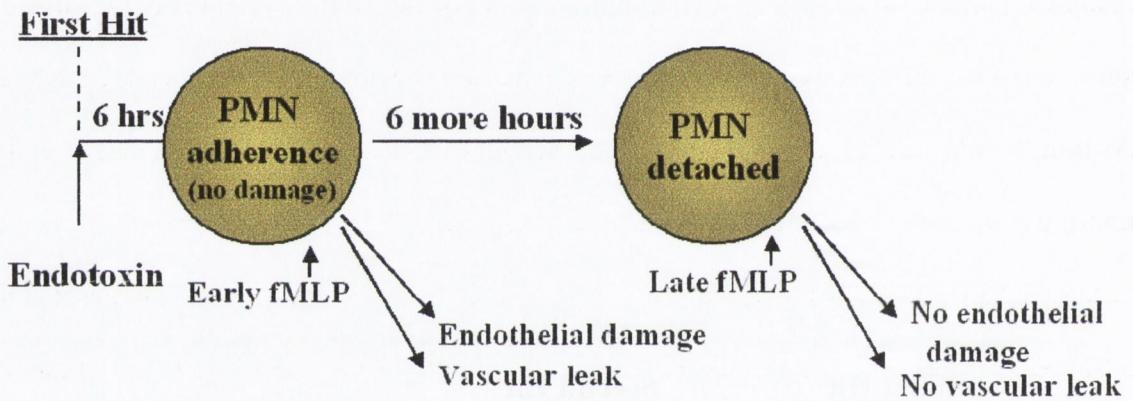
Figure 1.16   Neutrophil priming (Meldrum et al, 1997)

(iii) The Gut Hypothesis

Failure of the gut barrier to contain endogenous bacteria and endotoxins within the gastrointestinal tract, allowing their translocation to mesenteric lymphatics and the portal and systemic circulations is central to the gut hypothesis of multiple organ failure. This hypothesis incriminates bacteria and endotoxins derived from the gastrointestinal tract as triggers, which initiate, perpetuate or exacerbate a systemic inflammatory response resulting in the development of SIRS. During haemorrhage or shock, a decrease in splanchnic blood flow occurs, resulting in mucosal hypoxia, which is then accompanied by a fall in mucosal pH. Necrosis commences at the tips of the villi and extends progressively outwards. The extent of the necrosis is dependant on the degree and duration of the ischaemia, but can develop within minutes of the onset of hypoxia. This damage to the mucosa disrupts the physiological gut barrier, intestinal permeability is increased and translocation of bacteria and endotoxins from the gut can occur, resulting in activation of the systemic immune system. This hypothesis of bacterial translocation can provide an explanation as to why no septic focus can be found in 30% of bacteraemic MODS patients or how patients may develop a SIRS in the absence of infection or another recognizable cause (Deitch, 1992; Fiddian-Green, 1988; Reynolds, 1996; Saadia, 1995; Swank et al, 1996; Vatner, 1974).
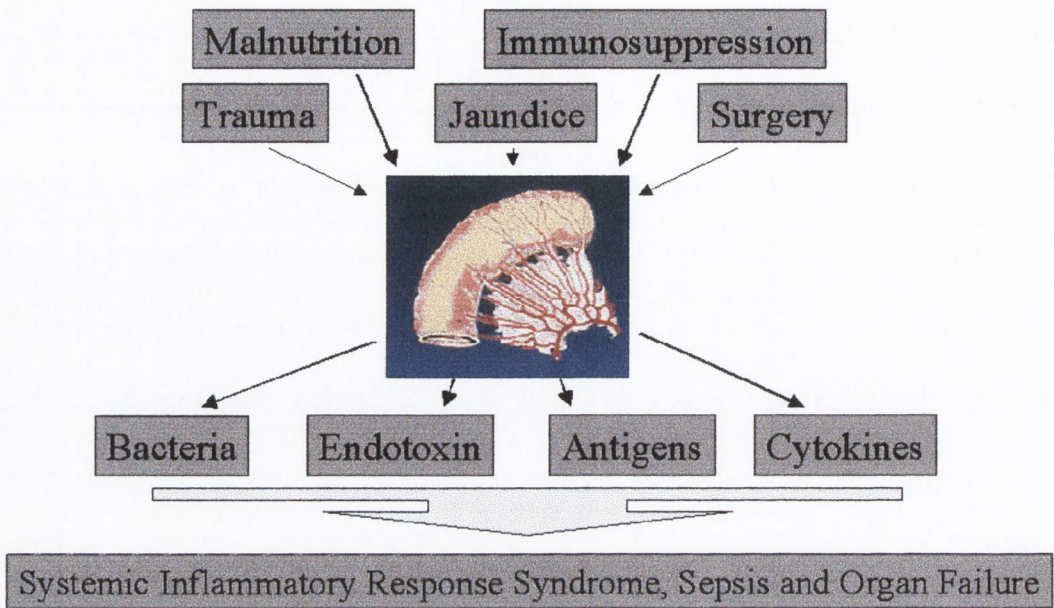
Figure 1.17   The gut hypothesis

The evidence supporting the above hypothesis are as follows: (1) a large body of experimental data documenting that intestinal bacteria can escape from the gut and induce both lethal and non-lethal systemic infectious syndromes; (2) clinical studies indicating that loss of the intestinal barrier to bacteria and endotoxin contributes to the development of systemic infections or MODS; and (3) human studies showing that intestinal permeability is increased during sepsis, jaundice and pancreatitis, and shortly after injury, trauma or major surgery. In addition, new evidence is implicating the gut as a cytokine-generating organ, suggesting that the "stressed" gut may contribute to the development of MODS by direct release of systemic mediators as well as by the process of bacterial translocation (Deitch, 1992; Kompan et al, 2001; Rahman et al, 2003; Riddington et al, 1995; Roumen et al, 1993; Swank et al, 1996; Welsh et al, 1998).

1.2.7 IMMUNE RESPONSES TO SURGERY & MAJOR TRAUMA

Major trauma results in global immune dysregulation leading to systemic inflammatory response, sepsis and death. In the past, it was thought that the predominant effect of surgery on immune function was suppressive; numerous studies on T cells in postoperative patients have documented post-operative anergy, delayed hypersensitivity response and an increase in suppressor cells. It is now recognized, however, that some components of the immune system are instead activated after surgery or trauma, and may be predictive of subsequent recovery or response (Guillou, 1993; Tashiro et al, 1999; Windsor et al, 1995).

(i) C-Reactive Protein

As stated previously, C-reactive protein was the first acute phase protein to be described, as early as 1930, and is an exquisitely sensitive systemic marker of inflammation and tissue damage. Its plasma concentration may increase by several hundred fold within 24 to 48 hours after tissue injury, from a normal resting CRP concentration of <0.8 mg/l. These levels persist for the duration of the acute phase response, giving an excellent correlation between circulating CRP concentration with the severity, extent and progression of inflammation (Foglar et al, 1998; Karayiannakis et al, 1997; Pepys et al, 2003; Shine et al, 1981; Vigushin et al, 1993).

The inflammatory response to surgical procedures has been described in numerous studies. The CRP level of a patient undergoing surgery who then later develops a septic complication might be expected to follow one of two possible courses: (1) the CRP level rises post-operatively and fails to fall to normal as expected; or (2) the CRP level rises following an operation, falls normally as would be anticipated, and then rises again as sepsis becomes established (Foglar et al, 1998). In studies evaluating the efficacy of serial post-operative CRP in predicting postoperative septic complications, CRP has been confirmed as being highly predictive; for example one study showed a sensitivity of 63% and a specificity of 82%, resulting in a positive predictive value of 69% (Mustard et al, 1987). In recent years, the possibility of using preoperative CRP levels to predict subsequent postoperative outcome has been investigated, with contradictory results. Franson et al, in a study of 593 patients undergoing cardiac operations, showed that pre-operatively increased CRP levels are associated with an increased risk of post-operative infections; this conclusion was also reached by Biancari et al in a study of 1048 patients undergoing coronary artery bypass surgery (Biancari et al, 2003; Franson et al, 1999). In contrast, however, Gaudino et al failed to find any correlation between preoperative CRP value and postoperative clinical course (Gaudino et al, 2002).

(ii)   Leucocyte responses

In normal situations, proinflammatory mediators are released locally in response to a stimulus, such as infection, injury and/or ischaemia, in order to eliminate pathogens and to promote healing and recovery. This response is then down-regulated by the release of anti-inflammatory mediators, resulting in the restoration of homeostasis. Severe injury, such as trauma, burns or surgery, sets in motion a systemic proinflammatory host immune response which is then followed by a counter-inflammatory reaction, that may leave the patient highly susceptible to opportunistic infections and subsequent infections. These two responses are referred to respectively as the systemic inflammatory response syndrome (SIRS) and the compensatory anti-inflammatory response syndrome (CARS). The latter syndrome is a cytokine antagonist cascade, which results in an immunosuppressed and/or lymphopenic state and has been recognized for over 40 years. The balance between pro- and anti-inflammatory responses is frequently lost (Muller Kobold et al, 2000; Windsor et al, 1995).

Previously, it was thought that major surgery led to a general immunosuppression of T cell function, as characterized by delayed hypersensitivity reactions, decreased T helper cell activity and increased suppressor T-cell activity. With a few exceptions, however, this T-cell hyporeactivity was not found to possess any clinical correlation with post-operative infection. It is now believed that a polarization of the lymphocyte response to a Th2 phenotype occurs, rather than a generalized T-cell suppression, resulting in down-regulation of cell-mediated immunity and up-regulation of antibody-mediated immunity. Indications for this include reduced secretion of Th1 cytokines IFN-γ and IL-2 and increased or unchanged secretion of Th2 cytokine IL-4 by stimulated PBMC from post-

operative patients (Berguer et al, 1999; Cheadle et al, 1993; Christou et al, 1995; Decker et al, 1996; Guillou, 1993).

The initial cytokine milieu is one of the main factors determining Th1 and Th2 differentiation post-operatively and it is paradoxical that an injury-induced cytokine response, which should promote a proinflammatory immune response, instead produces the Th2 phenotype. One possible explanation is that this shift in phenotypes may be due to endogenous corticosteroid release following stress. In general, it is accepted that surgical stress induces a systemic endocrine-metabolic response, which includes stimulation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. This results in production of glucocorticoids and androgens. Glucocorticoids enhance Th2 activity and synergize with IL-4 to attenuate Th1 activity. In addition, corticosteroids can reduce monocyte transcription of IL-1, IL-6 and TNF, and so may be responsible for the 24-hour decline in circulating monokine levels. Monocytes also release prostaglandin E_2, a known suppressor of T cell function and several studies have demonstrated the presence of PGE_2-secreting monocytes after injury (Berguer et al, 1999; Decker et al, 1996; Faist et al, 1996; Guillou, 1993).

Within the monocyte/macrophage lineage, immediate hyperactivation with the excessive release of proinflammatory cytokines is followed in most patients by substantial paralysis of cell function, which is then overcome after 3 to 5 days with newly recruited macrophages. The importance of the monocyte in the postoperative immune response was emphatically demonstrated with studies examining the level of expression of MHC class II antigen (HLA-DR) postoperatively and the subsequent development of complications or sepsis. In patients with an uneventful recovery, HLA-DR expression falls, but rises to

normal again within one week; in patients who develop infection or complications, this return to normal levels is delayed (Cheadle et al, 1991; Ditschkowski et al, 1999; Guillou, 1993; Lin et al, 1993; Wakefield et al, 1993).

It has also been observed that T cells show an early up-regulation of HLA-DR post-operatively, which is abrogated in patients who subsequently develop sepsis in a manner similar to that observed on monocytes. This response may be related to the nutritional status of the patient (Wakefield et al, 1993; Welsh et al, 1996).

The notion has evolved in recent years that tissue injury associated with MODS and SIRS may be mediated by secretion of reactive oxygen species and proteases by activated neutrophils (Meldrum et al, 1997; Schlag et al, 1996). It is known that neutrophils are activated during shock and trauma and then become sequestrated into vital organs, such as the lung and liver. When activated, neutrophils upregulate their expression of the integrin CD11b/CD18, which is necessary for adherence to vascular endothelium (Guillou, 1993). Following uncomplicated major abdominal surgery, the expression of this integrin was unchanged throughout the postoperative period. In contrast, however, mean CD11b expression on the first postoperative day was greater in those patients who subsequently developed postoperative sepsis (Wakefield et al, 1993). Similarly, in a study of patients undergoing major thoracic surgery, it was found that preoperative neutrophil NF-κB levels were greater in patients who developed postoperative MODS than those who did not (Foulds et al, 2001).

(iii)   Intestinal permeability

As described previously, a non-invasive method of investigating the gut hypothesis is that of measuring intestinal permeability. Previous studies have shown that intestinal permeability increases following such insults as surgery, radiation therapy, chemotherapy, malnutrition and obstructive jaundice. However, these studies have to date failed to show a correlation between elevated postoperative permeability and the subsequent development of post-operative complications (Kanwar et al, 2000; Kohout et al, 1999; Nejdfors et al, 2000; Roumen et al, 1993; Sinclair et al, 1995; Welsh et al, 1998).

(iv)   Splanchnic blood flow

Assessment of pHi in both intensive care and intraoperatively has been shown to correlate with morbidity and mortality of critically ill patients. While a small number of studies have examined the relationship between intraoperative pHi and clinical outcome measures, there is an absence of data assessing the specific effects on immune function, such as acute-phase response, leucocyte activation and intestinal permeability (Bass et al, 1985; Björck et al, 1994; Gutierrez et al, 1992; Olsen et al, 1995).

1.2.8   IMMUNE RESPONSES TO NEOADJUVANT CHEMORADIOTHERAPY

Oesophagectomy remains a standard treatment for patients with resectable oesophageal carcinoma, and long-term survival exceeds 20% in some surgical series, showing steady improvement, but this may be more a reflection of advances in preoperative staging and selection in recent years, rather than effectiveness of therapy. Many patients may have micrometastatic spread at the time of their surgery, leading to later recurrence.   In theory, neo-adjuvant chemoradiotherapy offers early treatment of this micrometastatic disease and downstaging of the primary tumour, leading to easier resection at surgery.   In addition, neoadjuvant therapy has been shown to be better tolerated than post-operative therapy.   However, chemoradiotherapy has associated treatment toxicity, and may contribute to post-operative morbidity and mortality, but there is as yet no conclusive evidence to prove or disprove this hypothesis (Swisher et al, 1996; Urschel et al, 2002).

(i)   C-Reactive Protein

Little research to date has been performed assessing specific immune responses, such as CRP, to chemoradiotherapy, or to postoperative response in patients who have undergone neoadjuvant therapy. Staal-van den Brekel and Milroy et al found contradictory results in their studies of immunological response to chemotherapy for small cell lung carcinoma; Staal-van den Brekel et al found that chemotherapy significantly reduced CRP levels in the immediate post-treatment period, but Milroy et al determined that CRP was elevated following chemotherapy in their study. Endo et al examined a number of immune parameters in a group of 38 patients undergoing lung resection for carcinoma, 19 of whom

had undergone neoadjuvant chemotherapy. They observed a postoperative increase in serum CRP levels in both groups, but no significant difference between the two (although serum concentration of the cytokines IL-6 and G-CSF rose significantly in the neoadjuvant group in comparison to those who received surgical therapy alone). It should be noted however, that these immune parameters were only recorded until the third post-operative day (Endo et al, 2004; Milroy et al, 1989; Staal-van den Brekel et al, 1997).

(ii)   Leucocyte responses

As with CRP, there is very little research to date regarding the postoperative leucocyte response in patients who have first undergone neoadjuvant therapy. In one study of 30 patients undergoing treatment for rectal carcinoma, half of whom received neoadjuvant chemoradiotherapy, postoperative monocyte and granulocyte counts were significantly lower in the neoadjuvant group. In addition, after preoperative chemoradiotherapy, a significant depression of T cells, T helper cells, T suppressor cells and natural killer cells was observed when compared to pretherapeutic values, and this persisted in the postoperative period, with significant differences visible between the two study groups (Wichmann et al, 2003).

Similarly, a further study investigating the effect of neoadjuvant therapy prior to surgery for oesophageal carcinoma also concluded that suppression of T cell function was observed in the neoadjuvant group. Specifically, they observed a reduced proliferative response following stimulation with superantigen in patients who had undergone pre-operative chemoradiotherapy in comparison to those who had surgical therapy alone. Of note, the HLA-DR expression on T lymphocytes was significantly elevated in those who underwent neoadjuvant treatment as compared to those who did not (Heidecke et al, 2002).

(iii)   Intestinal permeability

While alterations in intestinal permeability following the administration of chemotherapy or radiotherapy has been studied in the past, no data was obtainable as regards postoperative permeability in patients who had received neoadjuvant therapy as compared to those who did not. Multiple studies have confirmed that intestinal permeability is increased following chemotherapy for a number of conditions, in both paediatric and adult populations (Keefe et al, 1997; Kohout et al, 1999; Melichar et al, 2001; Pledger et al, 1998). Similarly, radiation therapy has been shown to increase permeability in irradiated, as compared to nonirradiated, large bowel (Nejdfors et al, 2000).

1.3   STATEMENT OF HYPOTHESIS & AIMS

The systemic inflammatory response syndrome (SIRS), sepsis and multiple organ dysfunction syndrome (MODS) are recognized sequelae of complex major surgery and underlie significant morbidity and mortality.   Some studies estimate that MODS is responsible for as many as $50 - 80\%$ of all surgical intensive care deaths, with little improvement in mortality rates over the past 20 years (Deitch, 1992; Guillou, 1993). Patients at high risk include those undergoing major invasive resectional surgery, such as for upper gastrointestinal malignancy.

The systemic immunoinflammation underlying SIRS and MODS in the presence or absence of infection is not fully understood, but two of the current hypotheses are the 'two-hit' and gut hypotheses. The 'two-hit' hypothesis proposes that if the host immune system has been primed by an initial insult, that the immune response to any subsequent or second insult will be greatly amplified. The gut hypothesis incriminates bacteria and endotoxins derived from the gastrointestinal tract as triggers which initiate, perpetuate or exacerbate a systemic inflammatory response resulting in the development of SIRS.

The aim of this research was to examine the effect of reduced intraoperative splanchnic blood flow on gastrointestinal and systemic immune function, by assessing portal and systemic acute phase responses, cellular activation and alterations in postoperative gut function. In addition, systemic immune responses in those who have received neoadjuvant chemoradiotherapy were investigated, to assess if any 'priming' of immune components is identifiable, which would support the 'two-hit' hypothesis.

The main aims of this research were as follows:

1. To determine whether the development of the Systemic Inflammatory Response Syndrome or Multiple Organ Dysfunction can be predicted by immune parameters in the immediate post-operative period following major upper gastrointestinal surgery for malignancy.

2. To determine whether a decrease in splanchnic blood flow is observed during major surgery and describe its association with subsequent intestinal permeability, cellular immune activation and development of SIRS/MODS.

3. To differentiate portal and systemic immune responses during major upper gastrointestinal surgery for malignancy.

4. To determine whether neo-adjuvant chemotherapy and radiation therapy prime the immune system, thus leading to exaggerated post-operative immune activation following oesophageal resection in comparison to patients who receive surgical treatment alone.

CHAPTER 2: MATERIALS & METHODS

2.1   PATIENT POPULATION

This study was performed in a tertiary referral centre and was approved by the hospital ethics committee for research involving human subjects according to the Helsinki agreement. In addition, informed consent was obtained from all patients pre-operatively. Patients undergoing surgical resection for upper gastrointestinal malignancy were prospectively recruited to the study and informed consent was obtained from all patients pre-operatively.

In total, 29 patients were recruited to the study, all of who underwent upper gastrointestinal resection for malignancy, namely gastrectomy (n = 7), two-stage oesophagectomy (n = 12), three-stage oesophagectomy (n = 6) and pancreatic resection (n = 4). All patients underwent initial endoscopic and radiological staging and no patient had evidence of metastatic disease. One surgeon (JVR) performed the operations and obtained intraoperative portal venous blood samples. Total gastrectomy and pancreatic resections were performed by laparotomy. Oesophagectomy was performed either by laparotomy (two-stage oesophagectomy) or by laparotomy, thoracotomy and neck incision (three-stage oesophagectomy). Insertion of a jejunostomy feeding tube was part of the standard procedure for oesophagectomy, enteral feeding was commenced on the first postoperative day at a rate of 30mls an hour, and increasing to a maximum of 100mls an hour after 72 hours. All patients received intravenous broad-spectrum antibiotics at induction of anaesthesia and this was continued for a minimum of 3 days.

Table 2.1   Basic Patient demographics

Number of Patients	29
Age [Median (interquartile range)]	66.0 (53.2 – 72.0) years
Sex	Male n = 18 Female n = 11

Two consultant anaesthetists (NH, MC) were involved in the general anaesthesia and intraoperative management of these patients.   Crystalloid, rather than colloid, resuscitation was used in all cases and all patients received intrathecal morphine (1-1.6 mgs) and 2% lignocaine via a thoracic epidural.   Postoperative analgesia was with L-Bupivicaine administered via the epidural for up to 72 hours in the High Dependency Unit followed by a morphine PCA either in the HDU or ward.   The mean arterial pressure (MAP) and central venous pressure were monitored during surgery (CVP).   If the MAP fell below a value considered appropriate by the anaesthesiologist for the patient despite adequate intravascular volume, a phenylephrine infusion (20µg/ml) was used to increase the pressure.   For the first 24 hours, all patients were initially managed in the high dependency or intensive care units, then returned to the wards as their progress permitted. Twenty-seven of these patients underwent flow cytometric analysis of T-cell and monocyte surface markers and twenty had intraoperative pHi measurements performed using a tonometric catheter, as described later in this section.

A second group of 100 patients were included in some sections of this research (chapter 6, sections 6.4.3 and 6.4.5 only). These patients also underwent upper gastrointestinal resection for malignancy in this institution, over a period of 3 years, all with curative intent. Half of these patients received neoadjuvant chemoradiotherapy prior to their surgery. Data such as white cell count, albumin and clinical outcome measures were recorded retrospectively. Operative and postoperative management protocols were as described above, but this group of patients did not undergo intraoperative tonometric monitoring or specific immunological intra- and post-operative investigations.

2.2 DETERMINATION OF POST-OPERATIVE OUTCOME MEASURES

Clinical outcome measures recorded prospectively in all patients included length of stay in hospital or ICU/HDU, and development of post-operative complications, such as sepsis, systemic inflammatory response syndrome (SIRS) or multiple organ dysfunction syndrome (MODS). SIRS was defined as two or more of the following criteria: a body temperature greater than 38°C or less than 36°C; pulse rate greater than 90 per minute; respiratory rate above 20 per minute or hyperventilation, as indicated by a $PaCO_2$ of less than 32mmHg; white cell count greater than 12,000 or less than 4,000 cells/mm^3 (Bone et al, 1992). Sepsis or a septic complication was defined as two or more of the above criteria present (as for SIRS) as a result of infection (confirmed by laboratory culture). The definition of multiple organ dysfunction applied was that proposed by Dietch (Ann Surg, 1992), that of dysfunction in two or more organ systems, as detailed in table 1.4.

2.3   PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

Venous blood was collected into Vacutainer tubes containing lithium heparin (Becton Dickenson, Oxford, U.K.) and then transported to the laboratory on ice and prepared for analysis within one hour of collection. Intraoperative blood samples were also obtained in all patients, one sample withdrawn from peripheral access (i.e. central line) and the second sample aspirated by the operating surgeon from the portal vein. In order to isolate peripheral blood mononuclear cells, a 5ml blood sample was diluted 50:50 with Hanks balanced salt solution (HBSS, Gibco, U.K.) supplemented with 2% HEPES buffer (Gibco, U.K.). The diluted blood was layered over 5ml lymphoprep (Nycomed, Oslo, Norway) before centrifugation at 1500rpm for 30 minutes at 4°C. Interface cells were removed by pipette, washed twice in HBSS/HEPES at 1500rpm for 10 minutes before resuspension in RPMI 1640 with glutamine (Gibco, U.K.), supplemented with 10% heat-inactivated fetal calf serum (Gibco, U.K.), L-glutamine, penicillin and streptomycin (Sigma, U.K.). Cell counting and viability were assessed using trypan blue (Sigma, U.K.). PBMC's were then resuspended at a concentration of 2×10^6/ml and viability was always greater than 95%.

2.4   T-CELL AND MONOCYTE SURFACE MARKER EXPRESSION

Expression of surface markers on T-cells and monocytes was performed using commercially available fluorescent monoclonal antibodies. A double staining technique with mouse anti-human monoclonal antibodies, directly conjugated to fluourescin isothiocyanate (FITC) or phycoerythrin (PE) was used: CD3-FITC (555332/30104X), CD4-FITC (30154X), CD8-FITC (555366/30324X), CD14-FITC (555397/30544X),

CD25-PE (555432/30795X), CD62L-PE (555544/32235X), CD69-PE (555531/31955X), CD71-PE (555537) and HLA-DR-PE (555812/34235X) were obtained from Pharmingen (BD Pharmingen, Oxford, U.K.). IgG1-FITC, IgG2a-FITC, IgG1-PE and IgG2a-PE (Pharmingen) were used as controls.

$100\mu l$ of PBMC's at 2 x 10^6/ml were added to 1ml of FACSFlow^{TM} (Becton-Dickenson, Oxford, U.K.) for cell staining. Cells were incubated with $5\mu l$ of each appropriate antibody in the dark for 30 minutes at 4^oC. The cells were then washed 3 times in FACSFlow^{TM} (Becton Dickenson, Oxford, U.K.) at 1200rpm and finally resuspended in 100ml FACSFlow^{TM} and then fixed with 100ml CellFIX^{TM} (Becton Dickenson, Oxford, U.K.). Flow cytometric analysis was performed within 24 hours of fixing the samples on a FACSort^{TM} analyser using CellQuest^{TM} software (Becton Dickenson, Oxford, U.K.). For each sample, a total of 10,000 events were recorded and isotype-matched controls were used to establish non-specific staining of samples.

T-cells were identified using their morphological characteristics of size and granularity (using forward vs. side scatter) and gated to exclude granulocytes and monocytes (figure 2.1). Non-specific fluorescence was quadranted out using the control antibody and T-cell subpopulations were identified by expression of surface CD3, CD4 or CD8 along the axis of an X plot (figure 2.2). Expression of CD25, CD69, CD71 and HLA-DR was read along the y-axis and expressed as the percentage of T-cells staining positive for the surface activation marker.
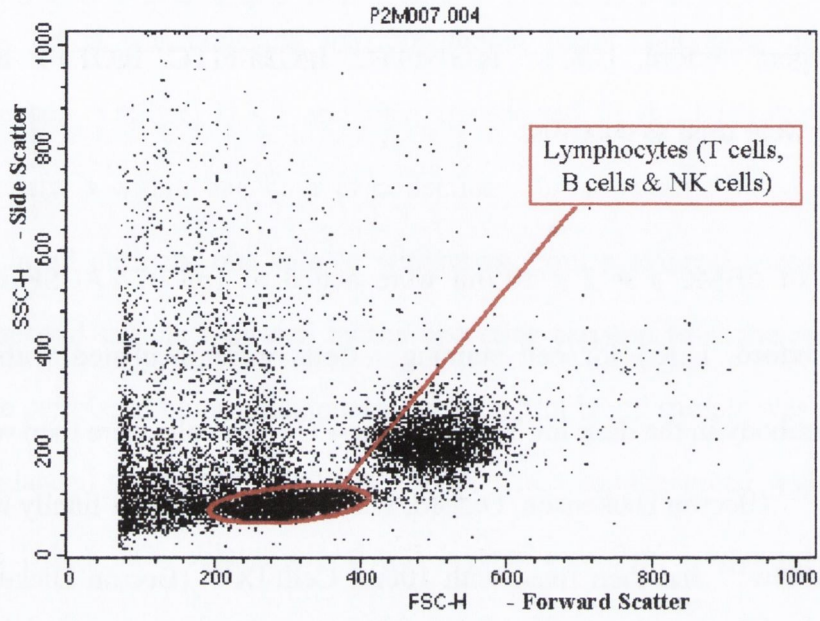
Figure 2.1   Identification of lymphocyte population (including T cells)

FITC staining for CD4
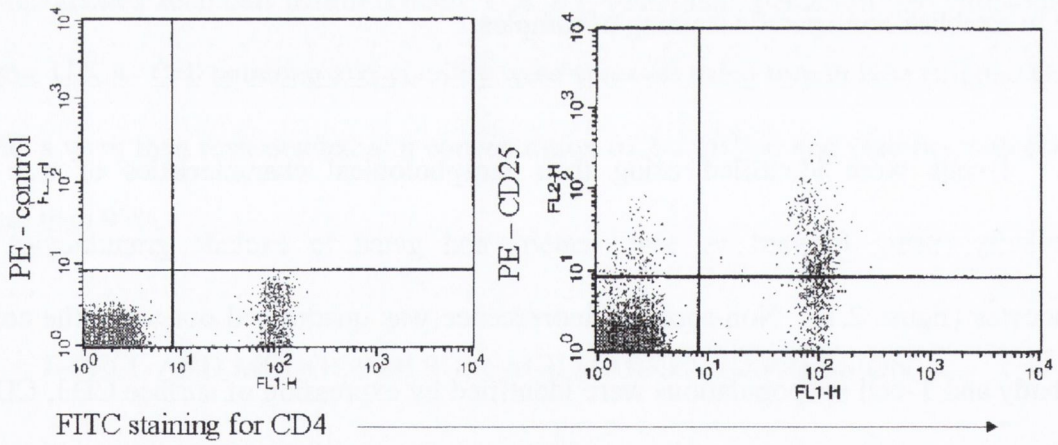
Figure 2.2   Sample dot plot of CD25 expression on CD4+ T-cells

Monocytes were identified and gated according forward light scatter characteristics and CD14-FITC staining (figure 2.3). Non-specific fluorescence was quadranted out using the control antibody (figure 2.4). Results were expressed as the percentage of monocytes staining positive for the surface activation marker.
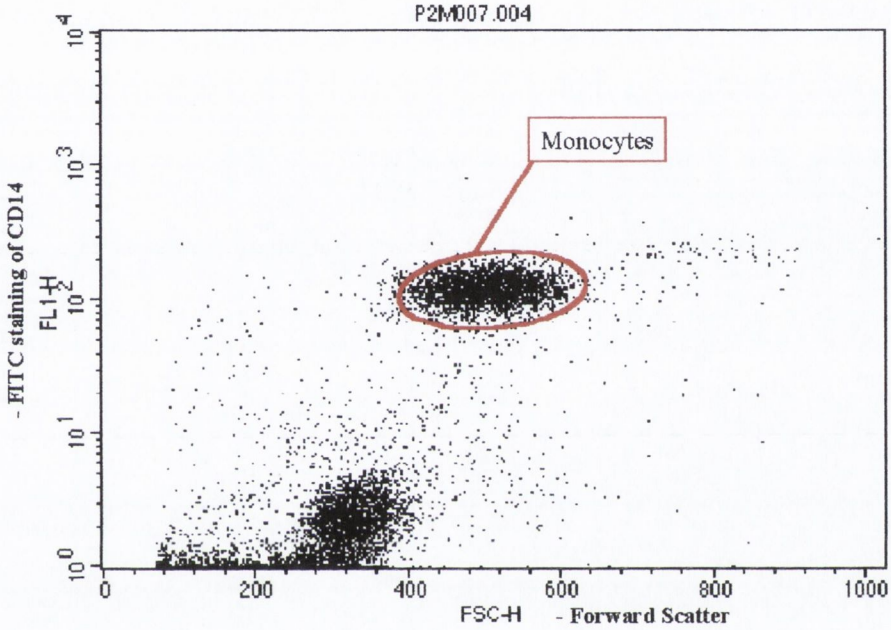
Figure 2.3   Identification of monocyte population
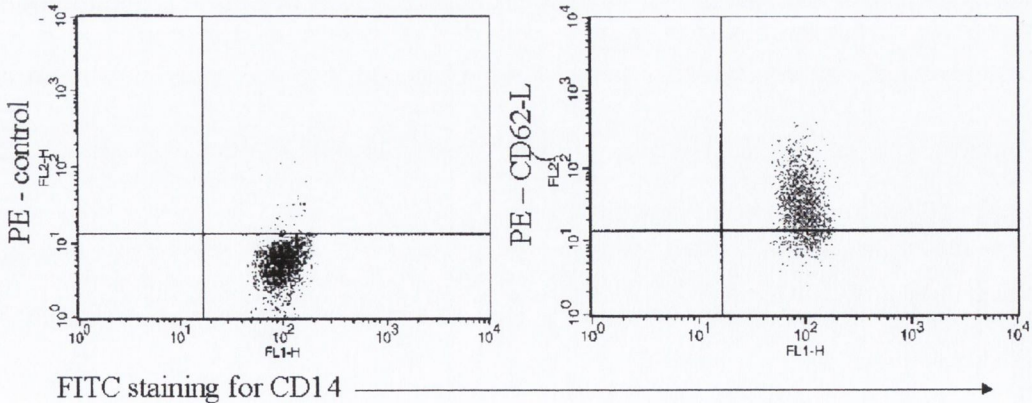
Figure 2.4   Sample dot plot of CD62-L expression on CD14+ monocytes

2.5   INTRANUCLEAR NF-KAPPA B EXPRESSION

A modification of a technique for quantitative analysis of nuclear-bound NF kappa B was employed (Foulds, 1997). Venous blood was collected into Vacutainer tubes containing EDTA (Becton Dickenson, Oxford, U.K.) and then transported to the laboratory on ice and prepared for analysis within one hour of collection. Intraoperative blood samples were also obtained in all patients, one sample withdrawn from peripheral access (i.e. central line) and the second sample aspirated by the operating surgeon from the portal vein.

In the laboratory, 2ml of FACS lysing solution (Becton-Dickenson, Oxford, U.K.) was added to a 100μl aliquot of blood, and incubated at room temperature for 30 minutes. The sample was then centrifuged at 900rpm for 5 minutes and the supernatent was discarded. The cells were then washed once with phosphate buffered saline (PBS) at 900rpm for 5 minutes. The sample was then prepared for staining using reagents from the Cycletest Plus DNA reagent kit (Becton-Dickenson, Oxford, U.K). Next, the sample was resuspended in 4mls of citrate buffer and centrifuged at 1100rpm for 5 minutes. The supernatent was discarded and then the cells were resuspended in 1.5mls citrate buffer and centrifuged for a further 5 minutes at 1100rpm. The supernatent was then carefully discarded and any residual fluid blotted dry.

The pellet was then very gently resuspended in 250μl solution A (trypsin in a spermine tetrahydrochloride detergent buffer) and 200μl solution B (trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine tetrahydrochloride) for 10 minutes at room temperature. A blocking step of 20 minutes incubation with 50μl normal human serum (NHS) was then used, prior to staining. Next, 40μl of rabbit polyclonal NF kappa B antibody (RelA) or control antibody (Santa Cruz Biological, Heidelberg, Germany) was then added for 10 minutes and incubated at room temperature, followed by a further 10 minute incubation with 2.5μl FITC-conjugated swine anti-rabbit monoclonal antibody (DAKO, Denmark). 200μl of cold propidium Iodide (PI) was then added and the sample was incubated for a further 10 minutes. The sample was then brought to the flow cytometer and analysed within 3 hours of the final preparation step.

Flow cytometric analysis was performed within 3 hours of incubation with propidium iodide on a FACSort™ analyser using CellQuest™ software (Becton Dickenson, Oxford, U.K.). For each sample, a total of 20,000 events were recorded and isotype-matched controls were used to establish non-specific staining of samples. The FACSort settings were determined using the DNA Quality Control Particles kit (Becton-Dickenson, Oxford, U.K) (Foulds, 1997). Singlet nuclei were first identified using propidium iodide staining characteristics, namely area (FL2-A) and width (FL2-W) (figure 2.5).
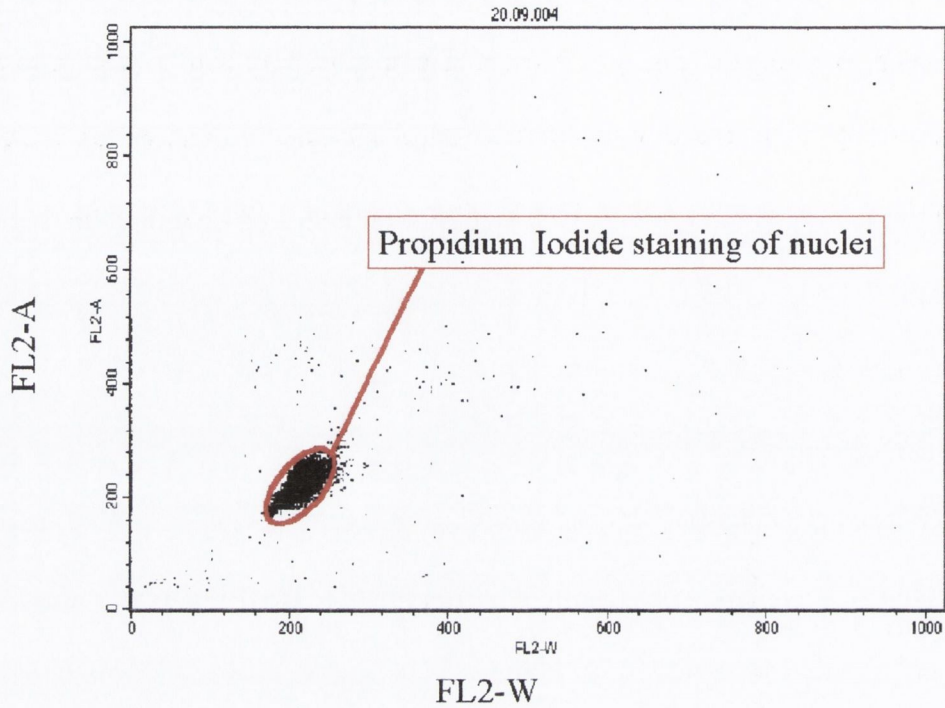
Figure 2.5   Identification of singlet nuclei

These singlet nuclei were then analysed according to forward and scatter side scatter characteristics, where nuclei from polymorphonuclear and mononuclear cells have distinctly different characteristics (Foulds, 1997). A gate was then set around either the mononuclear cells or the polymorphonuclear cells for analysis of FITC staining, using histogram analysis of the FL1-H parameter to determine the mean channel fluorescence (MCF) (Figure 2.6). In order to compensate for non-specific staining and autofluouresence, the MCF of the control sample was then subtracted from that of the NF-kappa B sample.

As this technique used polyclonal NF-kappa B antibody, direct comparison between samples analysed using different batches of antibody was inappropriate. Therefore, intraoperative and postoperative levels of NF-kappa B expression were defined as percentages of the patient's initial, preoperative, value for analysis purposes.
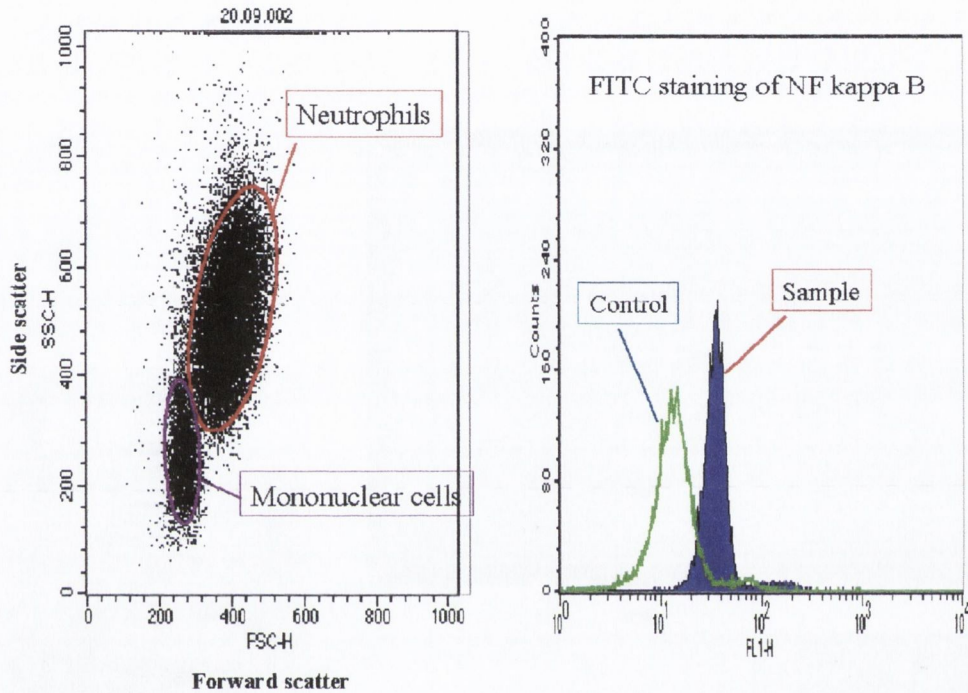
Figure 2.6   Identification of nuclei and measurement of mean channel fluourescence

2.6 INTRAOPERATIVE TONOMETRIC MEASUREMENT OF SPLANCHNIC BLOOD FLOW

Intraoperative splanchnic blood flow was monitored by means of a tonometer, a modified nasogastric tube with a silicone balloon incorporated into its tip and a sampling line along its length (Tono-16F, Datex-Ohmeda, Finland – figures 1.13 & 2.7). All patients received 50mg ranitidine pre-operatively, in order to inhibit intrinsic gastric acid production (Kolkmann et al, 1994). In order to standardise the study, only 2 consultants

were involved in the general anaesthesia and intraoperative management of these patients. Crystalloid, rather than colloid, resuscitation was used in all cases and all patients, bar two, had an epidural placed prior to induction, the other two receiving intrathecal morphine.
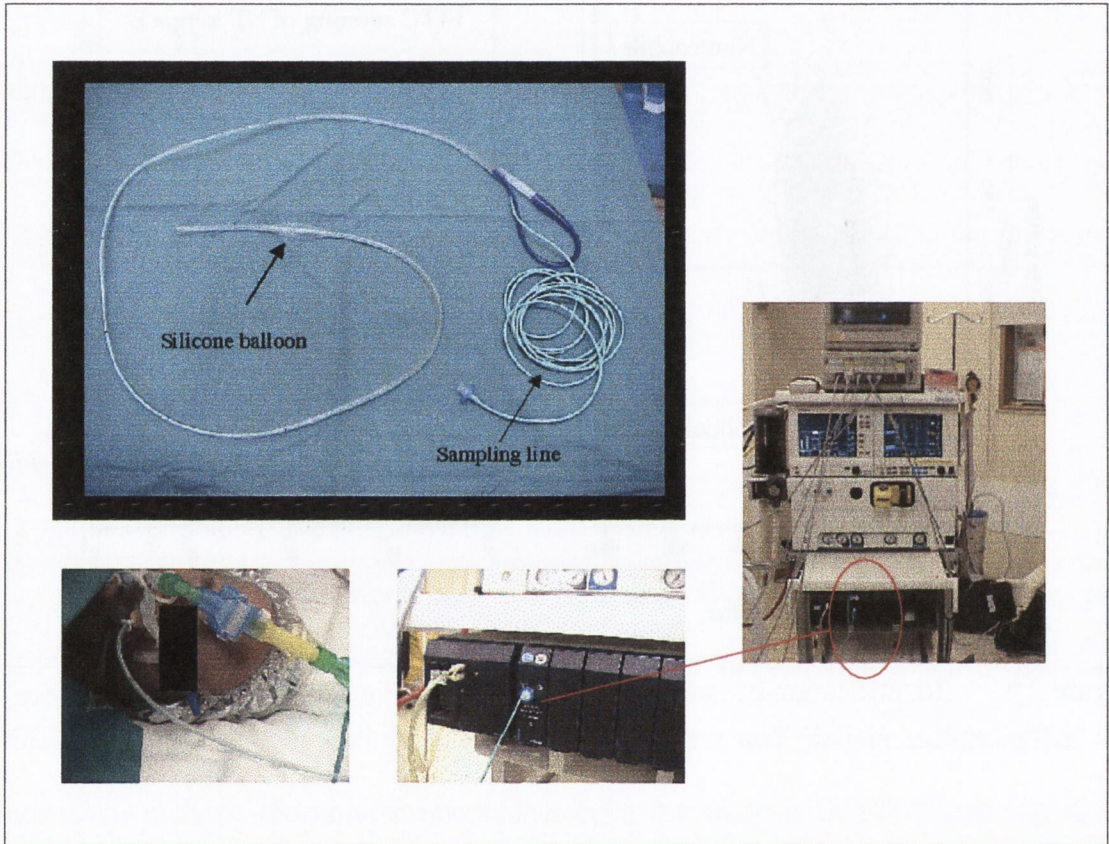
Figure 2.7   Intraoperative splanchnic tonometry

Following induction of anaesthesia, the tonometer was introduced into the stomach of all patients. The sampling line was then connected to a specialised tonometry module, which is incorporated into the anaesthetic machine (M-TONO, S/5 Tonometry Module, Datex-Ohmeda, Finland). The silicone balloon of the tonometer is permeable to carbon dioxide and the concentration of the gas equilibrates with the mucosal carbon dioxide concentration over a period of ten minutes. After this period of time, the gas within the balloon is withdrawn by means of the sampling line into the tonometry module and the carbon dioxide concentration is measured and recorded by the anaesthetic machine. Data ($PaCO_2$, PaO_2, arterial pH, SaO_2 and haemoglobin) from an arterial blood gas (ABG) is then inputted into the anaesthetic machine, which automatically calculates the mucosal pH by means of the Henderson-Hasselbach Equation (Clark et al, 1992). PHi measurements were obtained every 10 minutes throughout the procedure, along with the patients systolic, diastolic and mean arterial blood pressure, heart rate and central venous pressure. A fresh ABG for data input was obtained every hour, or following any alteration in the patient's condition.

2.7   ASSESSMENT OF INTESTINAL PERMEABILITY

Intestinal permeability was measured pre-operatively and on the first and seventh post-operative days by means of the lactulose/mannitol test (Juby et al, 1989). The test solution was made in batches by the Biochemistry Department of St. James' Hospital. Each solution contained 22.4g glucose, 2g mannitol and 5g lactulose in 100mls of distilled water. These were then stored at -20°C and thawed at room temperature prior to use.

Patients fasted from midnight and fully voided the bladder to provide a pre-test 'blank' urine sample. The test solution was then administered. In pre-operative patients, the solution was taken orally; post-operatively, it was administered by means of a jejunostomy tube. All urine passed within the subsequent 5 hours was collected into a polythene bottle containing 0.2g chlorhexidine (Medlock Medical Ltd, UK) as preservative. The volume of this sample was recorded and 20ml aliquots of both this and the pre-test urine were then frozen at -70°C until analysis.

Urinary lactulose and mannitol concentrations were determined by automated enzymatic assay on the Hitachi Modular (Roche, Lewis, U.K.) using the SAT reagent kit (Instruchemie, Hilversum, Netherlands) (Lunn et al, 1989; Northrop CA et al, 1990). Results are expressed as a ratio of the percentage recoveries of the two molecules. The normal lactulose-mannitol ratio is $0.004 - 0.028$ (Juby et al, 1989).

2.8   STATISTICAL ANALYSIS

Statistical analysis was computed using the Statview computer program (Abacus Concepts, Inc., Berkeley, California). For analysis of continuous variables, the Wilcoxon rank test was applied for non-parametric data. For comparison between two groups, the Mann-Whitney U test was applied, as the data was again of a non-parametric distribution. The Chi Square test with Yates's continuity correction was used for analysis of categorical variables.

CHAPTER 3: MAJOR SURGERY PRIMES THE IMMUNE SYSTEM, RESULTING IN ACTIVATION OF MONOCYTES & T CELLS & ALTERATION IN NUCLEAR NF-KAPPA B EXPRESSION

3.1   INTRODUCTION

In the past, it was thought that the predominant effect of trauma on immune function was suppressive; numerous studies on T cells in postoperative patients have documented post-operative anergy, delayed hypersensitivity response and an increase in suppressor cells (Faist et al, 1986; Pietsch et al, 1977). It is now recognized, however, that events such as trauma, burns or surgery set in motion a systemic proinflammatory host immune response which is then followed by a counter-inflammatory reaction, that may leave the patient highly susceptible to opportunistic infections and subsequent infections. These two responses are referred to respectively as the systemic inflammatory response syndrome (SIRS) and the compensatory anti-inflammatory response syndrome (CARS). The latter syndrome is a cytokine antagonist cascade, which results in an immunosuppressed and/or lymphopenic state and has been recognized for over 40 years. The balance between pro- and anti-inflammatory responses is frequently lost. Several components of the immune system have been implicated in this immunocompetency, of both the innate and adaptive immune systems (Aguilar et al, 1998; Giannoudis et al, 1998; Muller Kobold et al, 2000; Schaffer et al, 1998; Sweeney et al, 2005; Windsor et al, 1995).

Absolute numbers of leucocytes have been shown to decrease in the circulation following severe injury, with a decrease in overall T cell numbers. More specific T cell responses following trauma or injury depend to a large degree on subtype. A decrease in absolute numbers of CD4+ helper cells in sepsis or following trauma has been documented in a number of studies. Furthermore, the degree of depression has been shown by some authors to predict subsequent outcome, being most pronounced in those who develop sepsis or fail to survive (Aguilar et al, 1998; Cheadle et al, 1993; Cioffi et al, 1993; Holub et al, 2000; Lin et al, 1993; Menges et al, 1999; Schaffer et al, 1998; Walsh et al, 2000).

Changes in the CD8+ subtype following injury have shown a more variable pattern. Some studies show a decrease in absolute numbers of CD8+ helper cells in sepsis or following trauma, but not to the same degree as the CD4+ subtype, so that the CD4/CD8 ratio is typically elevated (Cheadle et al, 1993; Holub et al, 2000; Walsh et al, 2000). It is essential, therefore, to examine the expression of activation markers on various T-cell subtypes when assessing immune responses.

Classically, T cell activation by exposure to antigens is characterized by changes in surface marker expression, which can be quantifiably measured by flow cytometry. IL-2 is both a product of T cells and their growth factor. Binding of IL-2 to the IL-2 receptor (of which CD25 is a component) on the cell membrane induces cellular proliferation. This autocrine mechanism is of critical importance to the cell-mediated immune response and is dependant on both expression of functional IL-2 receptors on the cell surface and normal production and secretion of IL-2 itself. While the IL-2 receptor (IL-2R) was originally thought only to be expressed on activated T-cells, it is now known to be a non-lineage specific activation marker, which is also present on activated B-cells and monocytes/macrophages. It is a relatively early activation marker, appearing after 1 to 3 days, with studies showing expression on up to 80% of cells 48 hours after stimulation induction (Burmester et al, 2003; Knapp et al, 1989; Mason et al, 2002; Nelson et al, 1998; Schlossman et al, 1995). While some studies investigating IL-2R expression (CD25) on T cells following trauma or sepsis have found no significant alteration in levels of expression (Teodorczyk-Injeyan et al, 1990; Lin et al, 1993), there is evidence to dispute this, with some studies showing elevation of IL-2R expression following thermal and non-thermal injury (Schluter et al, 1991; Walsh et al, 2000).

Two other surface activation markers visible on T cells following stimulation are CD69 and CD71. CD69 is undetectable on resting peripheral blood T cells and is visibly expressed within 30 minutes of activation by mitogens, cytokines or contact with target cells. This expression peaks at 6 hours and then typically declines 48 hours after stimulation. While CD69 is an early marker of activation on B cells and NK cells, in T-cells it induces the production of IL-2 and IL-2R, which in turn result in T-cell proliferation (Craston et al, 1997; Knapp et al, 1989; Schlossmann et al, 1995; Simms et al, 1996).

The correlation between expression of CD71 and cell proliferation is well established; a rapid increase in expression is observed after stimulation of lymphocytes with mitogens. Thus, CD71 is a prototype activation marker associated with increased proliferative activity in most tissues, regardless of lineage. As with CD69, CD71 is usually upregulated on the cell membrane within a few hours of activation (Burmester et al, 2003; Knapp et al, 1989; Mason et al, 2002; Ponka et al, 1999; Schlossman et al, 1995).

HLA-DR molecules are required for antigen presentation and activation of helper T cells and so play a major role in the immune response. It has been proposed that reduction in HLA-DR expression following surgery may be associated with postoperative infection. Impaired function of phagocytic cells, including peripheral blood monocytes, after injury has been well demonstrated. Several studies have shown that HLA-DR expression on monocytes decreases after major trauma and surgery. Furthermore, the degree of depression has been shown to be predictive of clinical course, being most pronounced in those who develop poor outcomes, such as sepsis or septic shock. In addition to exerting a regulatory role over T helper cell activation by antigen-producing cells, HLA-DR also

regulates the monocyte secretion of cytokines such as IL-1 and TNF (Ditschkowski et al, 1999; Lin et al, 1993; Schaffer et al, 1998; Wakefield et al, 1993). HLA-DR expression as a marker of T cell activation has been investigated in clinical studies in recent years. These studies have shown that those with poorer postoperative outcomes have decreased expression of HLA-DR on T cells following major trauma or surgery when compared to those with uncomplicated recoveries (Ditschkowski et al, 1999; Holub et al, 2000; Wakefield et al, 1993).

L-selectin has also been postulated as a possible predictor of posttraumatic or postoperative outcome. L-selectin (CD62-L) expression is limited to haemopoetic cells, with most classes of leucocytes expressing L-selectin at some stage of development. It is shed rapidly following major injury, resulting in the generation of soluble L-selectin (sCD62-L) which then circulates in plasma. An increase in monocyte CD62-L expression has been documented following mechanical trauma (Cocks et al, 1998; Rainer et al, 2001). Whether monocyte CD62-L expression is useful in prediction of complications such as sepsis or MODS is as yet uncertain and recent work suggests that interpretation of results may be further complicated by gender differences (vanGriensven et al, 2004; Kerner et al, 1999; Tedder et al, 1994).

Little research has been performed to date on neutrophil expression of NF-kappa B in surgical populations, but initial reports are of great interest. NF-kappa B is a transcription factor which plays a central role in activation and regulation of the immune system. It is found in an inactive form in the cytoplasm and can be induced by a wide variety of stimuli such as hypoxia, endotoxin, radiation and injury. Studies examining levels of NF-kappa B in peripheral blood mononuclear cells have shown significantly

higher levels of expression in non-survivors as compared to survivors. More recent studies have demonstrated enhanced expression in primed neutrophils of SIRS patients (Arnalich et al, 2000; Baeuerle et al, 1994; Blackwell et al, 1997; Bohrer et al, 1997; Nakamori et al, 2003).

Few studies to date have examined normal postoperative responses or patterns of expression of NF-kappa B, but in one surgical study examining NF-kappa B levels in neutrophils of patients undergoing thoracoabdominal aortic aneurysm repair, pre-operative elevation of NF-kappa B was associated with postoperative morbidity and development of MODS. There was also a significant difference in pre-operative NF-kappa B levels between patients with organ failure who survived their surgery and subsequent postoperative complications and those who did not (Foulds et al, 2001).

The relationship between nutrition and immunity has been investigated previously. Surgical stress can result in imbalance between the rates of whole-body protein synthesis and breakdown, due to increased protein catabolism or decreased protein synthesis. Protein synthesis is necessary for the production of many components of the immune system, such as neutrophils, monocytes and various lymphocyte subsets as well as other rapid turnover cells, such as those in gut mucosa. Clinical studies have shown that nutritional parameters decline sharply following major surgery with some studies indicating that the preoperative nutritional state appears to be predictive of postoperative complications (Nakamura et al, 1999; Tashiro et al, 1996; Wang et al, 1998).

3.2   AIMS OF STUDY

This chapter aims to examine the early effects of major upper gastrointestinal surgery on post-operative immune function.   In particular, it examines the effect on postoperative acute phase response (as measured by C-reactive protein levels) and leucocyte responses, specifically T-cell, monocyte and neutrophil activation, and investigates how these responses correlate to subsequent clinical outcome.

3.3   PATIENTS AND METHODS

Twenty-seven patients from the first group of patients described in section 2.1 were included in this section of the study.   Expression of the T-cell surface markers CD25, CD69, CD71 & HLA-DR were assessed by flow cytometry in all patients (section 2.3 & 2.4) as were the monocyte surface markers CD62-L and HLA-DR.   Specimens were obtained preoperatively and on the first, second, third and seventh post-operative days. Serum samples were also obtained at these time periods for assessment of C-reactive protein levels and serum albumin measurements. Nuclear expression of NF-κB in neutrophils was also assessed by flow cytometry (section 2.5) in a further subgroup of 8 patients.

3.4   RESULTS

3.4.1   PATIENT DEMOGRAPHICS

Patient demographics for this group of patients as a whole are described in table 3.1. A total of 27 patients were recruited to this section of the study, 16 men and 11 women. All underwent surgical resection for upper gastrointestinal malignancy. The median age of patients was 62 years (range 41 - 78). Seventeen of the patients underwent a 2-stage oesophagagectomy, 6 underwent a 3-stage procedure and 4 underwent pancreatic resection. All patients received jejunally administered enteral nutrition from the first post-operative day.

No. of patients	27
Age (years)*	62 (51 - 71)
Sex	
M	16
F	11
Procedure	
Three-stage oesophagectomy	6
Oesophagogastrectomy	17
Pancreatic resection	4
*All values are medians (interquartile range)	

Table 3.1   Patient Demographics (Ch. 3)

3.4.2   C-REACTIVE PROTEIN

C-reactive protein levels were recorded pre-operatively and on days 1, 2, 3 and 7 post-operatively; the results are shown in graph 3.1. An extreme rise was seen in this acute phase marker in the postoperative period, evident from the first postoperative day (preoperative vs. day 1: 3.9mg/l [3.4 – 10.5] vs. 161mg/l [129 – 181], p< 0.0001, wilcoxin rank test). This marked elevation persisted on the second and third postoperative days (p<0.0001, wilcoxin rank test). At one week, the C-reactive protein level could be seen to be falling, but was still significantly higher than the initial, preoperative, level (p<0.0001, wilcoxin rank test).

*- significant elevation compared to preoperative value
P < 0.0001 wilcoxin rank test
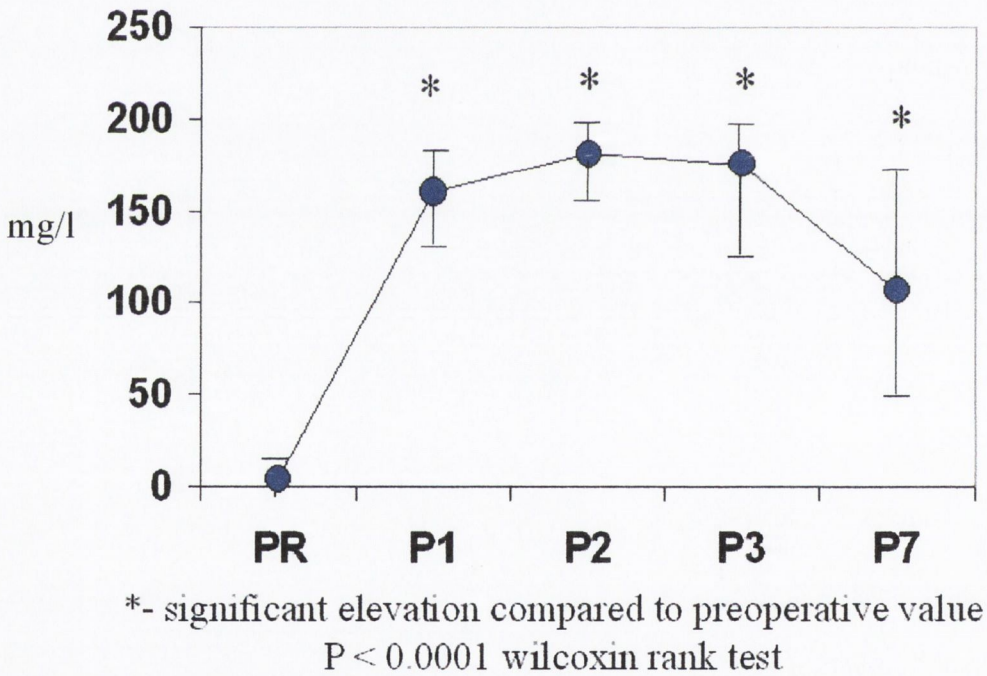
Graph 3.1   C-reactive protein

98

When the results between those who went on to develop septic complications in the postoperative period and those who did not were compared, no difference was found in the initial 48 hours following surgery. After this, however, the C-reactive protein level was visibly higher in those who developed complications, significantly so at one week postoperatively (99mg/l [54 – 138] vs. 198mg/l [152 – 229]; p< 0.05, Mann-Whitney U test).
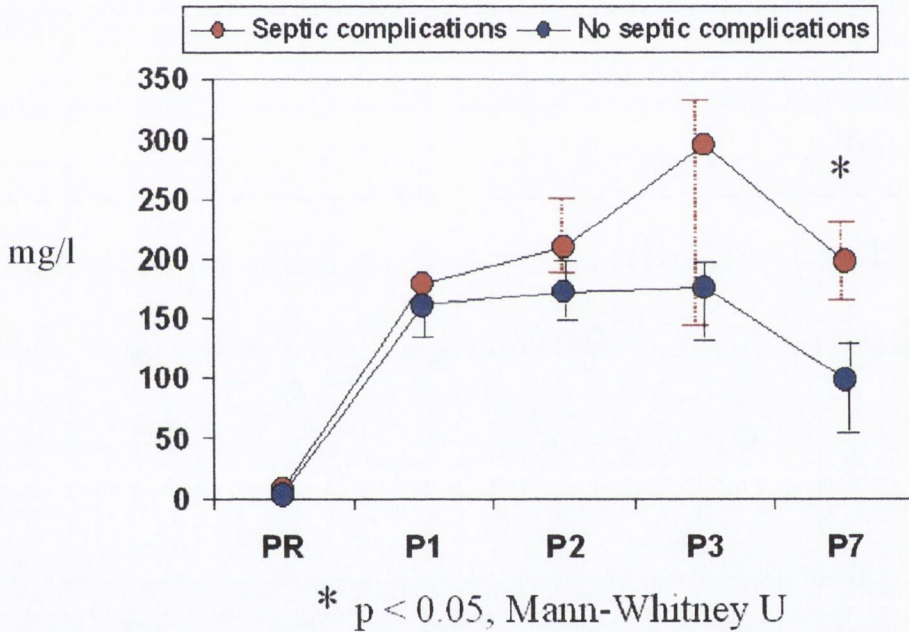
Graph 3.2   C-reactive protein and septic complications

3.4.3   LEUCOCYTE ACTIVATION

Initially, leucocyte counts both preoperatively and in the immediate postoperative period were examined. All patients had a normal white cell count preoperatively (6.4×10^9 cells/l [5.5 – 7.3]) as shown in graph 3.3. Postoperatively, a marked elevation in the white cell count was evident on the first postoperative day, which persisted for the duration of the study period.   While still within the normal range, this rise was highly significant as compared to the preoperative white cell count at all time points (p < 0.001, wilcoxin rank test).

*- significant elevation compared to preoperative value
P < 0.001 wilcoxin rank test
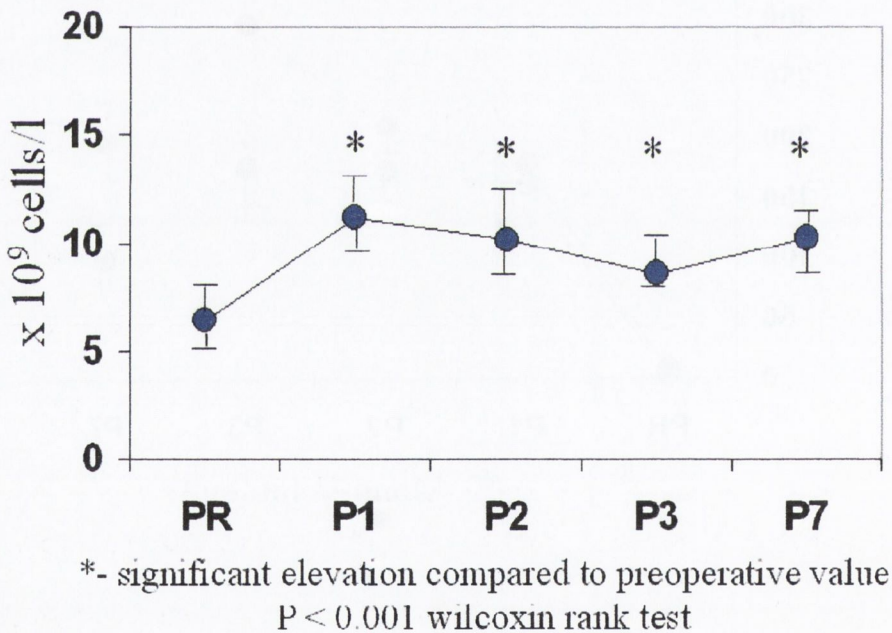
Graph 3.3   White cell count

(i)   T-cell activation and surface marker expression

Preoperatively, there was minimal expression of CD25 on CD3+, CD4 + and CD8 + cells (% cells expressing CD 25: 11[8 – 18], 20 [13 – 25] and 4 [2-8] respectively). There was an elevation in all subsets expressing this marker 48 hours postoperatively, significantly so in the CD3+ and CD4+ subtypes (graph 3.4). Levels then fell slightly on the third postoperative day, but were again significantly elevated as compared to the preoperative level in all 3 subtypes at one week (% cells expressing CD 25: 20 [13 – 25], 27 [16 – 38] and 7 [5 – 9] respectively; $p < 0.05$, wilcoxin rank test).

Next, T cell expression of CD 25 was examined and compared to postoperative outcome, looking at the development of septic complications initially. Preoperatively, there was minimal expression in both groups (Graph 3.5). There was an increase in CD25 expression in both groups after 24 hours, most prominent in those who did develop complications (n = 6), but with no significant difference evident on statistical analysis. On day 2, however, there was a significant difference between groups; those patients who had an uncomplicated postoperative outcome had significantly higher expression on this day as compared to those who developed septic complications in the CD3+ (% cells expressing CD 25: 18.1[12 – 24] vs. 8.7 [8 - 12]) and CD4+ subtypes (% cells expressing CD 25: 27 [18 – 34] vs. 13 [10 – 14]). This difference between the 2 groups persisted until day 7, those with septic complications expressing low levels of CD25, but did not reach significance at any other time period.
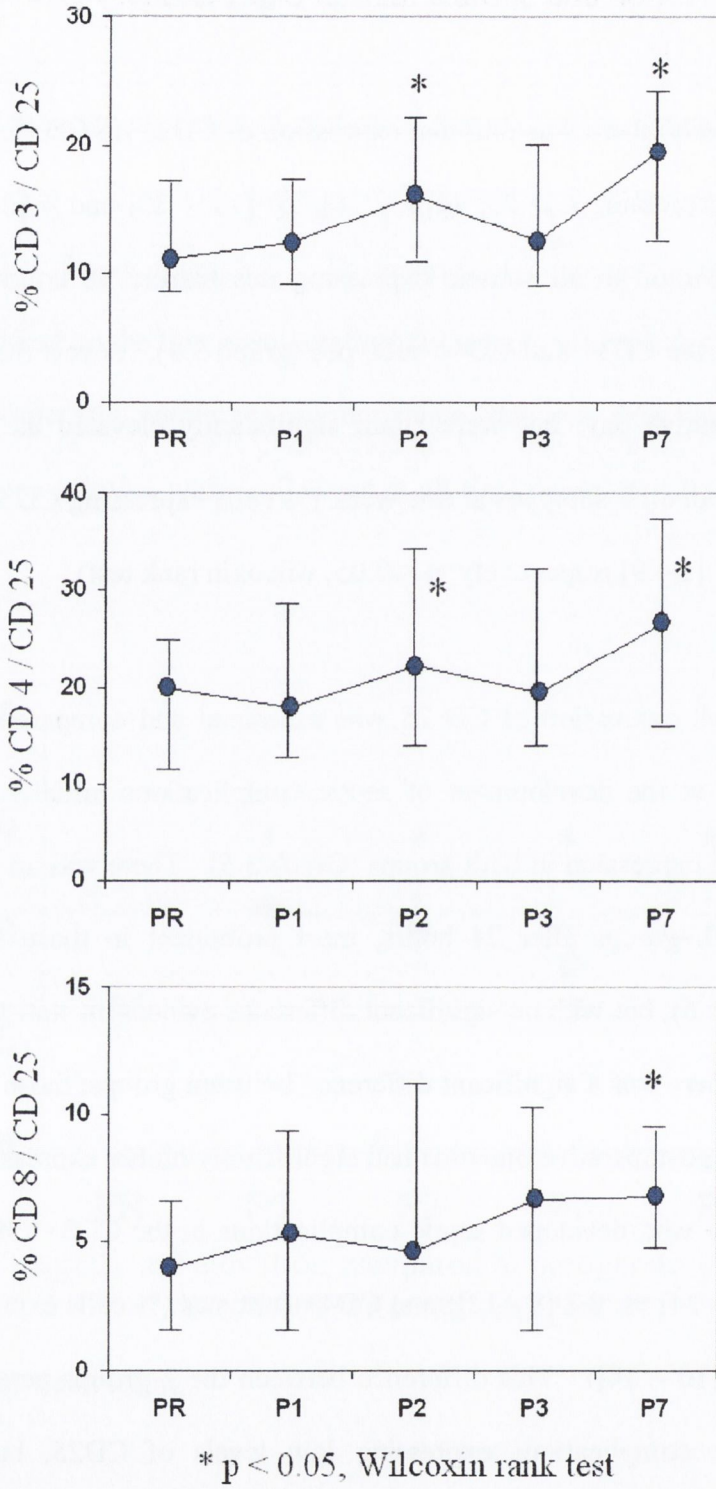
Graph 3.4   T-cell expression of CD25
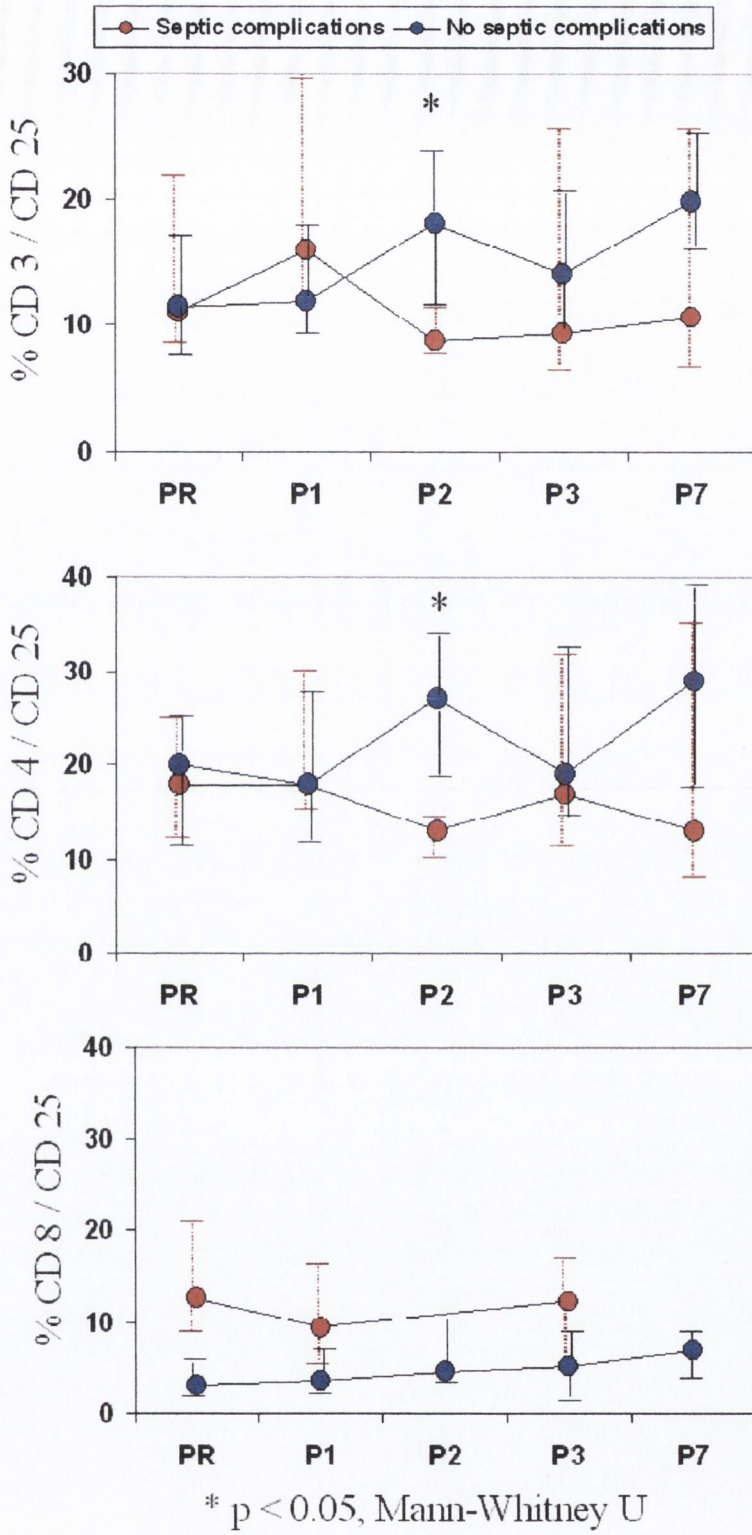
Graph 3.5   T-cell expression of CD25 and septic complications

Preoperatively, there was minimal expression of CD69 on CD3+, CD4 + and CD8 + cells (% cells expressing CD 69: 1.3 [0.8 – 2.3], 0.8 [0.4 – 1.3] and 5.1 [3 – 8] respectively). There was an elevation in all subsets expressing this marker 48 hours postoperatively, significantly so in the CD3+ and CD4+ subtypes (graph 3.6). Levels then fell slightly on the third postoperative day, but were again significantly elevated as compared to the preoperative level in all 3 subtypes at one week (% cells expressing CD 69: 2.4 [1 – 5], 1.3 [0.6 – 2.1] and 10.7 [7 – 13] respectively).

T cell expression of CD 69 was examined and compared to subsequent postoperative outcome, looking at the development of septic complications initially. Preoperatively, there was minimal expression in both groups (Graph 3.7). There was an increase in CD69 expression in both groups within 24 hours. On day 7, those who developed septic complications had higher levels of expression of CD69 than those who did not; this was only significant within the CD4+ subtype (% cells expressing CD 69: 1 [0.5 – 1.5] vs. 2.5 [1.7 – 4.8]).
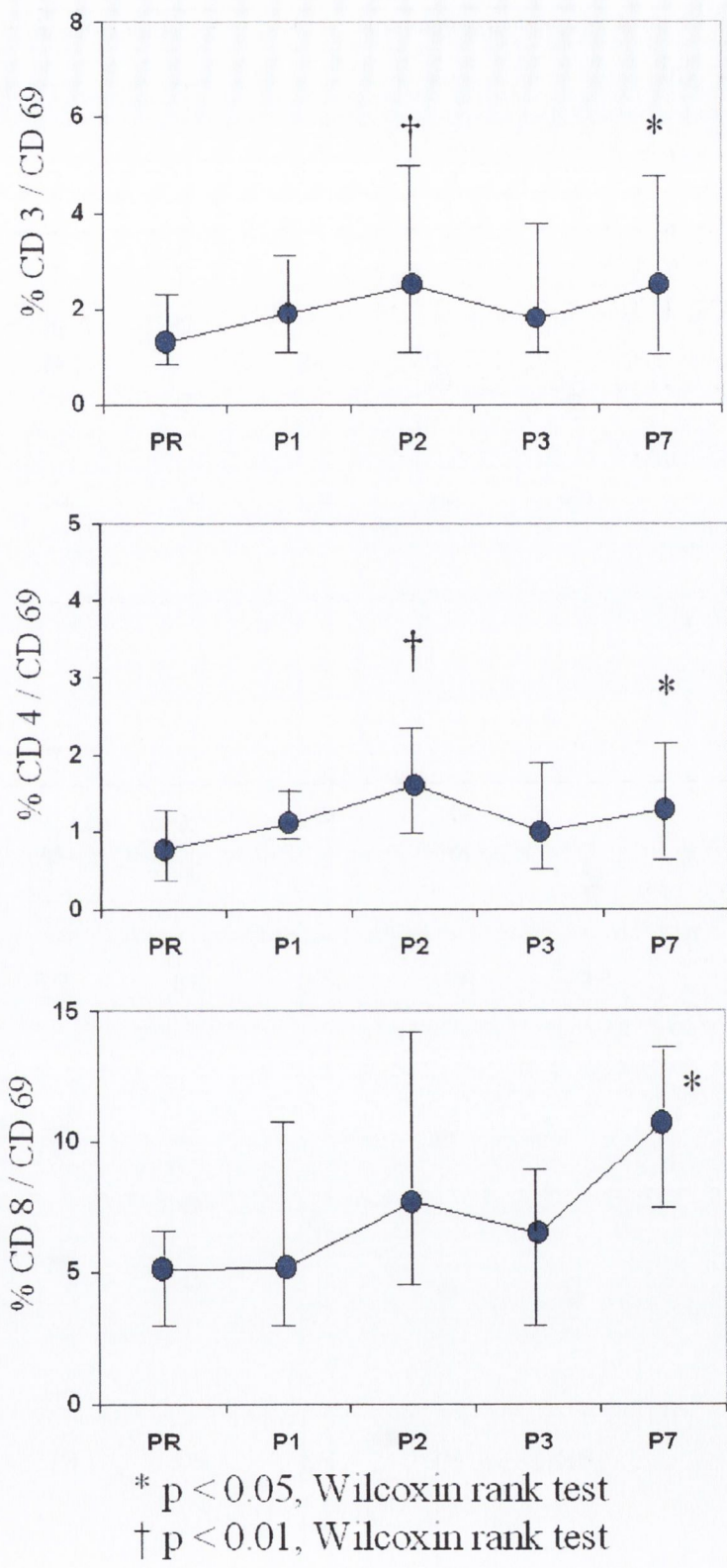
Graph 3.6   T-cell expression of CD69

105
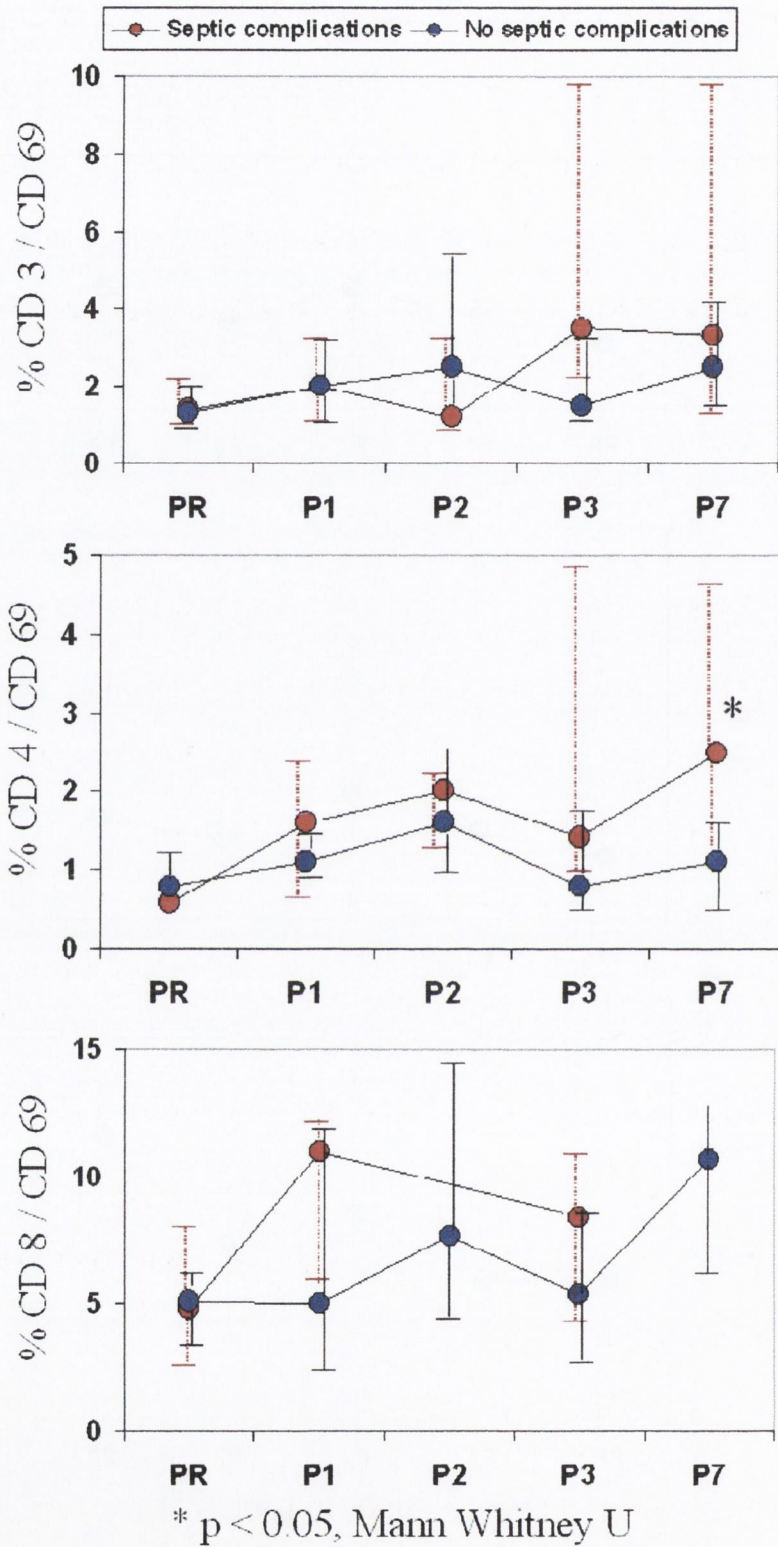
Graph 3.7   T-cell expression of CD69 and septic complications

Preoperatively, there was minimal expression of CD71 on CD3+, CD4 + and CD8 + cells (% cells expressing CD 25: 2.3 [1.3 – 4.8], 2.8 [2.1 – 5.2] respectively, graph 3.8). Expression was elevated at 24 and 48 hours postoperatively, but this was only significant within the CD4 subset (4.6 [3.3 – 7.3] and .3.8 [2.7 – 7.2]). The level of expression then rose again at one week in the CD3+ and CD4+ subsets, but this rise was only statistically significant within the CD4+ subset (% cells expressing CD 71: 3.2 [1.9 – 4.4] and respectively).

T cell expression of CD 71 was also examined and compared to postoperative outcome, looking at the development of septic complications. Preoperatively, there was minimal expression in both groups (Graph 3.9). There was an increase in CD71 expression in both groups within 24 hours. Within the CD3+ subtype, levels of expression were identical until day 3, with minor differences occurring thereafter (not significant). Within the CD4+ subtype, patients who developed septic complications had reduced levels of CD71 expression, which was significant at 48 hours postoperatively, as compared to patients who had an uncomplicated postoperative outcome (% cells expressing CD 71: 1.8 [1.7 – 2.1] vs. 4.8 [3.4 – 7.7]; p<0.05, Mann-Whitney U test). This difference between the 2 groups persisted at day 7, those with septic complications expressing low levels of CD71, but did not reach significance.
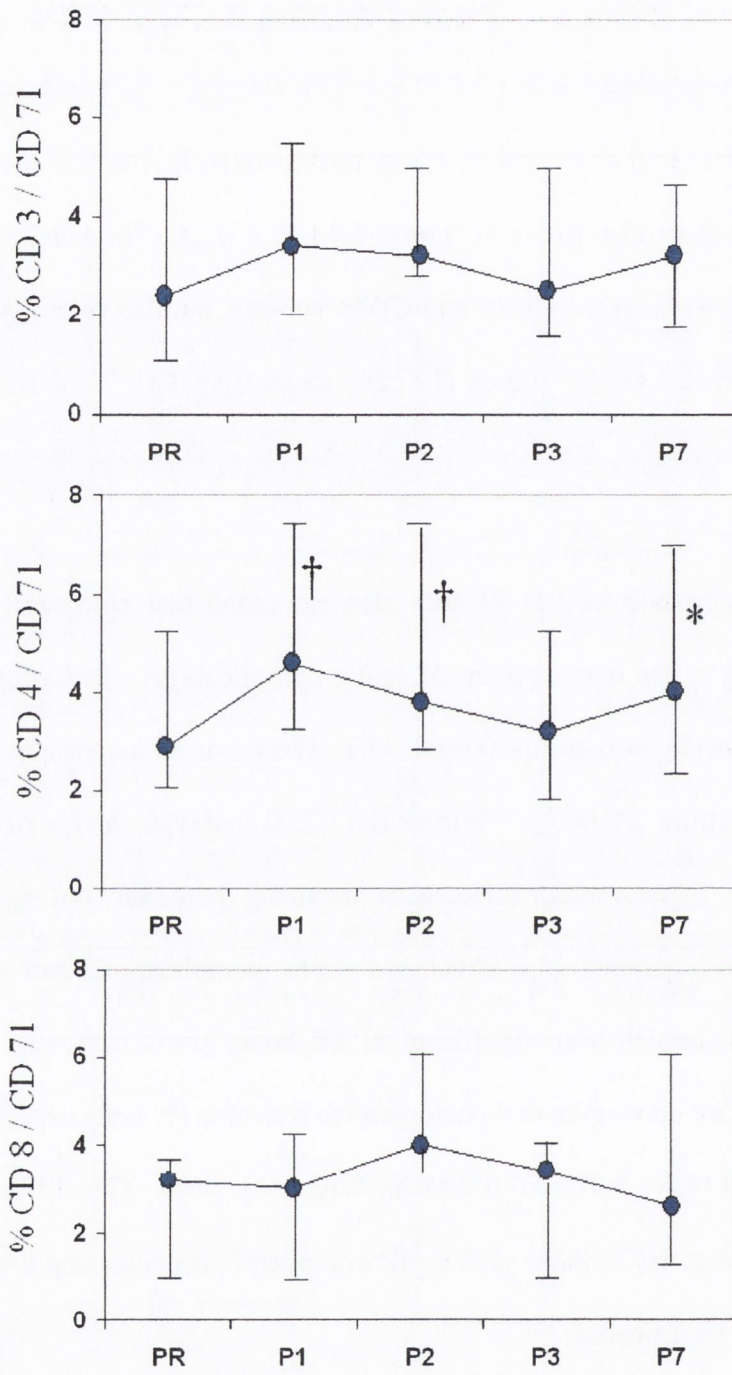
* p < 0.05, † p < 0.01, Wilcoxin rank test

Graph 3.8   T-cell expression of CD71
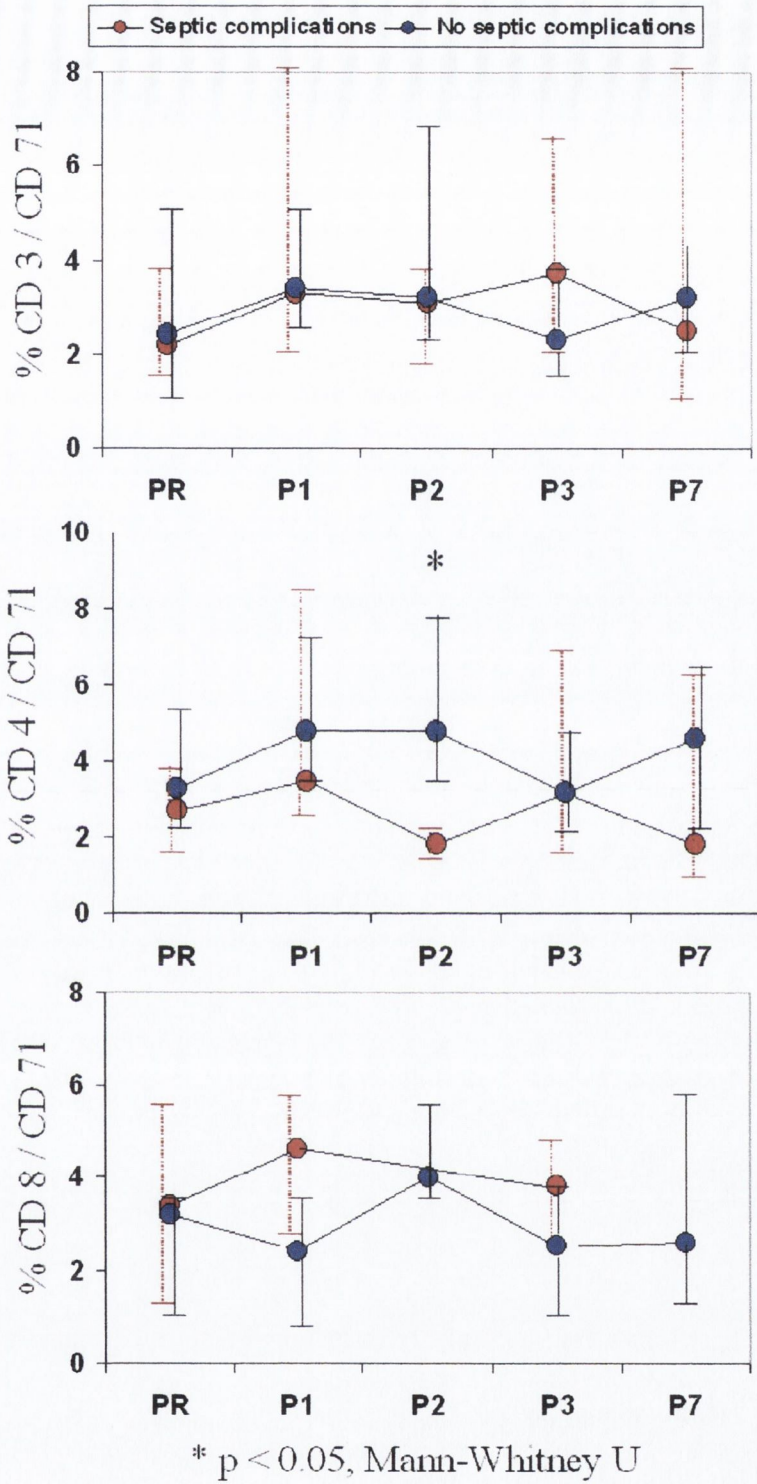
Graph 3.9   T-cell expression of CD71 and septic complications

Preoperatively, there was minimal expression of HLA-DR on CD3+, CD4 + and CD8 + cells (% cells expressing HLA-DR: 4.9 [3.3 – 7.3], 5.7 [3.1 – 9.4] respectively, graph 3.10). Within the CD3+ subtype, there was then an immediate rise postoperatively, significant on day 1 (8 [4.7 – 12.2]). The CD4+ subtype had the same pattern, with a significant rise (as compared to the preoperative value) being present on the first and second postoperative days (6.9 [5.3 – 12.1] and 7.1 [3.9 – 12.3]). The CD8+ subtype, however, showed a very different reaction. Here, there was an initial fall in levels of HLA-DR expression, which then came back to normal by one week (no levels significant on statistical analysis).

While initial examination of HLA-DR expression on the CD3+ T cells reveals no difference in expression between those who went on to develop septic complications and those who did not, there are significant differences within the CD4+ subtype visible. Those with an uncomplicated postoperative recovery still have mildly elevated levels of HLA-DR expression. In contrast, those with septic complications have a marked depression in expression, maximal at 48 hours, when expression is significantly different between the groupd on statistical analysis (% cells expressing HLA-DR: 1.7 [0.9 – 3.4] vs. 8.3 [5.6 – 15.7]; $p<0.05$, Mann-Whitney U test). Levels in those with septic complications then rise again, the level of expression in both groups being indistinguishable on the third and seventh postoperative days (graph 3.11).
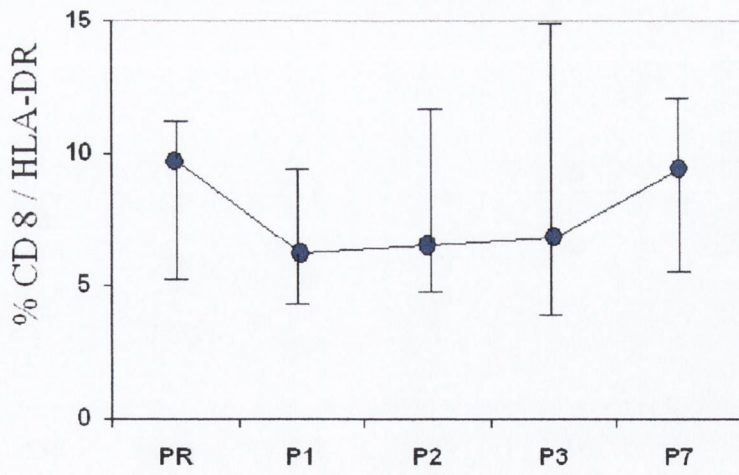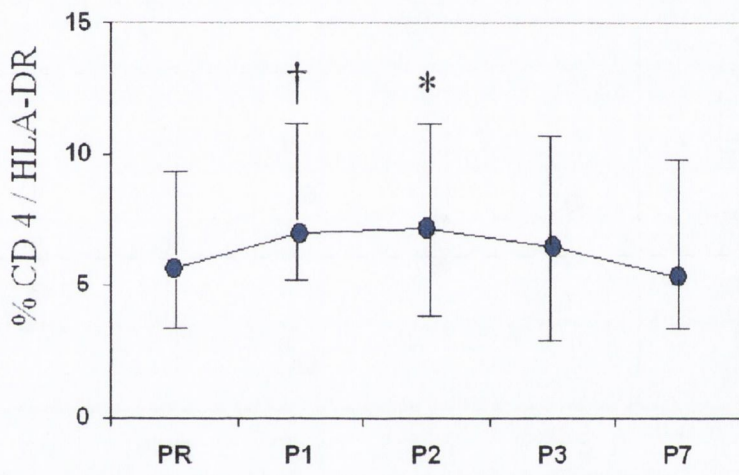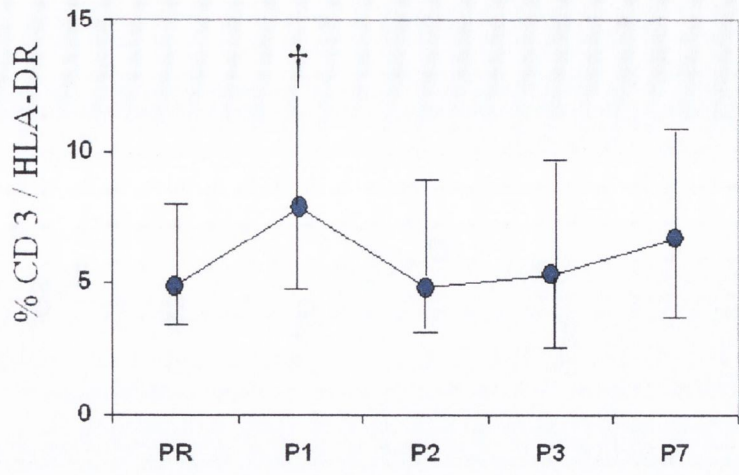
* p < 0.05, † p < 0.01, Wilcoxin rank test

Graph 3.10   T-cell expression of HLA-DR
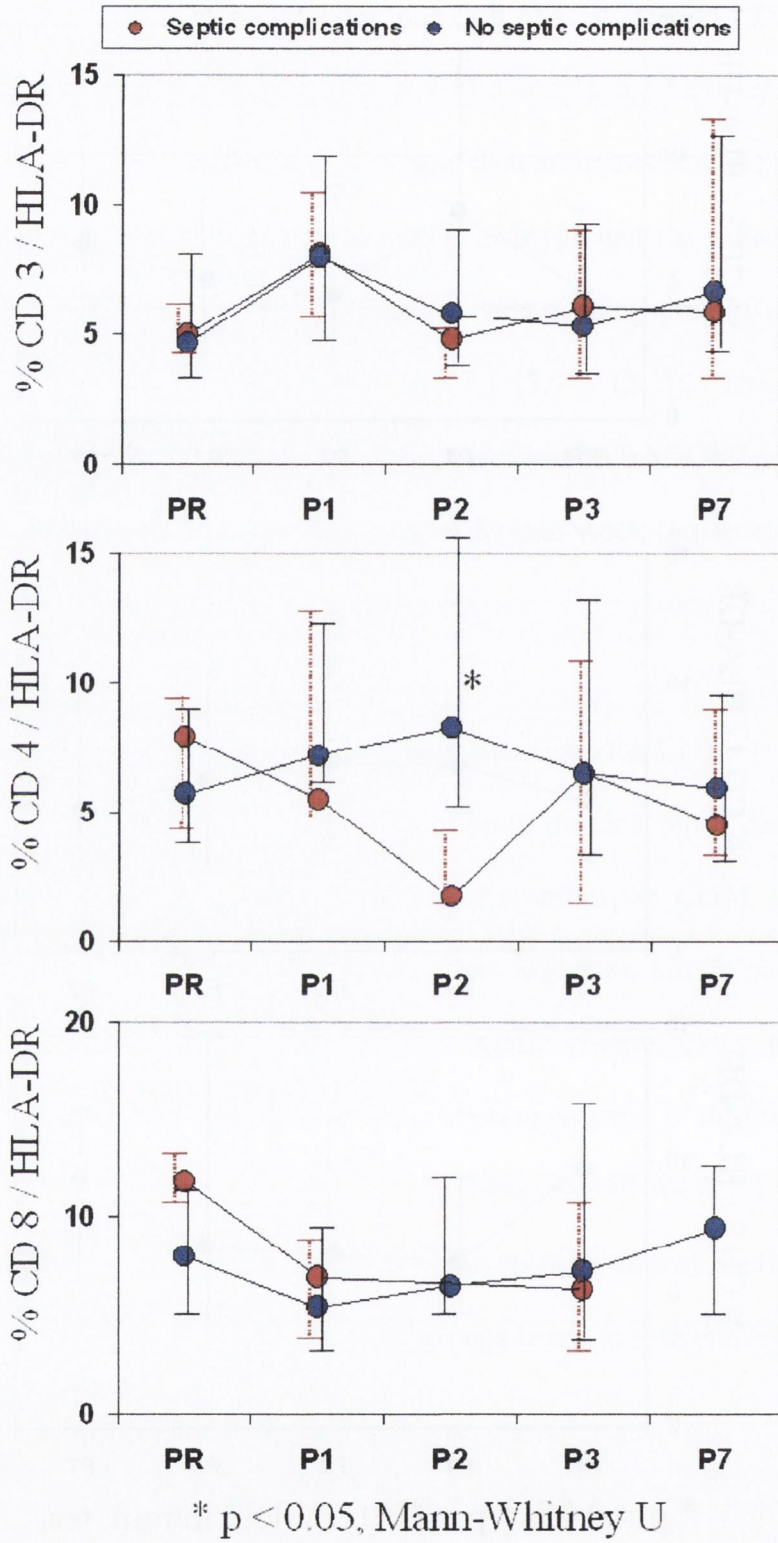
Graph 3.11   T-cell expression of HLA-DR and septic complications

(ii)   Monocyte activation and surface marker expression

The expression of HLA-DR on monocytes preoperatively and on postoperative days 1, 2, 3 and 7 is shown in graph 3.12. There is a rapid drop in monocyte HLA-DR, most evident on the first postoperative day, which was significant when compared to the preoperative level on analysis (% cells expressing HLA-DR preoperatively and on day 1: 55 [41 - 74] vs. 17 [14 - 23] respectively, $p < 0.0001$, wilcoxin rank test). This depression persists on the second and third post-operative days ($p < 0.0001$ on both days, wilcoxin rank test). The percentage of monocytes expressing HLA-DR then begins to rise again by day 7, but is still lower than the initial, preoperative, level (% cells expressing HLA-DR preoperatively and at one week: 55 [41 - 74] vs. 30 [16 - 42] respectively, $p < 0.001$, wilcoxin rank test).
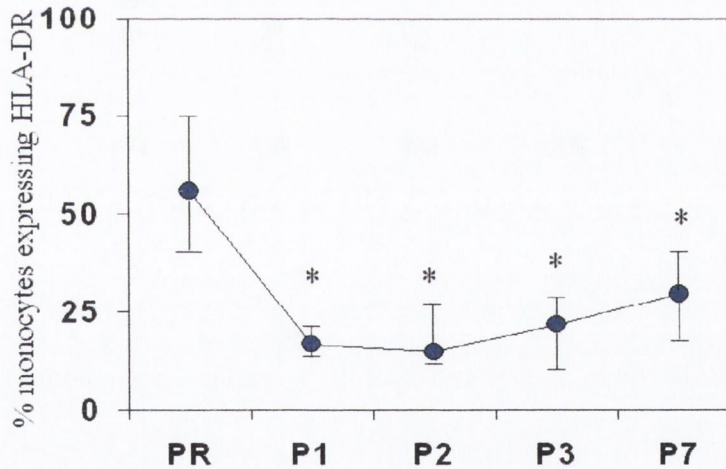
*- significant depression compared to preoperative levels
Wilcoxin rank test (see text for details)

Graph 3.12   Monocyte HLA-DR expression

All patients had an initial depression in monocyte HLA-DR expression, regardless of postoperative outcome. Those who developed septic complications had a more pronounced depression, the difference between groups only being significant at one week postoperatively (% cells expressing HLA-DR: 13.9 [10.8 – 16] vs. 36.1 [17.8 – 49.7]; p<0.01, Mann-Whitney U test).
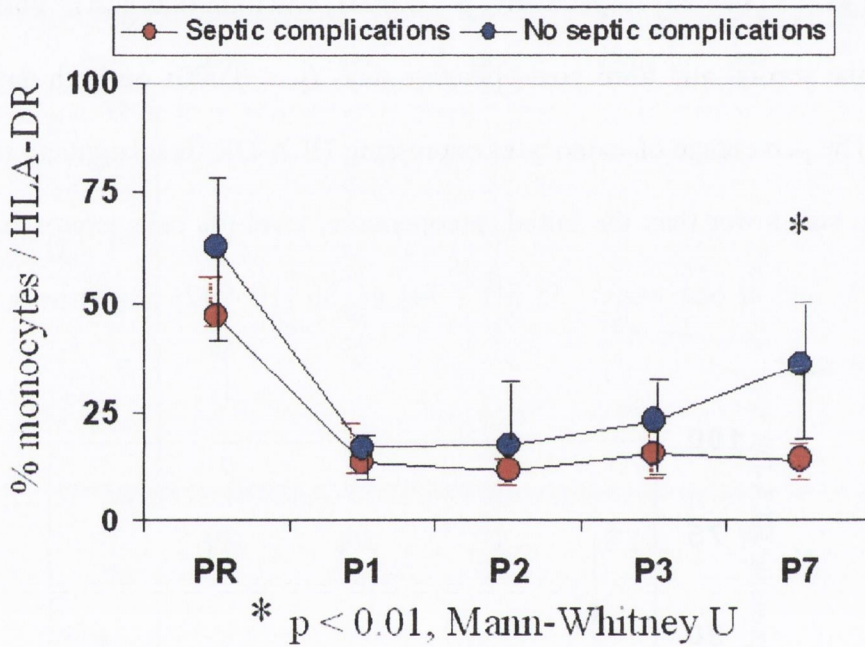
Graph 3.13   Monocyte HLA-DR expression and septic complications

Next, expression of the surface adhesion molecule CD-62-L (L-selectin) within the monocyte population was ascertained (graph 3.14). A significant elevation in levels of expression were seen on the first postoperative day, which was significant when compared to the preoperative level on analysis (% cells expressing CD-62-L preoperatively and on day 1: 62 [45 – 71] vs. 81 [74 – 87] respectively, p < 0.001, wilcoxin rank test). This elevation in expression continued throughout the study period until one week, and was significant at all time periods. With regard to development of septic complications, these patients had slightly reduced CD62-L expression compared to those with uncomplicated recoveries, but this was not significant (graph 3.15).
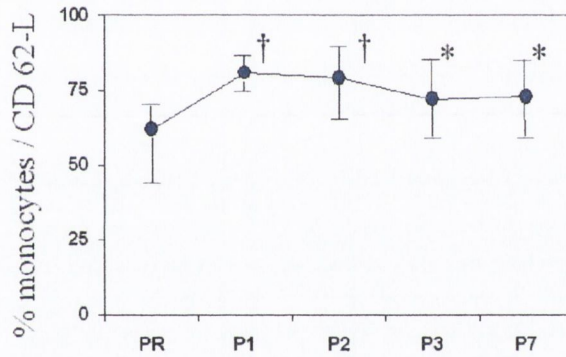
† p < 0.001, * p < 0.05 Wilcoxin rank test

Graph 3.14   Monocyte CD62-L expression
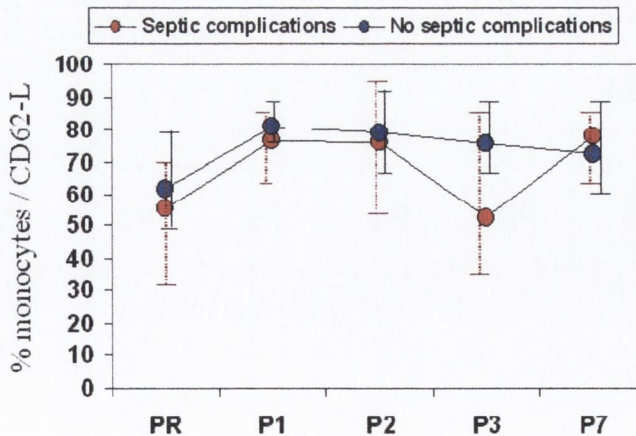
Graph 3.15   Monocyte CD62-L expression and septic complications

(iii) Neutrophil intranuclear NF-kappa B levels

The expression of neutrophil intranuclear NF-kappa B levels preoperatively and on postoperative days 1, 2, 3 and 7 is shown in graph 3.16. As described in section 2.5, all postoperative values are expressed as a percentage of the patient's initial preoperative, level of expression. A marked elevation in expression is evident in the immediate post-operative period, the level of NF-kappa B expression being double the preoperative value on day 1 (200% of preoperative value [122 – 236], p<0.04, wilcoxin rank test). This elevation was short-lived, however, with levels falling back to normal range on the second day and throughout the remainder of the study period. The relationship between NF-kappa B expression and clinical outcome measures such as sepsis was not analysed, as the patient numbers within this group were too small (n=8).

*- significant elevation compared to preoperative value
P < 0.05 wilcoxin rank test
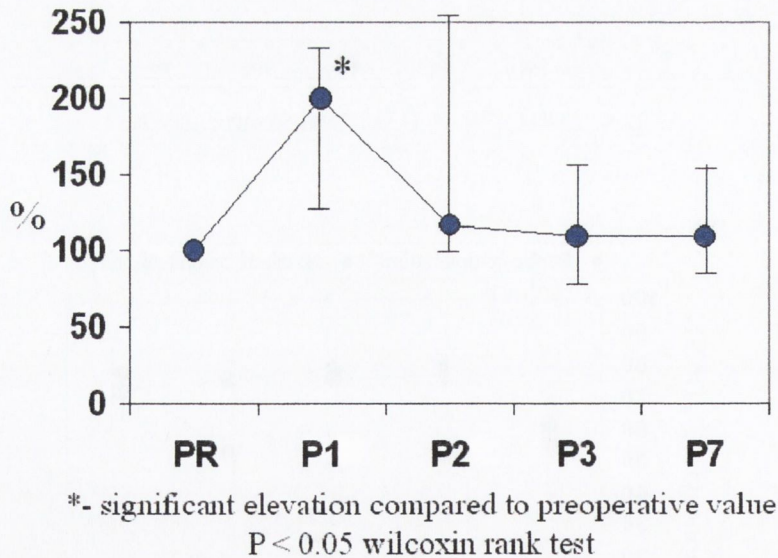
Graph 3.16   Neutrophil intranuclear NF-kappa B expression

3.5   DISCUSSION

Major trauma, whether controlled (i.e. major surgery) or uncontrolled (i.e. thermal or mechanical) results in global immune dysregulation, which may give rise to a systemic inflammatory response, sepsis and death. Several components of the immune system have been implicated in this immunocompetency, of both the innate and adaptive immune systems.

The role of T cells in the development of postoperative sepsis has still to be fully explained. In the past, it was thought that the predominant effect of trauma on immune function was suppressive; numerous studies on T cells in postoperative patients have documented post-operative anergy, delayed hypersensitivity response and an increase in suppressor cells. However, the majority of more recent studies have demonstrated activation of the adaptive immune system following surgery, with no evidence of overall functional paralysis.

These findings show that major surgery results in overall activation of CD3+, CD4+ and CD8+ T-cells in the early postoperative period, as would be expected. An elevation in postoperative expression of CD25 and CD69 was observed at 24 hours postoperatively, with maximal expression typically at 48 hours. These data agree with findings in other surgical populations but differ from those described after thermal trauma or blunt injury, where expression of activation markers has been delayed for up to three days. A postoperative elevation in CD71 expression was also observed, as would be expected with postoperative immune activation and cell proliferation.

With regard to T-cell HLA-DR expression, the majority of surgical studies to date have examined expression in the T cell population as a whole, with little data available on specific subtypes. The findings of this study show overall elevation in HLA-DR expression on CD3+ cells postoperatively, confirming previous reports. In addition, while it was found that the CD4+ subtype reflects this pattern of postoperative upregulation, the results of this study demonstrated that the CD8+ subtype has a very different response, showing a profound depression in levels of expression in the immediate postoperative period.

In addition to confirming the results of these previous studies, which did not discriminate between those patients who had either uncomplicated or septic outcomes, this study examines whether activation of T cell subtypes reflect clinical outcomes in the postoperative period. The findings suggest that those patients who have been shown to develop septic complications may demonstrate altered T cell function, which may be visible in advance of clinical sepsis.

IL-2 is both a product of T cells and their growth factor and is of critical importance to the cell-mediated immune response. A significant increase in postoperative expression of CD25 has previously been observed on T cells, in association with increased IL-2 production following superantigen stimulation in vitro, suggesting that these T cells are capable of normal mitogen-induced proliferation. However, this study shows an absence of IL-receptor upregulation in the CD3+ and CD4+ subtypes in the postoperative period in those patients who proceed to develop septic complications, perhaps reflecting an impairment of T cell-mediated immune reactions, thus predisposing to subsequent infection.

T cell HLA-DR expression has also been proposed as a predictor of postoperative sepsis. Previous studies have shown that patients who had uncomplicated postoperative recoveries have higher levels of T cell HLA-DR expression than those who develop infective complications. This research did not show any evidence of this in the overall CD3+ T cell population, with both groups of patients showing similar levels of HLA-DR expression. In the CD4+ subtype, however, a dramatic fall in HLA-DR expression was observed in patients who went on to develop septic complications, with a significant difference between groups being evident at 48 hours postoperatively.

Previous studies have shown that downregulation of monocyte HLA-DR expression is observed following a stress to the system such as surgery or trauma (Ditschkowski et al, 1999; Klava et al, 1997). Prolongation of this downregulation is seen as an early indicator of derangement of the immune response and is predictive of an increased risk of sepsis and morbidity (Cheadle et al, 1991; Ditschkowski et al, 1999; Wakefield et al, 1993). This research confirms these findings, showing that patients with reduced HLA-DR had an increased risk of post-operative septic complications.

An elevation in postoperative monocyte CD-62-L levels was also observed; this confirms previous studies which have shown expression to be elevated following trauma or surgery, possibly a reflection of post-injury leucocyte trafficking. While those who went on to develop postoperative septic complications had slightly lower levels of expression, there was no significance between the two groups. In addition, recent research has suggested that a gender related diamorphism exists in initial CD62-L expression following trauma. In one study, male patients were demonstrated to show significantly higher levels of polymorphonuclear CD-62L expression on admission as compared to female patients,

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with similarly dichotomous results concerning the development of posttraumatic MODS. No data is available as yet on possible gender difference in monocyte populations and our study failed to show any significant differences based on gender.

In summary, surgery drives immune activation. Systemic effects include an acute phase response, polyclonal T cell activation and activation of monocytes and neutrophils. In addition, this research supports the hypothesis that T cell activation markers and monocyte HLA-DR expression may be predictive of clinical outcome, perhaps reflecting an impairment of T cell-mediated immune reactions, thus predisposing to subsequent infection.

CHAPTER 4: MAJOR SURGERY CAUSES A SIGNIFICANT REDUCTION IN INTRAOPERATIVE SPLANCHNIC BLOOD FLOW, WITH A SUBSEQUENT EFFECT ON POST-OPERATIVE IMMUNE PARAMETERS AND CLINICAL OUTCOME MEASURES

4.1   INTRODUCTION

The systemic inflammatory response syndrome (SIRS), sepsis and multiple organ dysfunction syndrome (MODS) are recognized sequelae of complex major surgery and underlie significant morbidity and mortality.   The systemic immunoinflammation underlying SIRS and MODS in the presence or absence of infection is not fully understood, but a breakdown of gut barrier function has been incriminated (Deitch, 1992; Reynolds, 1996).   The intestine and liver, the splanchnic unit, behaves as a unit functionally, anatomically and metabolically, and houses a vast reservoir of immune cells, in particular Kupffer cells and the gut-associated lymphoid tissue (GALT).   There is emerging consensus that activated cells and proinflammatory cytokines within this splanchnic unit may influence regional and systemic immunoinflammatory responses under a variety of challenges to gastrointestinal defence, including the trauma of complex surgery (Moore et al, 1994).

During haemorrhage or shock, a decrease in splanchnic blood flow occurs, resulting in mucosal hypoxia and acidosis. The extent of the necrosis is dependant on the degree and duration of the ischaemia, but can develop within minutes of the onset of hypoxia (Fiddian-Green, 1988; Swank et al, 1996; Vatner, 1974). This damage to the mucosa disrupts the physiological gut barrier, intestinal permeability is increased and translocation of bacteria and endotoxins from the gut can occur, resulting in activation of the systemic immune system (Deitch, 1992; Saadia, 1995; Swank et al, 1996). This hypothesis of bacterial translocation can provide an explanation as to why no septic focus can be found in 30% of bacteraemic MODS patients or how patients may develop a SIRS in the absence of infection or another recognizable cause (Deitch, 1992).

Splanchnic blood flow can be determined non-invasively by means of a method called tonometry, which quantitatively measures the gastrointestinal mucosal pH (pHi) (Clark et al, 1992; Groeneveld et al, 1994). Experimental studies have shown that pHi decreases in parallel with decreasing blood flow, tissue PO_2 and oxygen consumption (Bass et al, 1985). In human studies, gastrointestinal pHi has been shown to correlate with morbidity and mortality of critically ill patients, and decreased morbidity has been observed in some reports from pHi-directed therapy (Gutierrez et al, 1992; Hameed et al, 2003; Marik, 1993).

The relationship of intraoperative pHi to gut barrier function and regional and systemic immune function has not been studied. Intestinal permeability to macromolecules is thought to reflect the functional integrity of the gut mucosa, and increased intestinal permeability has been reported following surgery, and is heightened in patients with malignant disease (Kanwar et al, 2000; Reynolds et al, 1997; Roumen et al, 1993; Welsh et al 1998). The cellular immune response following trauma and major surgery has been the subject of numerous studies, and impaired expression of the human leucocyte antigen-DR (HLA-DR) on monocytes has been consistently reported within 24 hours of surgery or trauma, and prolongation of this depression is associated with increased post-operative morbidity (Cheadle et al, 1991; Ditschkowski et al, 1999). The aim of this study was to examine whether intraoperative pHi, a surrogate marker of intraoperative splanchnic blood flow, was significantly associated with intestinal permeability and clinical and laboratory evidence of regional and systemic immunoinflammatory responses.

4.2   AIMS OF STUDY

This chapter examines the effect of surgery on splanchnic blood flow, as measured by gastric tonometry. It also considers whether alterations in other operative parameters, such as operative time, blood loss, or mean arterial blood pressure have any effect on the observed pHi. The consequences of reduced intraoperative blood flow on various components of the immune system are then considered. In particular, the effect on postoperative acute phase response (as measured by C-reactive protein levels) and leucocyte responses, specifically T-cell, monocyte and neutrophil activation. The effect of this reduced blood flow on the functional integrity of the gut barrier, as measured by intestinal permeability, is also tested.

4.3   PATIENTS AND METHODS

Twenty of the patients as described in section 2.1 underwent intraoperative tonometric measurement of splanchnic blood flow. Intraoperative pHi measurements were taken at 10 minute intervals throughout their surgical procedures, as detailed in section 2.6. In addition, data was obtained at these time periods regarding other haemodynamic variables, such as mean arterial blood pressure, central venous pressure and heart rate. The operative time, surgical blood loss and transfusions were also recorded.

All patients underwent analysis of T-cell (CD25, CD69, CD71 & HLA-DR) and monocyte (CD-62-L & HLA-DR) surface markers as described in sections 2.3 & 2.4 preoperatively and on the first, second, third and seventh days post-operatively. Serum samples were also obtained at these time periods for assessment of C-reactive protein levels and serum albumin measurements. Additionally, in a subsection of 11 of these, measurements of intestinal permeability (section 2.7) were taken preoperatively and on the first and seventh postoperative days.

4.4   RESULTS

4.4.1   PATIENT DEMOGRAPHICS

Patient demographics for this group of patients as a whole are described in table
4.1. A total of 20 patients were recruited to the study, 13 men and 7 women. All
underwent surgical resection for cancer of the esophagus or oesophagogastric junction. The
median age of patients was 66.5 years (range 41 - 84). Fifteen of the patients underwent a
2-stage oesophagagectomy, and 5 underwent a 3-stage procedure. All patients received
jejunally administered enteral nutrition from the first post-operative day. In no patient was
there difficulty maintaining MAP intraoperatively.

No. of patients	20
Age (years)*	66.5 (41 – 84)
Sex	
M	13
F	7
Procedure	
Three-stage oesophagectomy	5
Oesophagogastrectomy	15
*All values are medians (interquartile range)	

Table 4.1   Patient demographics (Ch. 4)

4.4.2 INTRAOPERATIVE SPLANCHNIC BLOOD FLOW

The median nadir pHi was 7.14, with a range from 6.9 to 7.35. Five patients developed severe splanchnic hypoperfusion intraoperatively, their pHi dropping below 7.1 intraoperatively. Seven patients had moderate hypoperfusion, the nadir pHi remaining between 7.1 and 7.2, while the remaining 8 patients maintained their pHi above 7.2 throughout their procedure. There were no significant differences between the groups as regards median age, operative time or intraoperative blood loss (table 4.2).

	pHi < 7.1	pHi 7.1 – 7.2	pHi > 7.2
No. of patients	5	7	8
Age (years)	70	69	59
Operative time (hours)	4.5	4.5	5.75
Median Intraoperative Blood Loss (mls)	800	1200	1125
Median Blood Transfusion (units)	2	0.5	1

Table 4.2   Patient demographics (Ch. 4), grouped by nadir pHi

The median preoperative mean arterial pressure (MAP) reading in patients in whom the pHi fell below 7.1 was 95mmHg, compared with 83mmHg in patients whose pHi remained above 7.1 (p = 0.12). Analysis of the minimum MAP, also as measured by mmHg, similarly showed no significant difference between these two groups (graph 4.1).
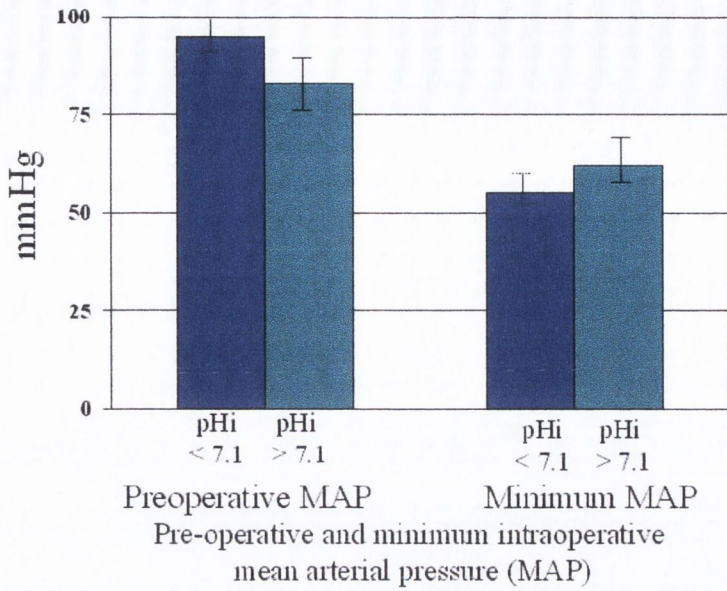
Graph 4.1   Nadir pHi vs. Mean arterial blood pressure (1)

When the median minimum MAP as a percentage of pre-incision MAP was analysed, however significant differences were seen between the two groups; those whose pHi fell below 7.1 had a percentage value of 63%, compared with 75% in those whose pHi remained above 7.1 ($p < 0.05$, graph 4.2).
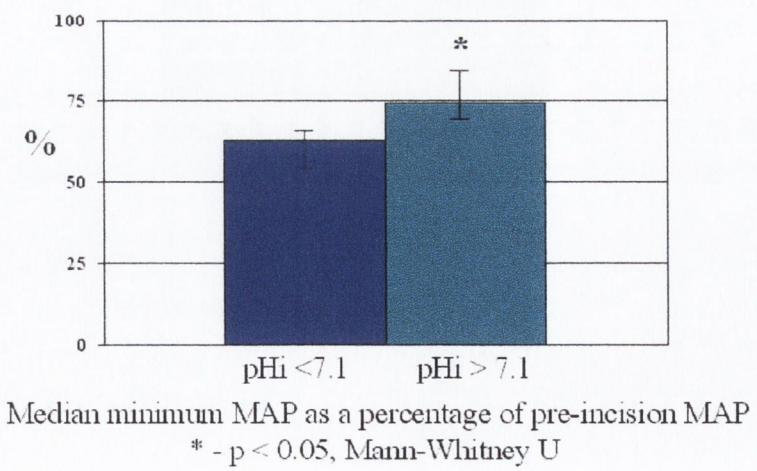
Graph 4.2   Nadir pHi vs. Mean arterial blood pressure (2)

With regard to central venous pressure (CVP), there was no difference between the 2 groups with regard to preoperative or minimum intraoperative CVP. When the maximal intraoperative CVP was examined, there was a significant difference between the 2 groups; those whose pHi fell below 7.1 had a median maximum CVP of 17 mmHg, while those whose pHi remained above 7.1 had a median value of 14.5 mmHg (graph 4.3). Of note, there was no difference in the maximal CVP between those patients who underwent thoracotomy as part of their surgical procedure and those who did not – the median maximal CVP in both groups being 15 mmHg.
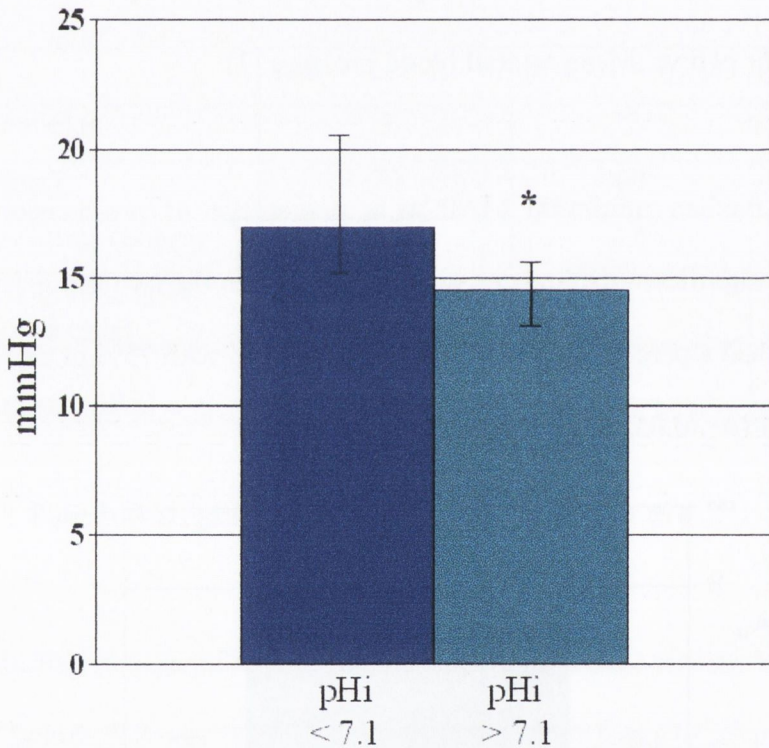
Graph 4.3   Nadir pHi vs. CVP

4.4.3 CLINICAL OUTCOME MEASURES

Thirteen patients (65%) manifested SIRS, 5 (25%) had sepsis, and 4 (20%) developed organ dysfunction. No patients developed gastric necrosis or an anastomotic leak. Patients were initially subdivided into three groups for comparison of clinical outcome measures, according to their nadir pHi reading intraoperatively (section 4.4.2, table 4.2).

No. of patients	20
Septic complications	5
SIRS	13
MODS	4
Hospital stay (days)*	17 (11 – 106)
ICU/HDU stay (days)*	4 (1 – 53)
*All values are medians (interquartile range)	

Table 4.3   Clinical outcome measures

Five patients developed septic complications post-operatively. These patients were found to have a median nadir of 7.04 compared with 7.2 in patients who did not develop septic complications ($p < 0.05$) (Graph 4.4). Those patients who developed SIRS or MODS also had lower nadir pHi than those who did not, but the differences here were not significant.
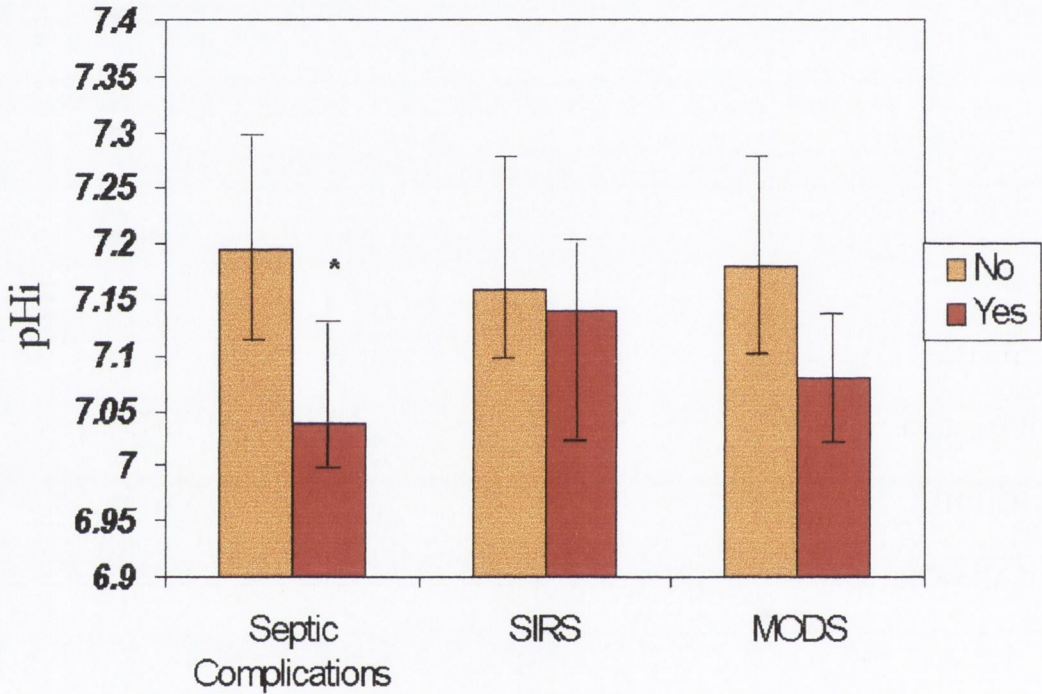
Comparison of median nadir pHi values in patients according to
development of septic complications, SIRS or MODS post-operatively
(* - $p < 0.05$, Mann-Whitney U)

Graph 4.4   Nadir pHi vs. Septic complications, SIRS & MODS (1)

The complication rate for each group (expressed as percentages) is shown in graph 4.5. Of those patients with a pHi of less than 7.1, 60% developed septic complications, compared to 29% of those in whom pHi fell to between 7.1 and 7.2 (p<0.05); however, no patient in whom pHi remained above 7.2 developed a septic complication (p<0.001). MODS was observed in 2 of 5 patients with pHi < 7.1, 2 of 7 patients with pHi 7.1 – 7.2, and in no patient with pHi > 7.2 (p=0.06). Clinical SIRS was common in all groups (p = 0.1).
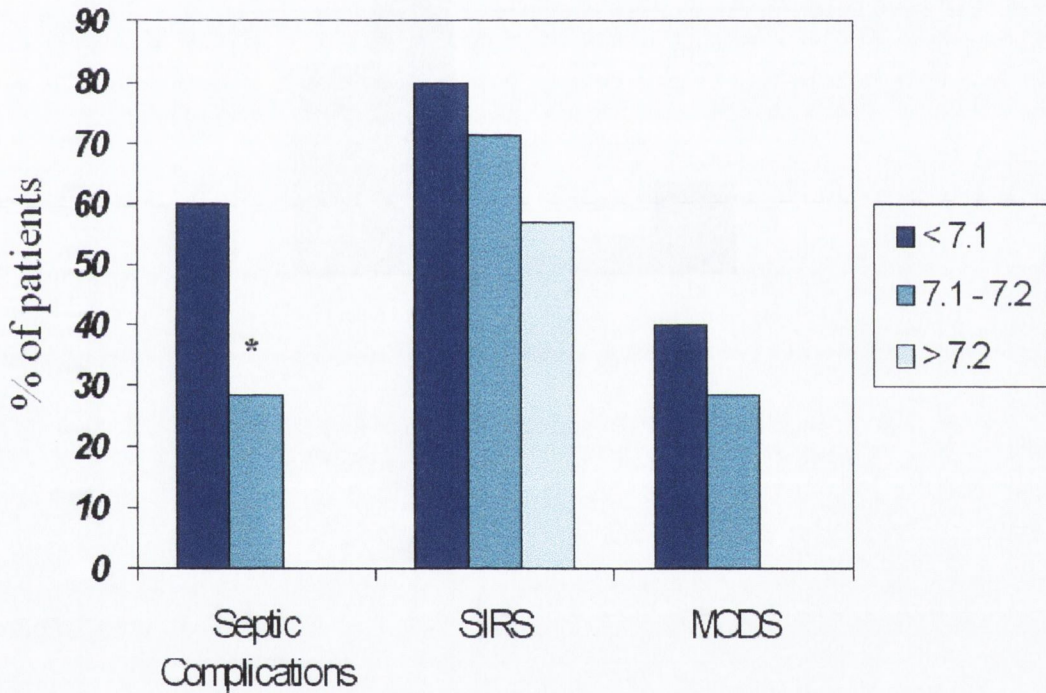
Percentage of patients in each group (pHi < 7.1, pHi 7.1 – 7.2, pHi > 7.2)
who developed septic complications, SIRS or MODS post-operatively
(* - p = 0.05, Chi-squared test)

Graph 4.5   Nadir pHi vs. Septic complications, SIRS & MODS (2)

The length of stay of patients within the high dependency or intensive care units (HDU/ICU) was also found to be related to intraoperative pHi. Patients whose pHi fell below 7.1 had a median length of stay of 7 days in the HDU/ICU, compared to a median of 3 days in patients whose pHi remained above 7.1 ($p < 0.05$, Mann-Whitney U).

Length of stay in HDU and overall length of hospital stay in discharged patients (LOS). (* - $p < 0.05$, Mann-Whitney U)
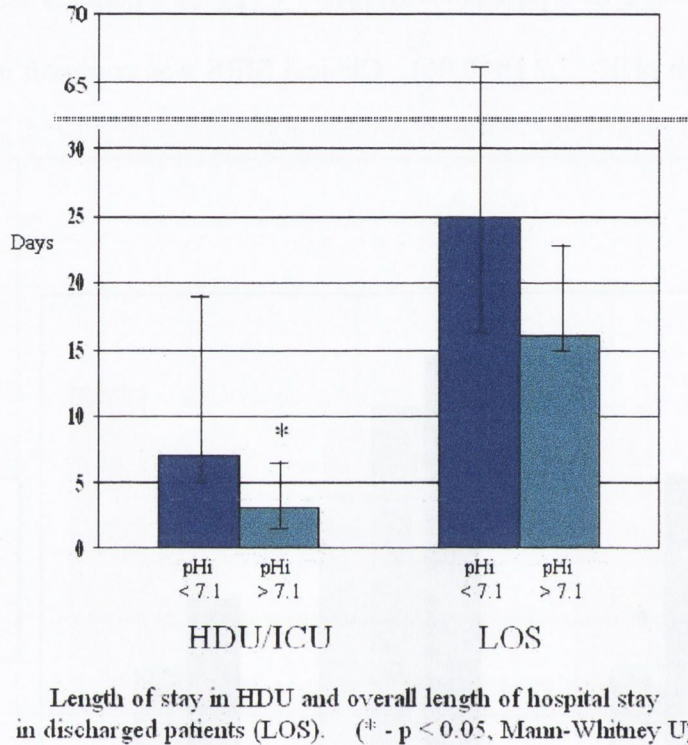
Graph 4.6   Nadir pHi vs. length of stay

There was no significant difference between the groups with regard to complications related to post-operative enteral nutrition. There was, however, a significant difference between the degree of nutritional support required by the two groups. Those whose pHi fell below 7.1 continued to receive nutritional support for a median of 24 days, while those whose pHi remained above 7.1 only required support for a median of 12 days ($p < 0.05$, Mann-Whitney U test).

4.4.4   C-REACTIVE PROTEIN

All patients showed an elevation in post-operative serum C-reactive protein levels (section 3.4.2). Comparison was then made between those whose nadir pHi fell below 7.1 and those whose pHi remained above this level. It was found that the postoperative elevation in C-reactive protein was greater in patients with a low pHi, the difference between the 2 groups being significant on the first and seventh post-operative days (Graph 4.7).
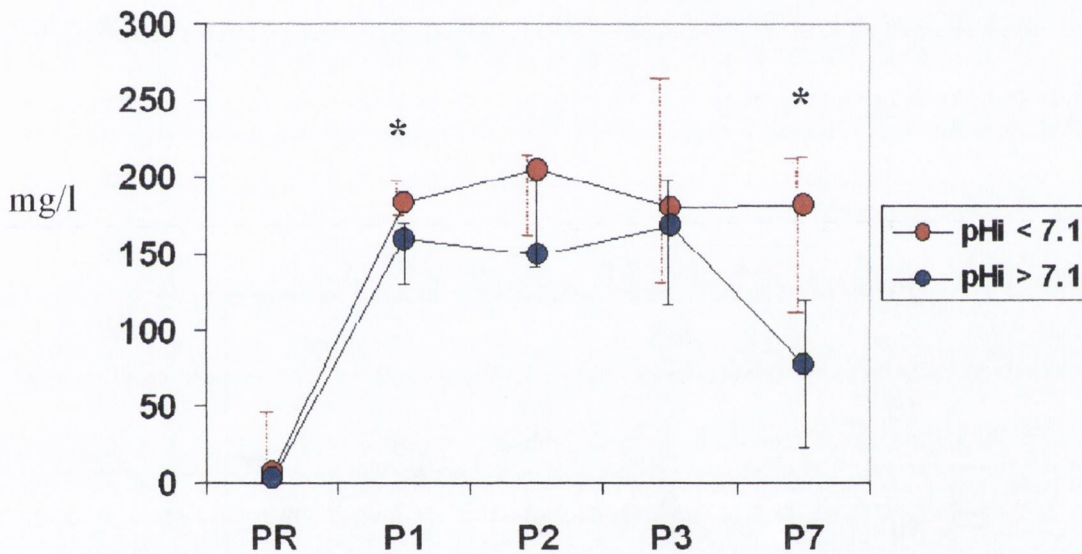
Median postoperative serum C-reactive protein levels
pre-operatively (PR) and on the first, second, third and seventh
post-operative days (P1, P2, P3 & P7 respectively)
(* - p<0.05 Mann-Whitney U Test)

Graph 4.7   Nadir pHi vs. postoperative CRP levels

4.4.5   POST-OPERATIVE LEUCOCYTE ACTIVATION

Both groups of patients, those with a pHi of less than 7.1 and those who maintained

a pHi of above 7.1, had a normal white cell count preoperatively (6 x 10^9 cells/l [5.7 – 6.7]

and 6.6 x 10^9 cells/l [5.5 – 7.5] respectively, shown in graph 4.8).   Postoperatively,

however, there were significant differences in the leucocyte counts in these two groups.

On the second day, those whose pHi fell below 7.1 had a significant leucocytosis (14 x 10^9

cells/l [11.7 – 16.3] vs. 10.1 x 10^9 cells/l [7.6 – 12.5]).   This difference was also significant

on day 3 (10 x 10^9 cells/l [9.3 – 13] vs. 7.6 x 10^9 cells/l [4.8 – 9.7] respectively), but had

resolved by one week after surgery.
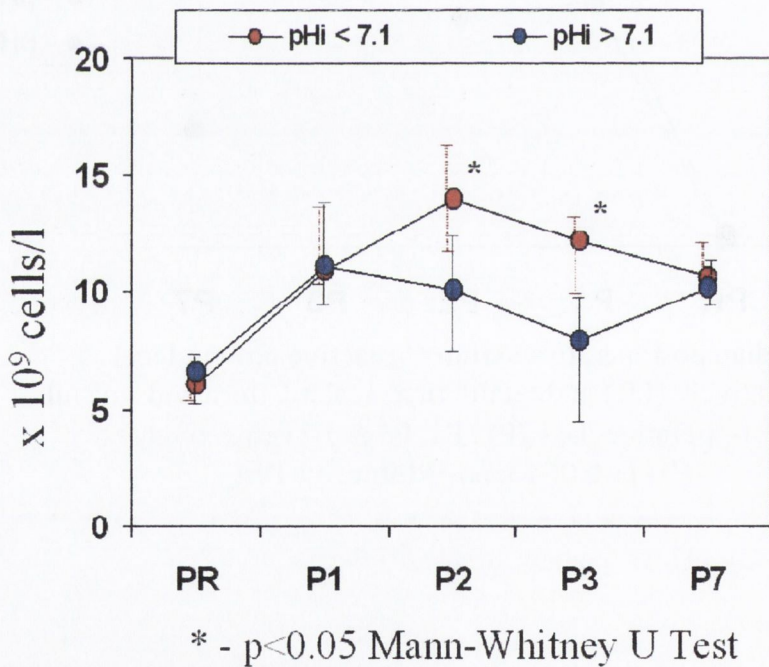
Graph 4.8   Nadir pHi vs. white cell count

(i)   T-cell activation and surface marker expression

In this section, the expression of the T-cell activation markers CD25, CD69, CD71 and HLA-DR on CD3+, CD4+ and CD8+ T-cells was investigated, comparing levels of expression in those who had severe intraoperative splanchnic hypoxia and those who maintained a pHi above 7.1.   CD25 was examined initially and it was found that preoperatively, there was minimal expression in both groups of patients, in all T-cell subsets (Graph 4.9).

The CD3+ and CD4 + subsets showed similar patterns of pre-and postoperative expression of CD25.   Postoperatively, those who maintained their pHi above 7.1 had the typical postoperative elevation in expression as shown in section 3.4.3 (i).   In those whose pHi fell below 7.1, however, no such rise was seen.   Indeed, the CD3+ and CD4+ subgroups showed no reaction to surgery in this group of patients, with no evident alteration of post-operative expression of CD25 as compared to the preoperative value.   In contrast, within the CD8+ subset, there were higher levels of CD25 expression both pre- and postoperatively in those patients whose pHi fell below 7.1.   This was still evident on the third postoperative day, and by one week, levels of expression were the same in the 2 groups.   None of these results were significant on Mann-Whitney U testing between groups.
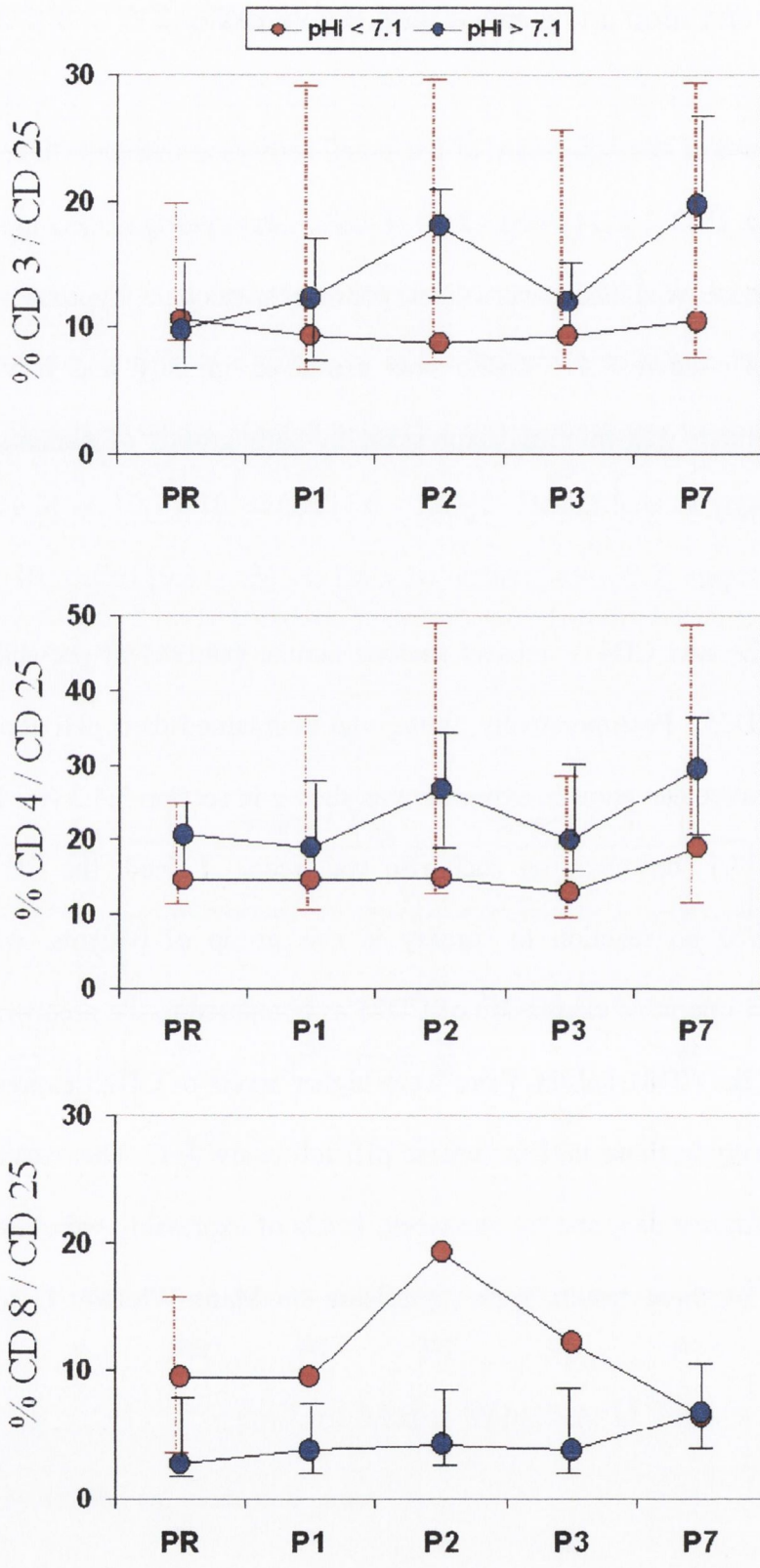
Graph 4.9   Nadir pHi vs. T-cell CD25 expression
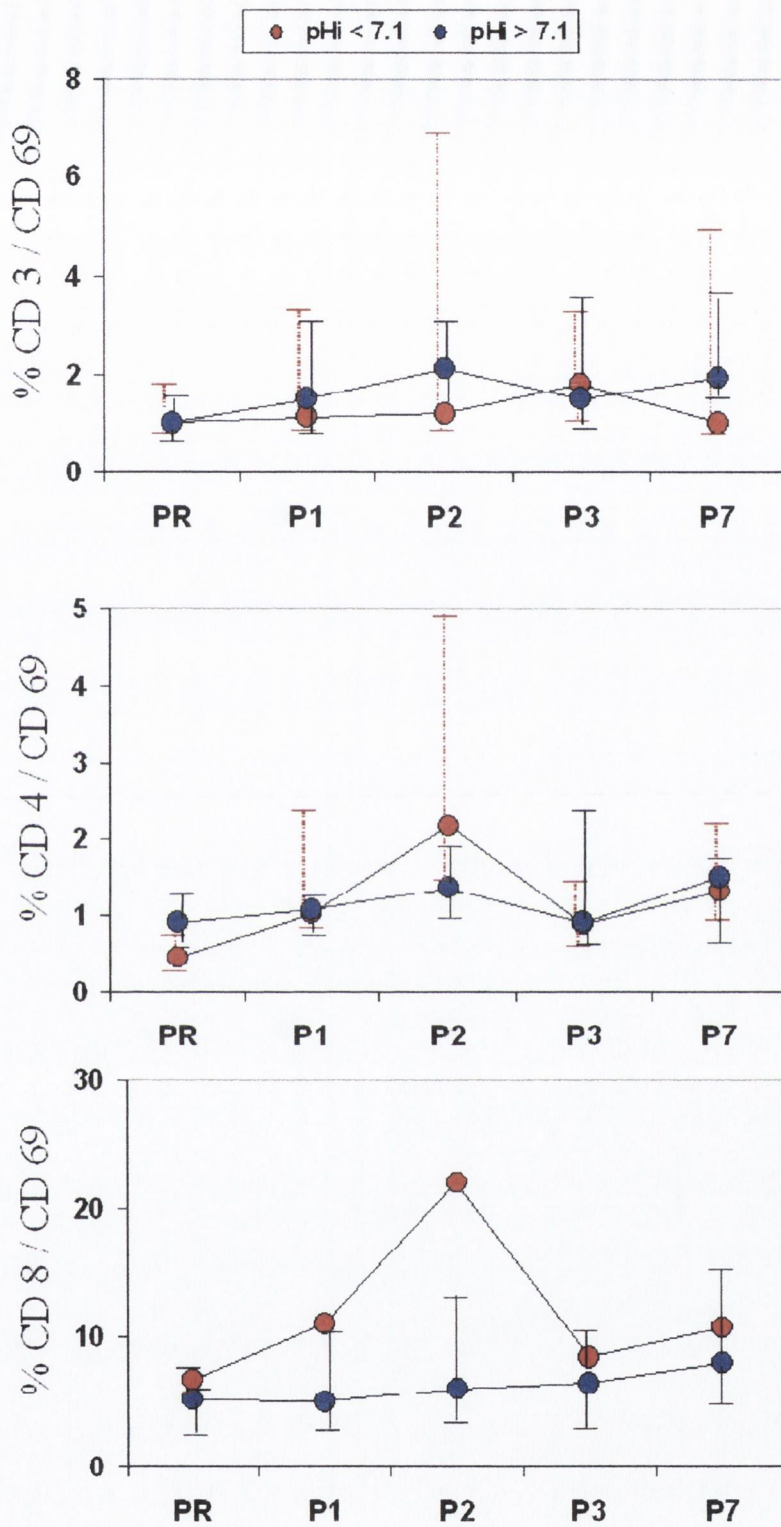
Graph 4.10   Nadir pHi vs. T-cell CD69 expression

Next, CD69 expression on all T-cell subtypes was examined, both preoperatively and on postoperative days 1, 2, 3 and 7 and very little difference between groups in the CD3+ and CD4+ subtypes was observed (graph 4.10). Within the CD3+ subtype, those who maintained a pHi greater than 7.1 had slightly higher postoperative levels of expression than those who did not, but this was not marked and did not reach statistical significance. Within the CD4+ subtype, both groups showed increased levels of expression postoperatively, this time greater in those whose pHi dropped below 7.1, but again this data failed to show any significance on statistical analysis. The CD8+ subtype also showed increased levels of expression postoperatively, higher in those whose pHi fell below 7.1 (maximal on the second postoperative day), but this again failed to reach significance on further analysis.

The expression of CD71 on the 3 T-cell subtypes preoperatively and on postoperative days 1, 2, 3 and 7 is shown in graph 4.11, comparing patients with nadir pHi < 7.1 with the cohort whose nadir pHi remained above 7.1. Within the CD3+ subtype, both groups of patients showed an elevation in postoperative expression. In patients whose pHi was maintained above 7.1, this elevation persisted to one week postoperatively (as seen in section 3.4.3 [i]) while the levels in those whose pHi dropped below 7.1 had returned to preoperative values. Within the CD4+ subset, those whose pHi remained above 7.1 showed increased levels of CD71 expression postoperatively, but those whose pHi fell below 7.1 had little or no increase as compared to their preoperative result. As with CD8+ expression of the other surface markers, there was an increased level of expression in those whose pHi fell below 7.1 as compared to those who maintained their pHi above 7.1.
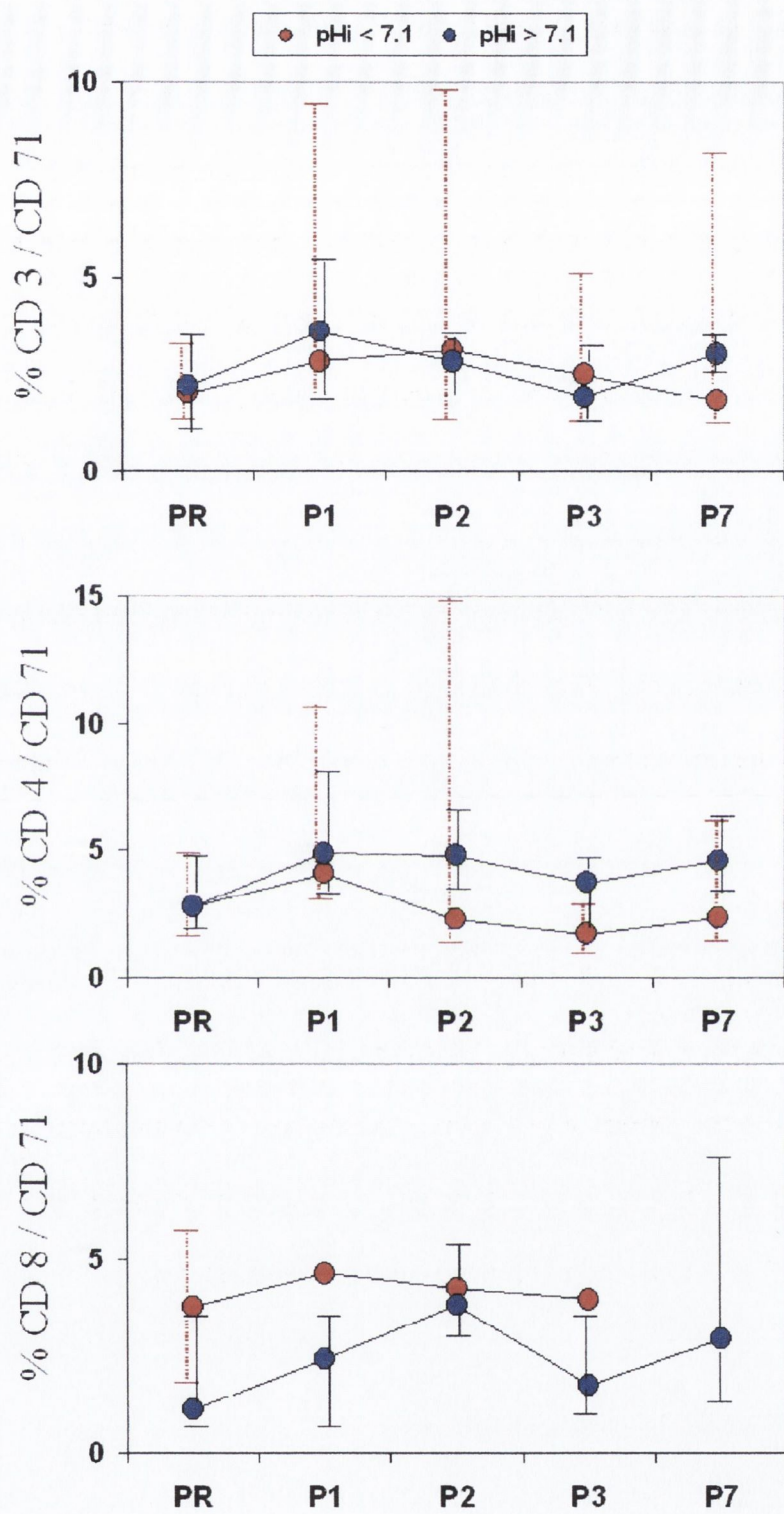
Graph 4.11   Nadir pHi vs. T-cell CD71 expression

HLA-DR expression on all T-cell subtypes was then investigated both preoperatively and on postoperative days 1, 2, 3 and 7 (graph 4.12). Within the CD3+ subtype, there was no difference in preoperative expression between the 2 groups. Both groups then showed an initial mild elevation in HLA-DR expression, which then decreased sharply in those whose pHi fell below 7.1, so that they had reduced expression on the second, third and seventh days as compared to those whose pHi remained above 7.1. These differences in HLA-DR expression were even more marked within the CD4+ subtype. Here, while those whose pHi remained above 7.1 had a mild post-operative rise in expression, those whose pHi fell below 7.1 had postoperative depression, persisting until the third post-operative day. This difference in HLA-DR expression between the 2 groups was significant on the second postoperative day (p < 0.05, Mann-Whitney U) and was close to significance on day 3 (p = 0.09).

With respect to the CD8+ subtype, HLA-DR expression, the 2 groups were initially similar, both showing a depression in HLA-DR expression postoperatively. There was a difference between the groups at one week, with persistent depression in those whose pHi had fallen below 7.1, but a marked elevation in expression in those who had maintained their pHi above 7.1, but this did not reach significance on analysis.
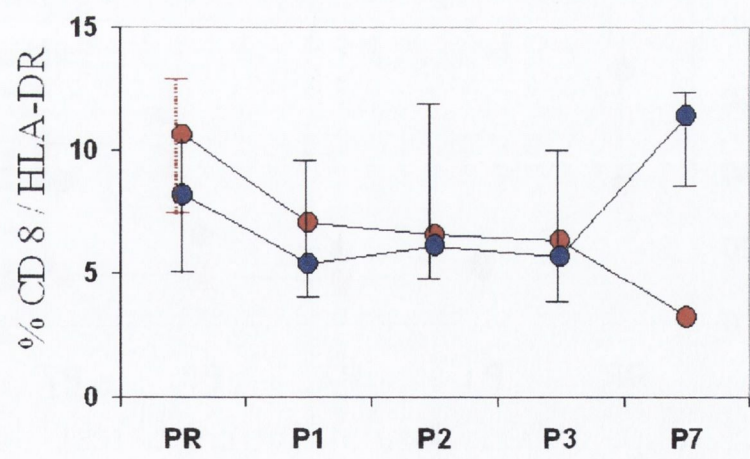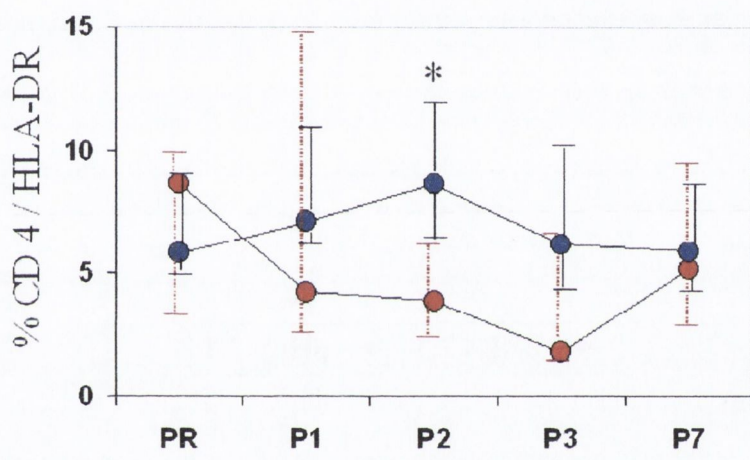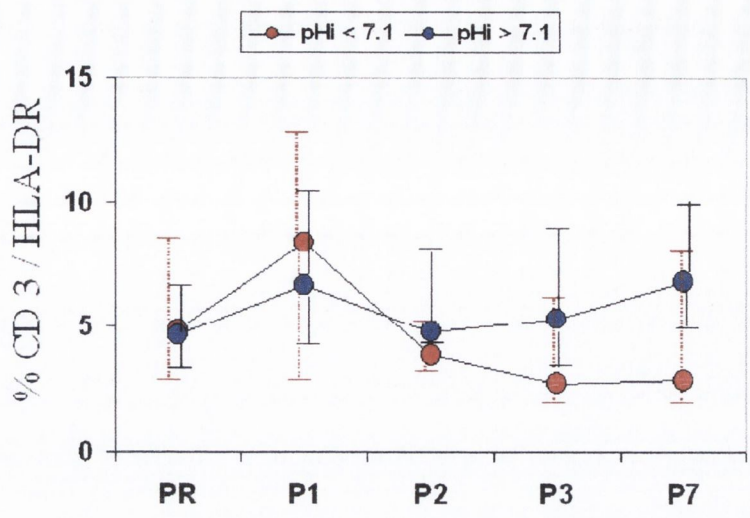
* p < 0.05, Mann-Whitney U test

Graph 4.12   Nadir pHi vs. T-cell HLA-DR expression

(ii)   Monocyte activation and surface marker expression

The expression of HLA-DR on monocytes preoperatively and on postoperative days 1, 2, 3 and 7 is shown in graph 4.13, comparing patients with nadir pHi < 7.1 with the cohort whose nadir pHi is > 7.1.   There was no difference between these groups preoperatively.   In both groups there was a significant decrease from preoperative expression to the first postoperative day.   Recovery of HLA-DR expression on monocytes was observed in succeeding days only in the pHi > 7.1 cohort, and this was significantly (p<0.05) greater than in the cohort with pHi < 7.1 on the seventh postoperative day. Notably, the five patients who developed septic complications also had decreased HLA-DR expression on the seventh post-operative day, as compared to those who did not (13% vs. 34 % expression, p < 0.05, Mann-Whitney U).
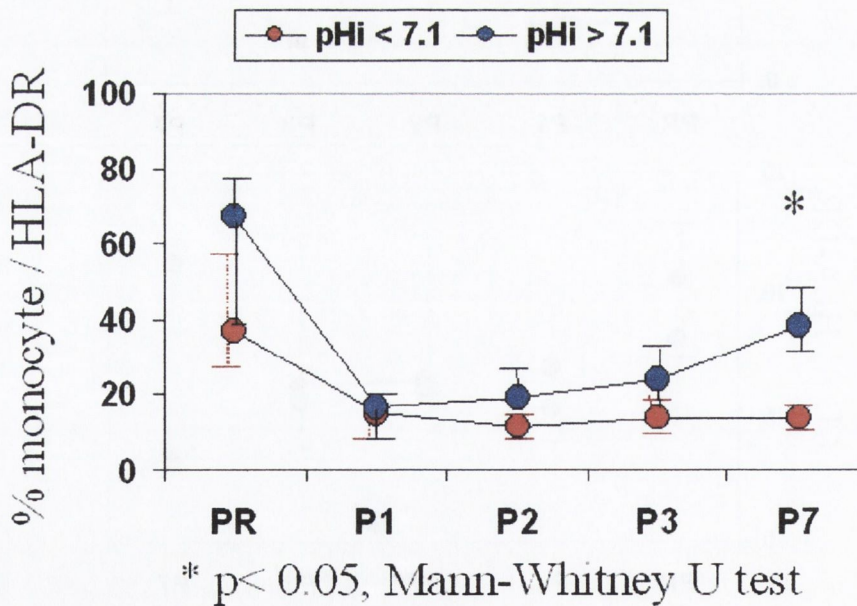
Graph 4.13   Nadir pHi vs. monocyte HLA-DR expression

While examining post-operative monocyte CD-62-L expression, it was observed that patients with a low pHi did not express the elevation in CD-62L levels usually seen after trauma or surgery (shown in section 3.4.3 [ii]), the difference in expression between the two groups being significant on the first and third post-operative days (graph 4.14).
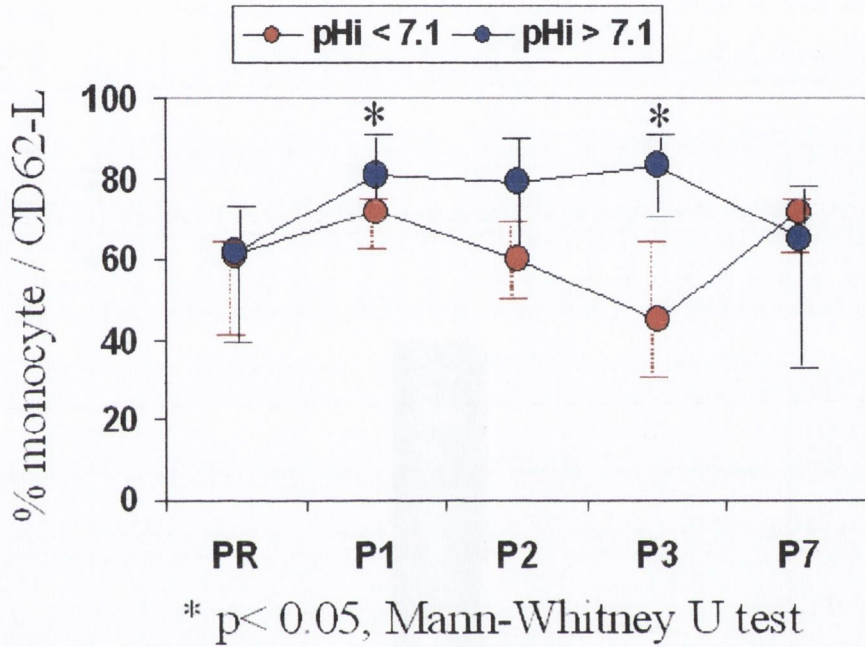
Graph 4.14   Nadir pHi vs. monocyte CD-62-L

4.4.6 INTESTINAL PERMEABILITY

Intestinal permeability, as measured by dual sugar testing (section 2.7) was performed preoperatively and on the first and seventh postoperative days; the results are shown in graph 4.15. There was a marked elevation present, from a median ratio of 0.024 preoperatively, to 0.279 on the first postoperative day (p< 0.01) before returning to normal by the seventh postoperative day.
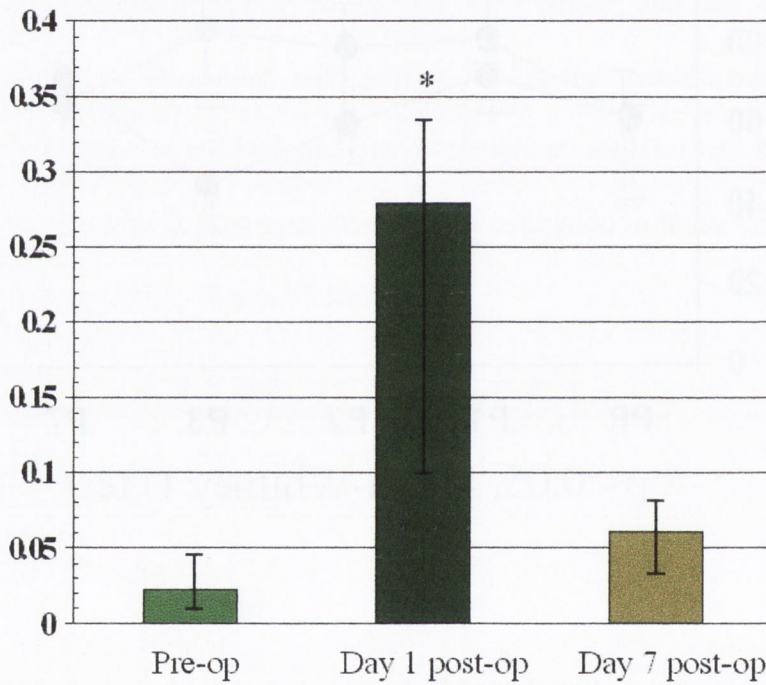
Post-operative changes in intestinal permeability, as measured by lactulose/mannitol ratio of the dual sugar test.
* - significant rise compared to pre-operative level, p < 0.01, Wilcoxin Rank Test

Graph 4.15   Intestinal permeability

Next, the results were assessed in order to determine whether there was any correlation between alteration in postoperative intestinal permeability and subsequent postoperative outcome. It was observed that those who went on to develop septic complications following their surgery had a higher elevation in lactulose:mannitol ratio as compared to those who had an uncomplicated recovery, but this was not significant on statistical analysis.
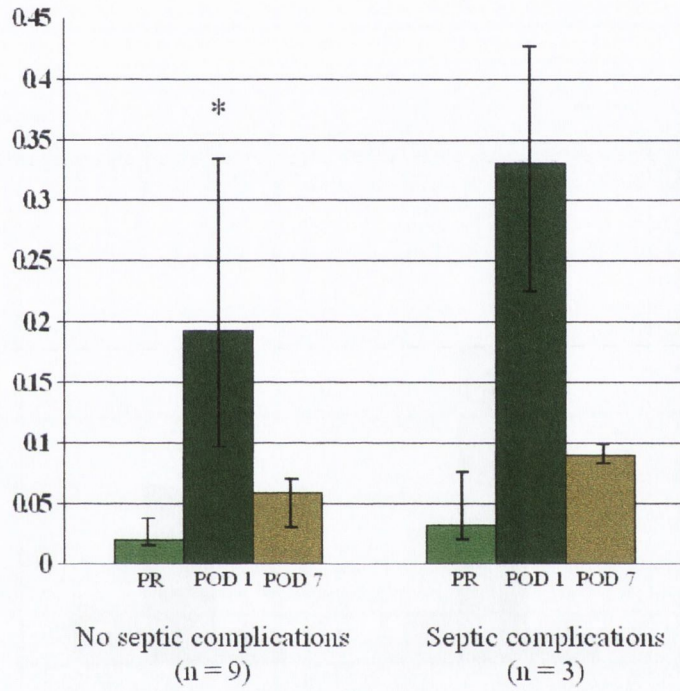
Post-operative changes in intestinal permeability, as measured by lactulose/mannitol ratio of the dual sugar test (PR = pre-operative, POD 1 – first post-operative day, POD 7 – seventh post-operative day) * - significant rise compared to pre-operative level, p < 0.05, Wilcoxin Rank Test

Graph 4.16   Intestinal permeability and septic complications

Finally, comparison was made between patients with a nadir pHi <7.1 with those with a pHi > 7.1, comparing intestinal permeability preoperatively and on postoperative days 1 and 7 (graph 4.17). The subgroup with severe mucosal acidosis showed a significant ($p<0.05$) near tenfold increase in permeability on the first postoperative day, which returned to normal by day 7. In the group with pHi > 7.1, the intestinal permeability was also increased on the first postoperative day, approximately fivefold, but this did not reach significance ($p=0.07$).
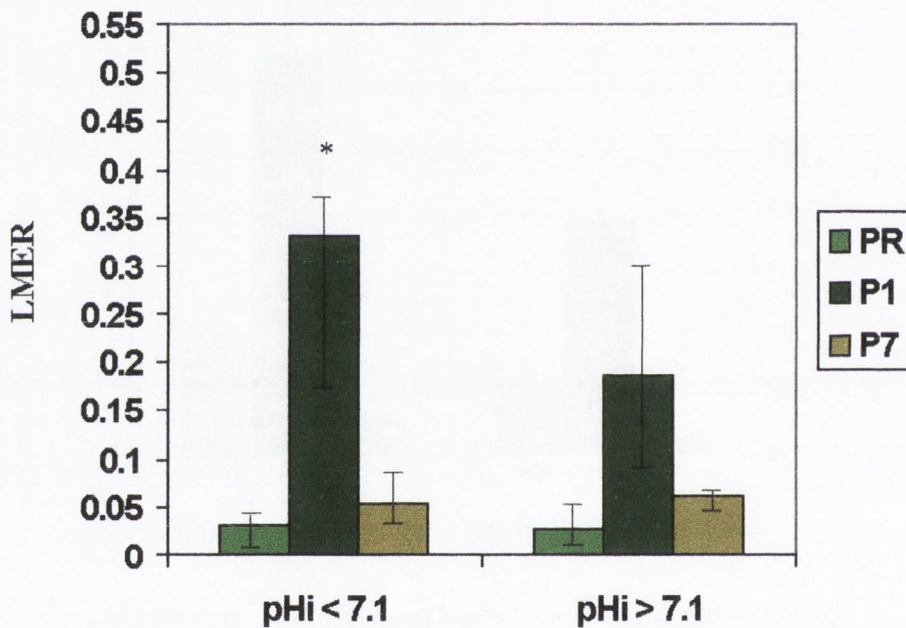
Median LMER ratio pre-operatively (PR) and on days 1 (P1)
and 7 (P7) post-operatively
(* - significant elevation compared to PR value,
$p<0.05$ Wilcoxon Signed Rank Test)

Graph 4.17   Nadir pHi vs. Intestinal Permeability

4.4.7   ALBUMIN

There was no difference in preoperative albumin levels between those whose pHi fell below 7.1 and those patients who maintained their pHi above this level (albumin g/l 39 [36.7 – 41] and 39 [38 – 40.7] respectively, graph 4.18). Both groups of patients showed a postoperative depression in albumin levels.   In those whose pHi remained above 7.1, albumin levels began to rise again at one week (33g/l [30 – 35]) while remaining low in the other group of patients (29 g/l [28.5 – 31.5]), but this did not reach significance (p = 0.29).
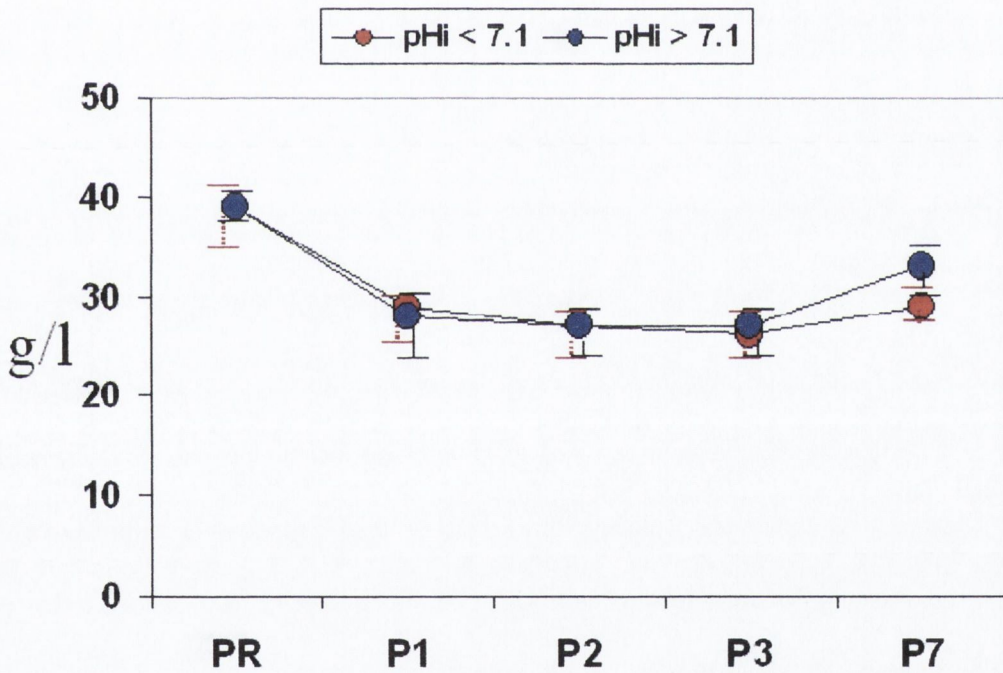
Graph 4.18   Nadir pHi vs. Albumin

4.5   DISCUSSION

The role of tonometric intramucosal pH monitoring in the prediction of clinical outcome measures in surgical patients is becoming increasingly established. The method was described in the 1960's, but widespread interest in the technique has developed mainly in the last 15 to 20 years (Bergogsky, 1964; Dawson et al, 1965). It relies upon the principle that the pCO_2 in the wall of the stomach equilibrates with that in the lumen of the stomach, which can be measured by means of the silicone-balloon tipped tonometer (Fiddian-Green et al, 1982). Mucosal pH is then calculated from the gastric pCO_2 and the serum bicarbonate based on the Henderson-Hasselbach equation (Clark et al, 1992).

With regard to the accuracy of this method of measuring pHi, some precautions must be observed. The underlying assumption that the serum bicarbonate is equivalent with that of the gastric mucosa is valid in well perfused tissue, but not in poorly perfused tissue, when the tonometer may underestimate the pHi (Antonsson et al, 1990). Also, the intraluminal gastric pCO2 may be altered by buffering of the gastric acid by salivary, duodenal or gastric bicarbonate, leading to a falsely low pHi reading (Fiddian-Green et al, 1982). Administration of intravenous ranitidine has been shown to ameliorate this effect, increasing accuracy of measurements (Kolkmann et al, 1994).

While the many ICU studies examining pHi in critically ill patients use a pHi of 7.35 or 7.32 as a predictor of clinical complications, there is some variance as to the cut-off level used in the literature examining pHi levels intraoperatively. In this study, there was only one patient who maintained their pHi above 7.35 for the entire operative period and only 3 above a level of 7.32. This is in agreement with the majority of operative studies, which report varying percentages of patients with nadir pHi below these "normal" levels, so that a fall in pHi is not necessarily predictive of complications. Bjork et al found that all patients (n = 26) within their study had a nadir pHi of below 7.32, as did the majority of patients undergoing oesophagectomy in the study by Boyle et al (Bjork et al, 1994; Boyle et al, 1998).

As noted above, the majority of patients in this study had a reduction in intraoperative pHi, below the level that would be considered "normal" in an ICU or ward setting. In 5 patients, the pHi fell below 7.1 and this group was classified as having severe splanchnic hypoperfusion. Seven patients had moderate hypoperfusion, the nadir pHi remaining between 7.1 and 7.2, while the remaining 8 patients maintained their pHi above 7.2 throughout their procedure. Similarly, Boyle et al studied 16 patients undergoing oesophagectomy using tonometry and scanning Doppler flowmetry and showed progressive decrease in perfusion in the stomach with gastric mobilization, with a decrease in mean gastric pH from 7.37 before mobilization to 7.18 following mobilization and 7.29 after the anastomosis.

With regard to correlating alterations in gastric pHi to haemodynamic parameters, data is sparse. Boyle et al make mention that no significant differences were found in heart rate, MAP or CVP between the three intraoperative measurement points described above and Welte et al also state that "haemodynamic and oxygenation variables remained unchanged thoughout surgery" (Boyle et al, 1998; Welte et al, 1996). In this study, the occurrence of hypoperfusion did not appear to relate to the duration of surgery or operative blood loss, but a significant association was evident when the nadir mean arterial pressure as a percentage of the pre-incision mean was compared, with more marked changes evident in patients with a pHi < 7.1 compared with patients whose pHi remained above 7.1.

As stated previously, initial studies performed in intensive care settings have shown that a low gastric pHi (defined as falling below 7.35 or 7.32) is significantly associated with an increase in subsequent complications, MODS and mortality (Marik et al, 1993; Gutierrez et al, 1992; Doglio et al, 1991). While operative studies suggest that a reduction in splanchnic blood flow during surgery is not unexpected, there does appear to be a correlation between pHi and subsequent recovery. A fall in sigmoid pHi intraoperatively has been shown to be predictive of postoperative morbidity and mortality in patients undergoing abdominal aortic aneurysm repair (Björck et al, 1994; Soong et al, 1998). With regard to the predictive value of intraoperative gastric pHi, this has also shown to be predictive of a number of clinical outcome measures, such as postoperative complication rate, mortality and anastomotic breakdown following upper gastrointestinal surgery (Poeze et al, 2000; Tarui et al, 1999). Björck et al found that a pHi of below 7.20 predicted major complications with a sensitivity of 100 per cent and a specificity of 81 per cent. Analysis of the raw demographics data within the paper by Roumen et al, reveals a positive

predictive value for complications of 56 in patients whose pHi fell below 7.2, and 100 when the level dropped below 7.1 (Björck et al, 1994; Roumen et al, 1994).

Of even greater interest than the predictive value of tonometry, is the possibility of improving patient outcome by pHi directed therapy. In an ICU study, Gutierrez et al performed a randomized trial to determine the effect of therapeutic interventions to maintain pHi above 7.35 or prevent a fall of greater than 0.10 units from the initial measurement (Gutierrez et al, 1992). Patients who were admitted to the intensive care unit with a pHi above 7.35 had significantly improved survival outcomes if therapeutic interventions were initiated as compared to the control group. There was, however, no difference between groups for those patients who were admitted to the ICU with a pHi of below 7.35 and this failure to improve outcome following pHi directed therapy has also been observed elsewhere (Gomersall et al, 2000).

In this chapter, a significant correlation was observed between low intraoperative pHi measurements and subsequent development of septic complications in the post-operative period. Patients with a low pHi also required a longer period of time in the high dependency or intensive care units postoperatively, before transfer to a ward setting. In examining the relationship between pHi and post-operative SIRS and MODS however, although a trend towards an increase in these conditions in the post-operative period was observed in those patients with a low pHi, the study failed to show that this was statistically significant, possibly a reflection of the number of patients included.

While analysis of clinical outcome measures as described above was included as part of this research, the main focus was on examining the effect of splanchnic hypoperfusion on post-operative gut and cellular immune responses. It has long been postulated that episodes of hypovolaemic ischaemia and splanchnic vasoconstriction compromise the integrity of the gut barrier function, thereby resulting in an increase in intestinal permeability and translocation of factors such as bacteria and endotoxin. These are then thought to trigger the release of inflammatory mediators such as cytokines, resulting in a subsequent systemic inflammation or development of organ dysfunction (Swank et al, 1996).

This research confirms the results of previous studies, showing an increase in postoperative intestinal permeability. It is still not known whether this increase in intestinal permeability correlates with an increased potential for bacterial translocation in humans or adverse clinical outcome. In this study, those who went on to develop septic complications following their surgery had a higher elevation in lactulose:mannitol ratio as compared to those who had an uncomplicated recovery, but this was not significant on statistical analysis and the numbers studied were small.

There are few clinical studies published to date that directly examine the relationship between splanchnic hypoxia (as measured by tonometry) and alterations in intestinal permeability. This study found that a reduced gastric intramucosal pH was associated with a significant rise in intestinal permeability on the first post-operative day, with levels returning to normal by the seventh post-operative day. Previous research has also demonstrated this relationship between intraoperative intramucosal acidosis and post-

operative intestinal permeability, but found that there was some overlap in results between those with a normal gastric pHi and those who did not (Sinclair et al, 1995). The explanation for this may be that gastric tonometry reflects alteration or reduction in blood supply to the celiac axis alone, while intestinal permeability is a reflection of small bowel function, which is supplied by the superior mesenteric artery. A number of small bowel tonometric studies, measuring jejunal pHi, have been performed, mainly to assess its predictive value with regard to clinical outcome measures and accuracy as compared to standard gastric and sigmoid pHi (Walley et al, 1998). Extending this research to examine whether alterations in jejunal pHi result in development of altered intestinal permeability in the post-operative period would seem to be a logical progression, but nothing has been published with regard to this to date.

In addition to the alteration in immune function seen at the gut level following a fall in gastric pHi, the leucocytosis and elevation in C-reactive protein levels observed in this study suggest that splanchnic hypoxia also results in a generalized activation of the systemic immune system. With regard to specific analysis of lymphocyte function, the effect of splanchnic hypoperfusion on systemic acute phase response and T cell and monocyte function was assessed, by examining expression of surface activation markers.

With regard to expression of IL-2 receptor (CD25), this study found that patients with splanchnic hypoperfusion had an absence of IL-R surface upregulation in the CD3+ and CD4+ subtypes in the postoperative period; this was similar to the anergy seen in chapter 3 in those patients who proceeded to develop septic complications, perhaps reflecting an impairment of T cell-mediated immune reactions, thus predisposing to subsequent infection. Evaluation of CD69 and CD71 expression failed to show any

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significant correlation with intraoperative splanchnic blood flow, although there was a tendency to reduced CD71 expression in the CD4+ subtype in those with pHi < 7.1.

With regard to T cell HLA-DR expression, depression of HLA-DR expression was observed in both the CD3+ and CD4+ subtypes of patients who experienced splanchnic hypoperfusion, although this was only significant within the CD4+ population. Again, this correlates with findings in this and other studies of reduced CD4+ expression in patients who develop septic complications postoperatively.

The effect of hypoperfusion on monocyte function, as measured by the monocyte activation markers HLA-DR and CD-62-L, was also specifically addressed by this research. Previous studies have shown that downregulation of monocyte HLA-DR expression is observed following a stress to the system such as surgery or trauma (Ditschkowski et al, 1999; Klava et al, 1997). Prolongation of this downregulation is seen as an early indicator of derangement of the immune response and is predictive of an increased risk of sepsis and morbidity (Cheadle et al, 1991; Ditschkowski et al, 1999; Wakefield et al, 1993). This research confirms these findings, showing that patients with reduced HLA-DR had an increased risk of post-operative septic complications. Furthermore, the data showed that patients with a reduced intraoperative pHi also have a significant prolongation of this downregulation in the post-operative period, confirming that a reduction in splanchnic blood flow is associated with an alteration in cellular immune responses.

This derangement of the normal monocyte response was also observed when analyzing CD-62-L expression. Monocyte CD-62-L levels have been shown to be elevated following trauma or surgery, possibly a reflection of post-injury leucocyte trafficking (Cocks et al, 1998; Ljunghusen et al, 1997). In this study, patients with a low pHi did not show this expected post-operative rise in expression, as compared to those with pHi of above 7.1.

In conclusion, this study confirms that patients undergoing major surgery have interruption or reduction in intestinal blood flow intraoperatively, reflected by a fall in gastric pHi. This reduction in splanchnic perfusion is associated with a subsequent increase in post-operative morbidity. Furthermore, this research demonstrates that this is also associated with detectable alteration in both local and systemic immune responses in the post-operative period, supporting the 'gut-origin' hypothesis of sepsis, that the stressed or damaged gut may become the trigger for systemic inflammation and subsequent development of MODS.

CHAPTER 5: INTRAOPERATIVE IMMUNE RESPONSES –

COMPARISON OF IMMUNE ACTIVATION IN SYSTEMIC AND

PORTAL VENOUS BLOOD SAMPLES

5.1   INTRODUCTION

Failure of the gut barrier to contain endogenous bacteria and endotoxins within the gastrointestinal tract, allowing their translocation to mesenteric lymphatics and the portal and systemic circulations is central to the gut hypothesis of multiple organ failure. This hypothesis incriminates bacteria and endotoxins derived from the gastrointestinal tract as triggers, which initiate, perpetuate or exacerbate a systemic inflammatory response resulting in the development of SIRS. This hypothesis of bacterial translocation can provide an explanation as to why no septic focus can be found in 30% of bacteraemic MODS patients or how patients may develop a SIRS in the absence of infection or another recognizable cause (Deitch, 1992; Fiddian-Green, 1988; Reynolds, 1996; Saadia, 1995; Swank et al, 1996; Vatner, 1974).

In addition, new evidence is implicating the gut as a cytokine-generating organ, suggesting that the "stressed" gut may contribute to the development of MODS by direct release of systemic mediators as well as by the process of bacterial translocation (Deitch, 1992 & 2002; Kompan et al, 2001; Rahman et al, 2003; Riddington et al, 1995; Roumen et al, 1993; Swank et al, 1996; Welsh et al, 1998).

While there is some documented evidence of bacterial translocation to the mesenteric lymph nodes in human populations, clinical studies investigating the gut-portal vein-liver axis following trauma or major surgery are extremely sparse. Portal venous studies have demonstrated gut ketogenesis in the initial 24 hours following major trauma; however, few immunological parameters, such as cellular immune activation and presence of endotoxin or cytokines within this portal circulation, have been studied to date. Clinical studies in trauma patients have to date failed to find bacteria in the portal vein of patients undergoing laparotomy, although cytokine studies suggest higher concentrations of IL-6 within the portal circulation, as compared to those in systemic venous samples, following cross-clamping of the aorta during aneurysm surgery (Baigrie et al, 1993; Moore et al, 1991; Pogetti et al, 1992; Swank et al, 1996).

5.2   AIMS OF STUDY

In this chapter, intraoperative blood samples were examined for evidence of early immune activation. Specifically, the aim was to assess whether there is any discernable difference in acute phase response or cellular activation levels between systemic and portal intraoperative blood samples, which would lend support to the hypothesis incriminating the gut as a main contributor in the development of SIRS and MODS following major trauma or surgery.

5.3   PATIENTS AND METHODS

Twenty-seven patients were included in this section of the study, the same group of patients as previously described in section 3.3. Expression of the T-cell surface markers CD25, CD69, CD71 & HLA-DR were assessed by flow cytometry in all patients (section 2.3 & 2.4) as were the monocyte surface markers CD62-L and HLA-DR. Specimens were obtained preoperatively and on the first, second, third and seventh post-operative days. In addition, intraoperative specimens were obtained from all patients. Systemic blood samples were obtained from a central venous catheter or arterial line, while a portal venous sample was obtained by direct aspiration of this vein by the operating surgeon; these two specimens were obtained simultaneously. Systemic and portal serum samples were also obtained for assessment of C-reactive protein levels.

5.4   RESULTS

5.4.1   PATIENT DEMOGRAPHICS

Demographic for the patients included in this chapter are described in detail in section 3.4.1 (including graph 3.1).

5.4.2   C-REACTIVE PROTEIN

There was no difference in C-reactive protein level in the intraoperative samples as compared to the preoperative levels and both the systemic serum sample and that taken from the portal vein were identical (3.36 [3.36 – 8.58] vs. 3.36 [3.36 – 5.54]).

5.4.3   INTRAOPERATIVE LEUCOCYTE ACTIVATION

(i)   T cell activation and surface marker expression

The expression of the T-cell activation markers CD25, CD69, CD71 and HLA-DR on CD3+, CD4+ and CD8+ T-cells intraoperatively was examined, comparing levels of expression in samples taken from a systemic access site (central venous catheter or arterial line) with samples aspirated directly from the portal vein by the operating surgeon. With regard to CD25, it was found that levels of expression were consistently higher in the systemic intraoperative blood samples in all T-cell subtypes. The differences between these levels and those of the portal venous samples were small, however, and did not reach statistical significance (graph 5.1)

Examining the expression of CD69 on the CD3+ T-cell subtype, both the systemic and portal intraoperative levels of expression were higher than the preoperative value ($p < 0.05$, wilcoxin rank test, graph 5.2), confirming that CD69 is an early activation marker, showing alteration in activation within 2 hours of stimulus. When the CD4+ subtype was specifically investigated, it was observed that both the systemic and portal samples were significantly higher than the preoperative level ($p < 0.05$, wilcoxin rank test). In addition, the difference between these two intraoperative samples was significant on analysis ($p < 0.05$, wilcoxin rank test), with higher levels of expression being evident in the portal venous blood sample than the systemic (% cells expressing CD 69: 1.65 [0.69 – 2.63] vs. 1.02 [0.76 – 1.57] respectively, $p < 0.05$, wilcoxin rank test).
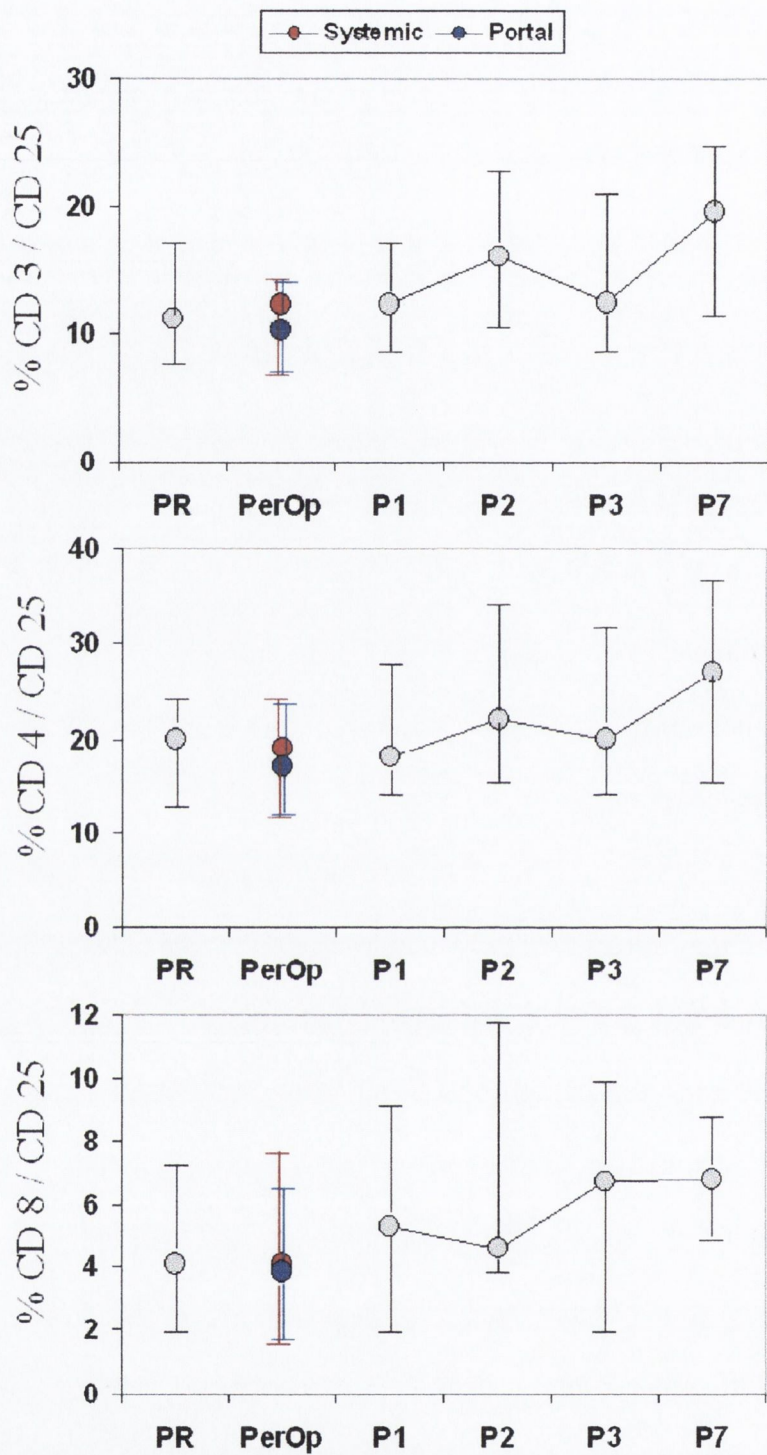
Graph 5.1   Intraoperative samples and T-cell CD25 expression
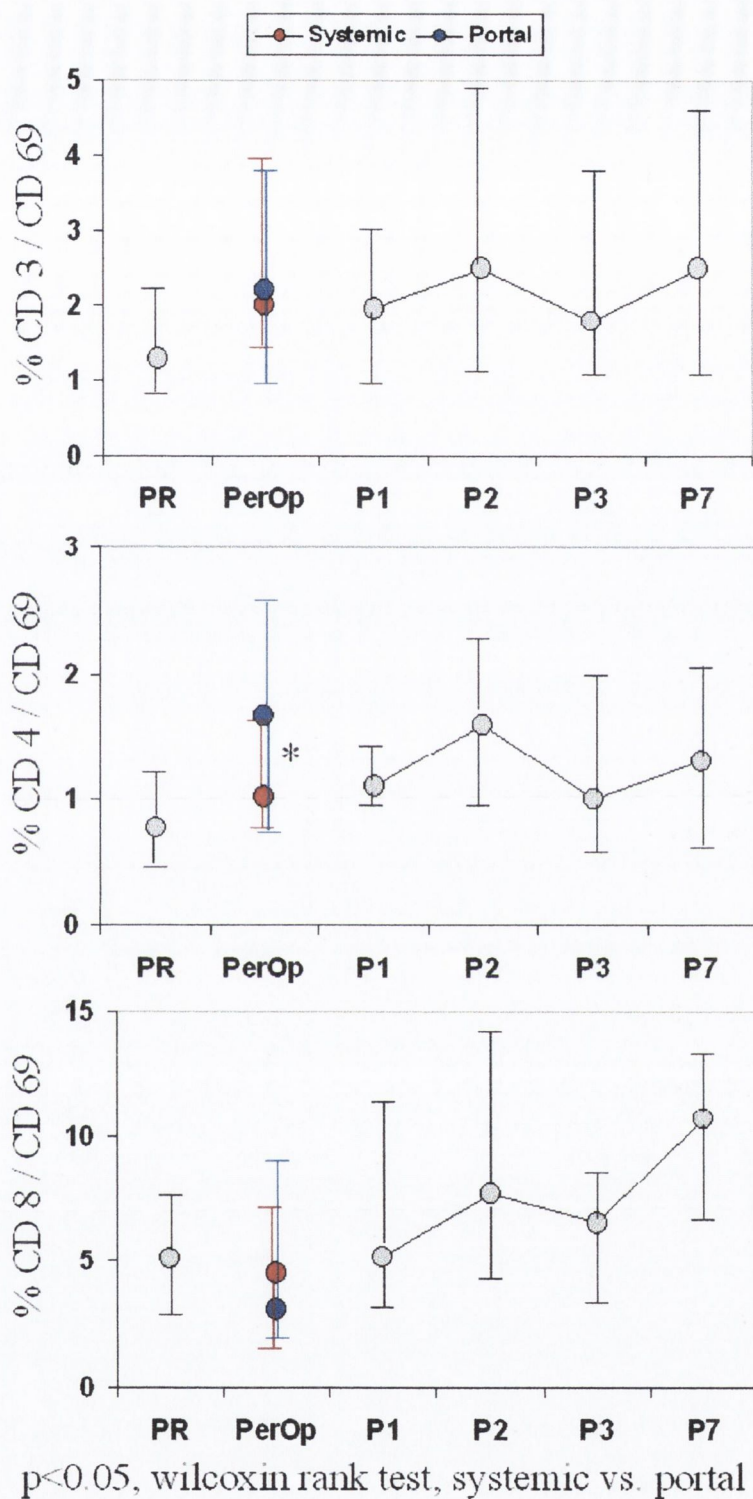
Graph 5.2　　　Intraoperative samples and T-cell CD69 expression

The surface expression of CD71 on the 3 T-cell subtypes in pre- intra- and postoperative samples is shown in graph 5.3. In the CD3+ T-cell subtype, both intraoperative samples appeared to be higher when compared to the preoperative level, but this elevation was not significant. When the CD4+ subtype was analysed, however, this elevation in intraoperative samples as compared to the preoperative level was significant, in both the systemic and portal sample ($p < 0.05$ and $p < 0.05$, wilcoxin rank test). With regard to differences between systemic and portal intraoperative samples, expression of CD71 appeared to be higher in the systemic sample within the CD3+ subtype, but this was reversed in the CD4+ and CD8+ subtypes (here, the portal samples had higher levels of expression); none of these differences were significant on analysis.

The surface expression of HLA-DR on the 3 T-cell subtypes in pre- intra- and postoperative samples is shown in graph 5.4. In the CD3+ and CD4+ subtypes, both the systemic and portal samples show higher levels of expression than preoperative levels, but this only reaches significance within the CD3+ subtype (preoperative vs. systemic and preoperative vs. portal $p < 0.05$ and $p < 0.05$ respectively). The CD8+ subtype showed the reverse, with both the systemic and portal samples having reduced levels of HLA-DR expression when compared to the preoperative level (this was not significant). In all 3 T-cell subtypes, the portal venous sample had slightly higher levels of HLA-DR expression than the systemic, but not significantly so.
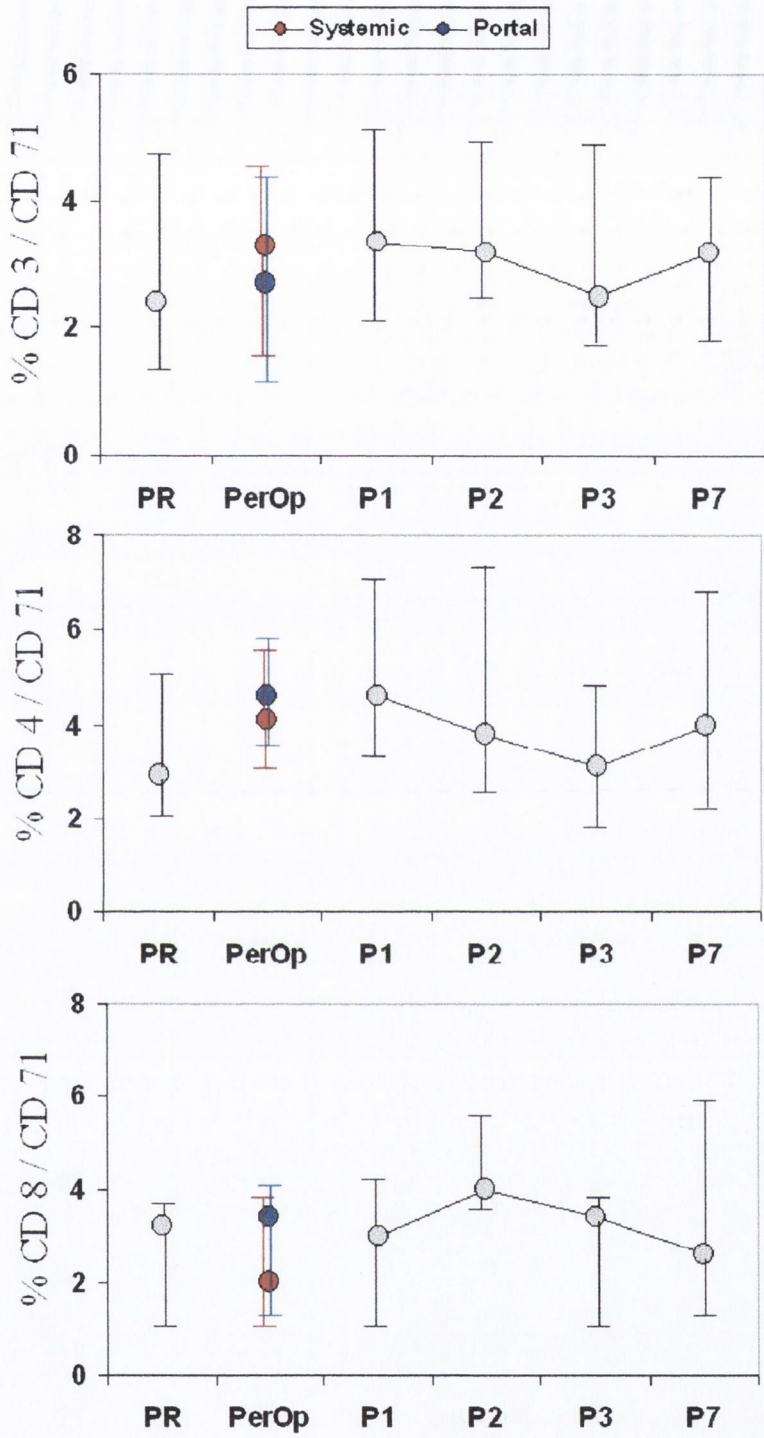
Graph 5.3   Intraoperative samples and T-cell CD71 expression
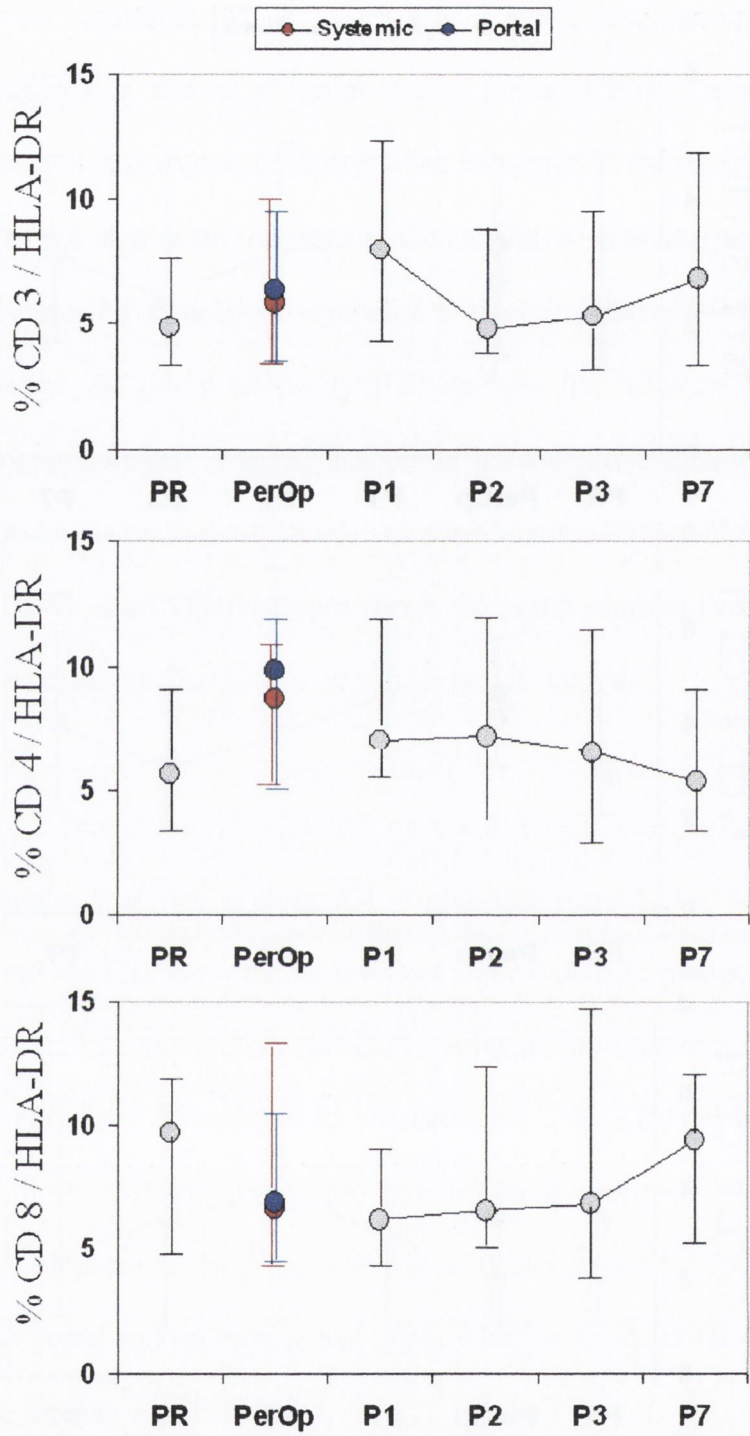
Graph 5.4　　　Intraoperative samples and T-cell HLA-DR expression

(ii) Monocyte activation and surface marker expression

The surface expression of HLA-DR on monocytes in pre- intra- and postoperative samples is shown in graph 5.5. Here, both the systemic and portal intraoperative samples are significantly reduced compared to the preoperative level of HLA-DR expression ($p<0.05$ and $p<0.001$, wilcoxin rank test respectively). The portal venous sample has slightly reduced expression as compared to the systemic sample, but this does not reach significance ($p=0.93$, wilcoxin rank test).
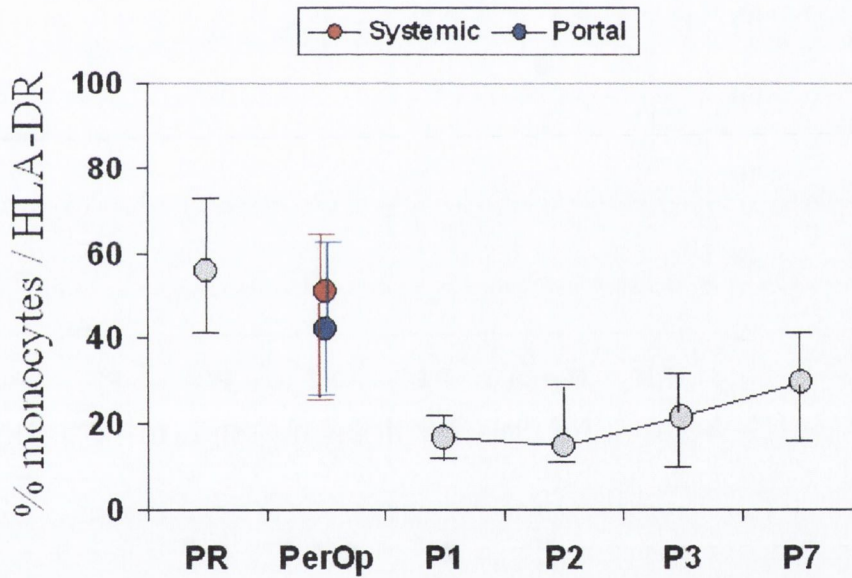
Graph 5.5   Intraoperative samples and monocyte HLA-DR expression

The expression of CD62-L on monocytes is shown in graph 5.6. Both systemic and portal levels of CD62-L expression are slightly elevated when compared to the preoperative value, but not significantly so. There was no difference in the level of expression between the two intraoperative samples, the levels being identical in the systemic and portal venous blood samples (% cells expressing HLA-DR: 68.6 [55.8 – 77.7] vs. 68.9 [51.7 – 77.2]).

(Systemic and portal values are superimposed)
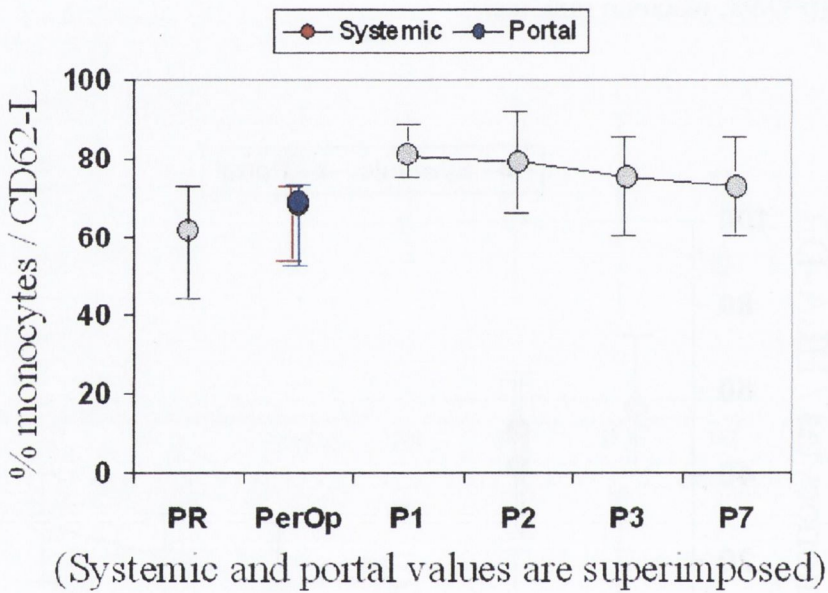
Graph 5.6   Intraoperative samples and monocyte CD62-L expression

(iii)   Neutrophil intranuclear NF-kappa B levels

The expression of neutrophil intranuclear NF-kappa B levels is shown in graph 5.7. The systemic samples have higher levels of intranuclear NF-kappa B expression when compared to the portal samples, but this is not significant. Both systemic and portal levels of intranuclear NF-kappa B expression are elevated when compared to the preoperative value, but only the systemic is significantly so (187% [137 – 222] of preoperative value, $p < 0.05$, wilcoxin rank test).
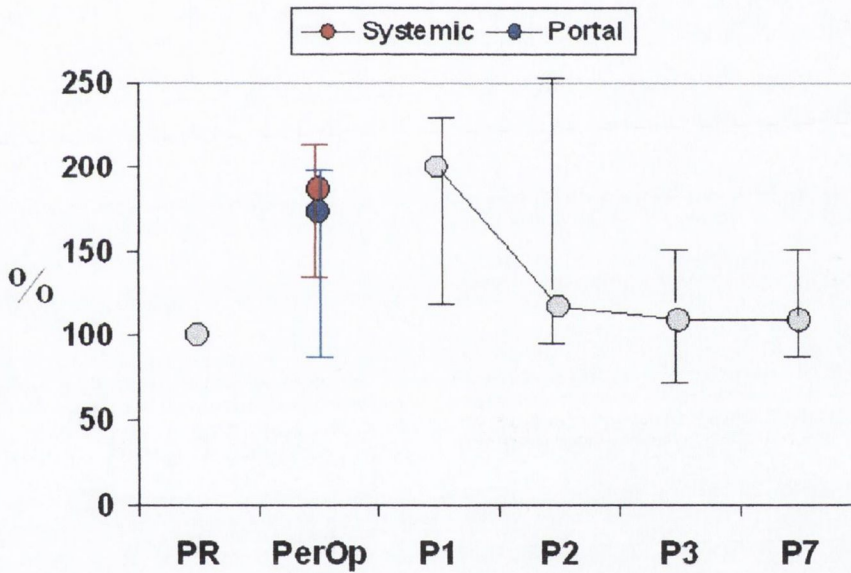
Graph 5.7   Intraoperative samples and Neutrophil intranuclear NF-kappa B

5.5   DISCUSSION

Analysis of intraoperative samples did show evidence of early immune activation. With regard to T cell function, there were significantly elevated levels of expression of CD69 on both systemic and portal venous samples when compared to the preoperative value. Expression of CD71 was likewise elevated, significantly so in the CD4+ subtype, again in both portal and venous samples, as was expression of HLA-DR in CD3+ T cells..

With regard to comparison of systemic and venous blood samples, small differences in T cell surface marker expression were visible. Expression of CD69 appeared to be higher in portal venous samples as compared to systemic, significantly so in the CD4+ subtype. This was, however, the only result of significance found. Indeed, the expression of CD71 appeared paradoxical; expression was higher in systemic samples in the CD3+ subtype, but both the CD4+ and CD8+ subtypes appeared to show slightly elevated levels of expression in the portal venous samples. HLA-DR expression on all T cell subtypes was consistently higher in portal venous samples, but only to a mild degree.

Examining monocyte function, no significant difference was seen with regard to portal and systemic venous samples. While portal venous blood samples appeared to have greater downregulation of HLA-DR expression than systemic samples, this was not significant; in addition, levels of CD62-L expression were identical in both intraoperative samples.

Analysis of neutrophil function suggested greater activation within the systemic circulation; both portal and systemic samples showed increased levels of intranuclear NF-kappa B expression when compared to preoperative values, with the systemic sample being significantly higher on statistical analysis.

As previously stated, there is as yet no hard evidence in human clinical studies to support the hypothesis of bacterial translocation via the portal vein. The failure to document this may be due to the fact that the systemic spread of bacteria and intestinally-derived immunoinflammatory mediators occurs mainly via the lymphatic route. In summary, while initial analysis of T cell and monocyte function in this chapter may show a trend towards earlier activation within the portal venous system, there is little statistically significant evidence and some results are contradictory. Further investigation with larger patient populations may determine whether this lack of significant data is due to type two error, or whether the theory of bacterial translocation via the portal vein is unsupportable by empirical evidence.

CHAPTER 6: NEOADJUVANT CHEMORADIOTHERAPY PRIMES THE PREOPERATIVE IMMUNE SYSTEM, ALTERING SUBSEQUENT POSTOPERATIVE OUTCOME AND IMMUNE PARAMETERS

6.1   INTRODUCTION

Oesophageal carcinoma is a highly aggressive malignancy with poor prognosis. Oesophagectomy remains standard treatment for patients with resectable oesophageal carcinoma, and although some series report long term survival rates in excess of 20%, median survival overall remains in the region of one year. Locoregional and systemic recurrences may develop after surgical resection alone, possibly as a result of the presence of micrometastatic disease at the time of diagnosis, which remains unaffected by surgical treatment. Chemotherapy has shown activity in advanced oesophageal carcinoma, particularly when used in conjunction with combination radiotherapy prior to surgical resection; this combination of neoadjuvant chemoradiotherapy followed by surgical resection is commonly referred to as 'multimodal' therapy. In addition, neoadjuvant therapy has been shown to be better tolerated than post-operative therapy (Heidecke et al, 2002; Swisher et al, 1996; Urschel et al, 2002).

Chemotherapy induces a transient, and possibly permanent, immune deficit in treated patients. Little information is available regarding the immunological effects of neoadjuvant chemoradiotherapy in patients undergoing multimodal treatment for oesophageal carcinoma. Preoperative or induction chemotherapy in patients undergoing resection for lung carcinoma has been shown to increase the risk of perioperative complications and lead to a demonstrable increase in IL-6 and GCSF production in the immediate postoperative period when compared to those who underwent surgical resection alone (Endo et al, 2004; Roberts et al, 2001).

With regard to assessment of cellular immune function, there is very little research to date documenting the postoperative leucocyte response in patients who have first undergone neoadjuvant therapy. Postoperative monocyte and granulocyte counts have been shown to be significantly lower in patients who have received neoadjuvant chemoradiotherapy prior to surgery, with evidence of significant depression of T cells, T helper cells, T suppressor cells and natural killer cells when compared to pretherapeutic values, persisting in the postoperative period (Heidecke et al, 2002; Wichmann et al, 2003).

With regard to splanchnic immune function, multiple studies have confirmed that intestinal permeability is increased following chemotherapy for a number of conditions, in both paediatric and adult populations. Similarly, radiation therapy has been shown to increase permeability in irradiated, as compared to nonirradiated, intestine. However, while these alterations in intestinal permeability following the administration of chemotherapy or radiotherapy are well documented, there is as yet no data documenting alterations in postoperative permeability in patients who have received neoadjuvant therapy prior to their surgery as compared to those who have not (Keefe et al, 1997; Kohout et al, 1999; Melichar et al, 2001; Nejdfors et al, 2000; Pledger et al, 1998).

6.2   AIMS OF STUDY

This chapter aims to examine the effects of neoadjuvant chemoradiotherapy on post-operative immune function. In particular, it examines the effect on postoperative acute phase response (as measured by C-reactive protein levels) and leucocyte responses, specifically T-cell and monocyte activation, and postoperative intestinal permeability.

6.3   PATIENTS AND METHODS

Twenty-seven patients were included in this section of the study, the same group of patients as previously described in section 3.3. Expression of the T-cell surface markers CD25, CD69, CD71 & HLA-DR were assessed by flow cytometry in all patients (section 2.3 & 2.4) as were the monocyte surface markers CD62-L and HLA-DR. Specimens were obtained preoperatively and on the first, second, third and seventh post-operative days. Serum samples were also obtained at these time periods for assessment of C-reactive protein levels and serum albumin measurements. Intestinal permeability was assessed in a further subgroup of thirteen patients.

In addition to this first group, a second group of 100 patients were also included for investigation in this chapter (group 2, section 2.1). Half of these patients received neoadjuvant chemoradiotherapy prior to their surgery. Data such as white cell count, albumin and clinical outcome measures were recorded retrospectively and are included in section 6.4.3 and 6.4.5 of this chapter (analysis of white cell count and albumin levels).

6.4   RESULTS

6.4.1   PATIENT DEMOGRAPHICS

Patient demographics for the first group of twenty-seven patients included in this chapter are described in detail in section 3.4.1 (including graph 3.1).

In addition to this first group, a second group of 100 patients were also included for investigation in this chapter (table 6.1). All underwent curative treatment for oesophageal carcinoma, fifty receiving neoadjuvant chemoradiotherapy prior to their surgery and fifty undergoing surgical resection alone.

No. of patients	100
Age (years)*	61.5 (53 – 68)*
Sex	
M	69
F	31
Treatment:	
Multimodal therapy	50
Surgery alone	50
*All values are medians (interquartile range)	

Table 6.1   Patient Demographics (Ch. 6, group 2)

6.4.2 C-REACTIVE PROTEIN

There was no difference in C-reactive protein levels before (PreMM) chemoradiotherapy or preoperatively (PR) in the multimodal group of patients (8.4mg/l [4.6 – 38] vs. 3.9mg/l [3.4 – 5.5], graph 6.1). Postoperatively, both groups of patients showed an increase in serum CRP levels. However, this elevation was more pronounced in those who received surgery alone, with the C-reactive protein levels then falling at one week. In those who had received neoadjuvant chemoradiotherapy, the postoperative elevation was still present, although not as high, and this elevation persisted throughout the study period. These differences between the two groups failed to reach statistical significance, but came close on the second postoperative day (p = 0.08, Mann-Whitney U test).
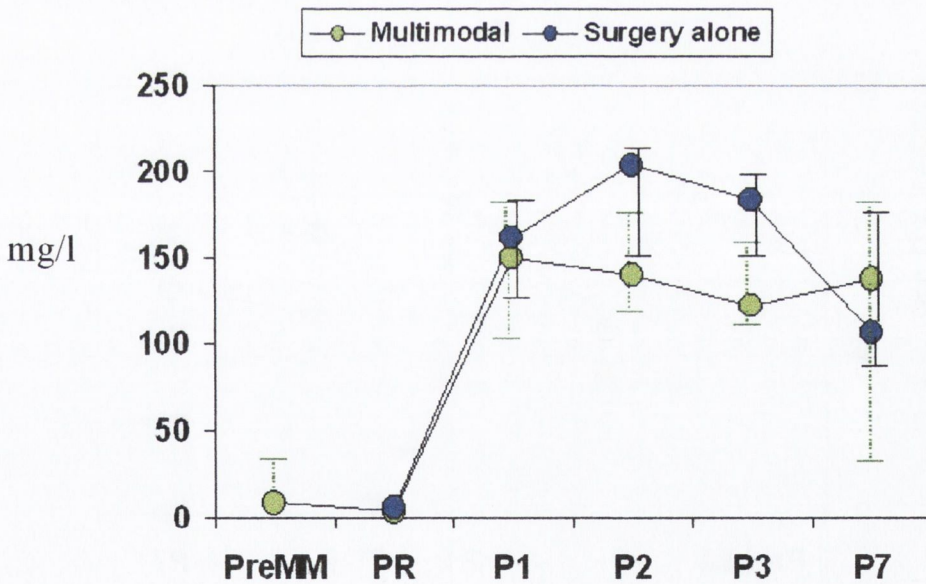
Graph 6.1   Multimodal therapy and C-reactive protein

6.4.3   POST-OPERATIVE LEUCOCYTE ACTIVATION

In order to assess absolute white cell counts, laboratory results were gathered retrospectively from the second group of 100 patients as described in section 2.1. Looking at patients who had received neoadjuvant multimodal therapy, there was a significant fall in absolute white cell count when their initial white cell count was compared to that taken after completion of their chemoradiotherapy (6.95 x 10^9 cells/l [5.6 – 7.7] vs. 4.75 x 10^9 cells/l [3.8 – 5.9], p<0.0001, wilcoxin rank test). When comparing the two groups, those who received multimodal therapy and those who had surgical treatment alone, those who underwent chemoradiotherapy have a significantly lower absolute white cell count at all time periods, both pre- and postoperatively.
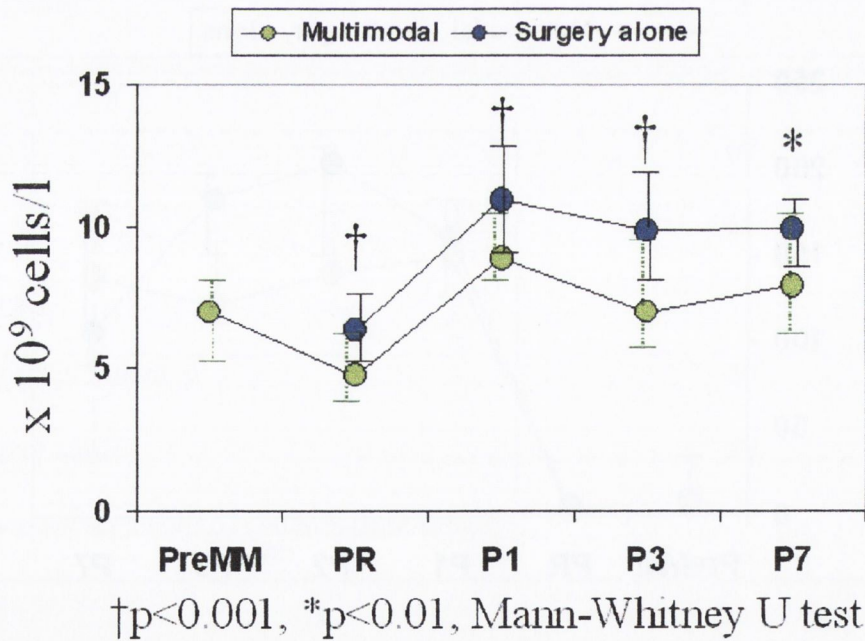
Graph 6.2   Multimodal therapy and white cell count

(i)   T cell activation and surface marker expression

In this section, the expression of the T-cell activation markers CD25, CD69, CD71 and HLA-DR on CD3+, CD4+ and CD8+ T-cells was investigated, comparing levels of expression in those who received multimodal therapy (neoadjuvant chemoradiotherapy followed by surgery, n=5) and those who received surgical treatment alone (n=22). With regard to expression of CD25 (Graph 6.3), there was a marked elevation in CD25% expression within the CD3+ subtype following neoadjuvant chemotherapy in patients who received multimodal therapy (% cells expressing CD 25: 10.1 [6.5 – 14.1] and 17.2 [11.9 – 19.6] respectively). This elevation persisted postoperatively at all time periods studied, the CD25% levels being notably higher in this group when compared to levels in those patients who had received surgery alone, significantly so on the second and seventh postoperative days (p<0.05, Mann-Whitney U).

When CD25 expression within the CD4+ subtype was examined, there were elevated levels of CD25 in the multimodal group as compared to those patients who had surgery alone, which were significantly higher at all of the time periods studied, both pre- and postoperatively (p<0.05, Mann-Whitney U). The same pattern was present again in the CD8+ subtype, with higher levels of expression present in the multimodal group, but this did not reach significance on statistical analysis.
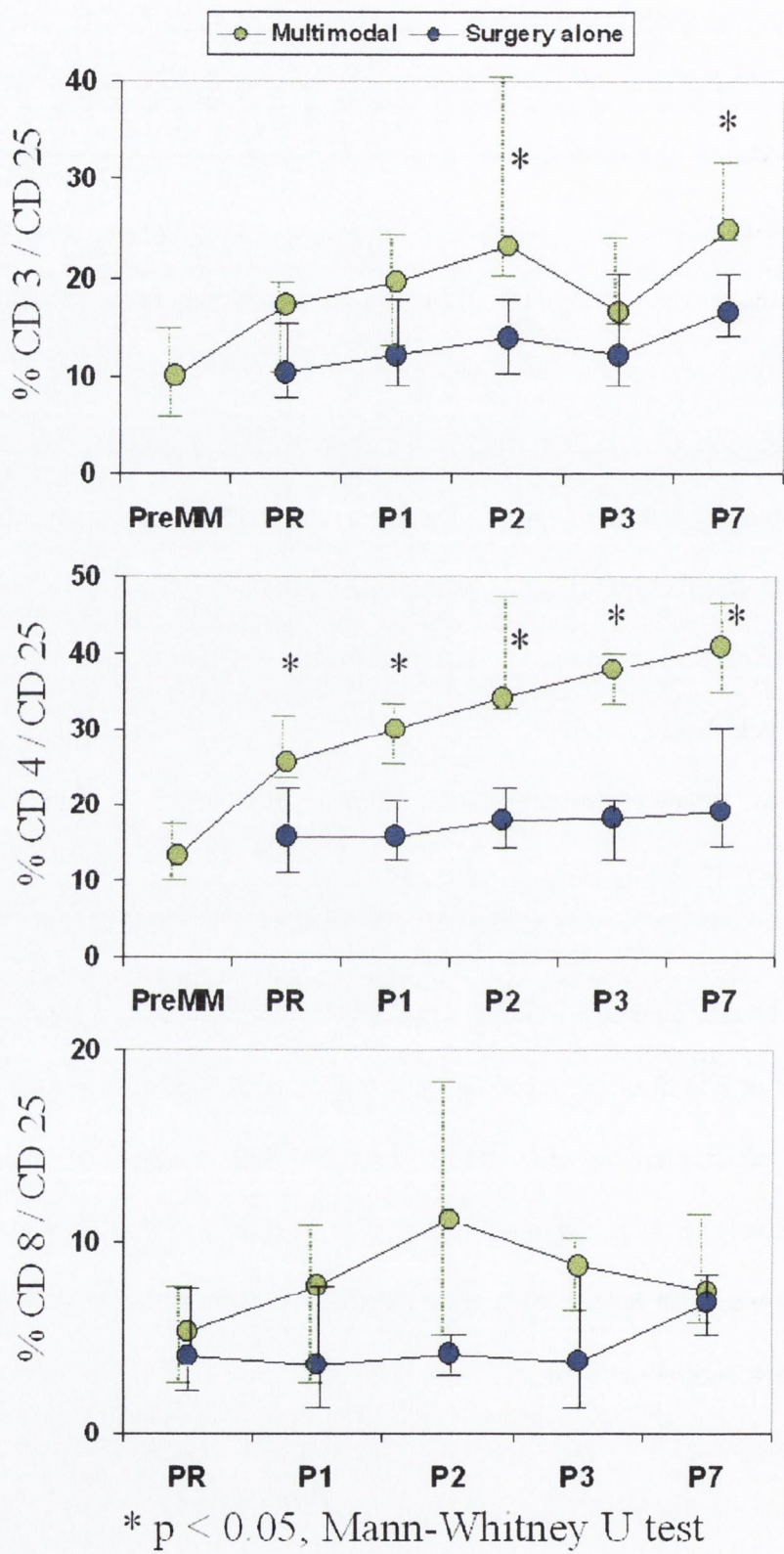
Graph 6.3   Multimodal therapy and T-cell CD25 expression
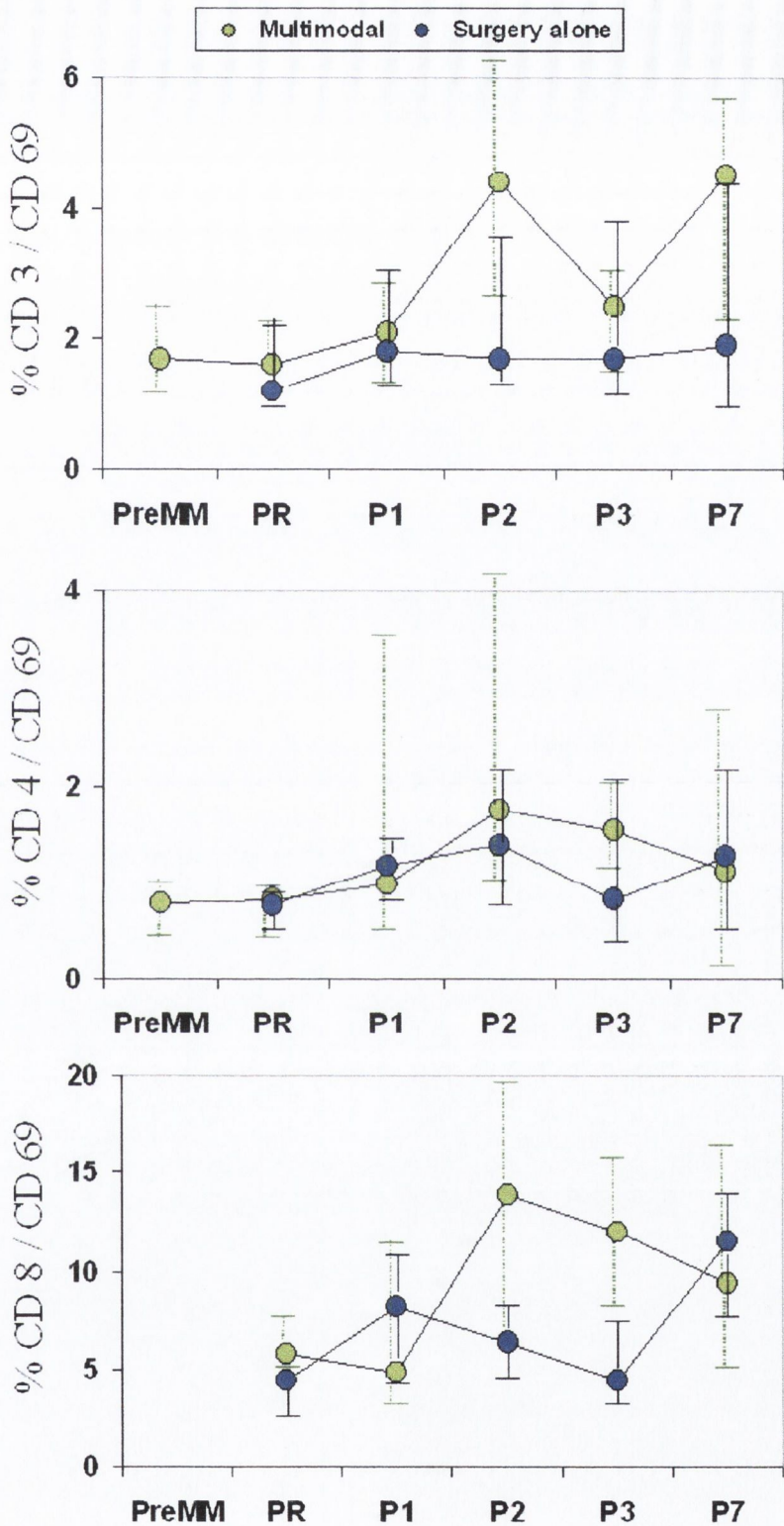
Graph 6.4   Multimodal therapy and T-cell CD69 expression

T-cell expression of CD69 was then assessed (graph 6.4). While there appeared to be no immediate elevation in levels of expression prior to chemoradiotherapy and after completing this treatment in the multimodal group, there were some differences in levels of expression between the two groups evident within the postoperative period. Those who had multimodal therapy had higher levels of CD69 expression evident in the CD3+ subset on the second, third and seventh postoperative days, but these differences failed to reach significance.

Analysis of the CD4+ subtype again showed a small difference in the level of CD69 expression between the two groups; those who received preoperative chemoradiotherapy having slightly higher levels of expression, but this failed to reach statistical significance. The CD8+ subtype was similar, showing higher levels of expression in the multimodal group on days 2 and 3 postoperatively, but these results were not significant on analysis.

The surface expression of CD71 on the 3 T-cell subtypes in multimodal patients and those who received surgery alone is shown in graph 6.5. In the CD3+ subtype, a rise in CD71 expression was observed following neoadjuvant chemoradiotherapy in the multimodal group (% cells expressing CD 71: 1.6 [1.5 – 4] vs. 5.4 [2.1 – 5.3] respectively, p=0.1, Mann-Whitney U test). Patients in the multimodal group have higher levels of CD71 expression both pre- and postoperatively, but this does not reach significance in this T-cell subtype (p=0.1 preoperatively and on days 1 and 2 postoperatively, Mann-Whitney U test).

When the CD4+ subtype is examined, however, these differences are significant. Expression of CD71 was very significantly elevated preoperatively and on the first postoperative day in the multimodal group as compared to patients who had surgery alone (p<0.01, Mann-Whitney U test). This elevated level of expression in multimodal patients was also significant between groups on the second day and at one week postoperatively (p<0.05, Mann-Whitney U test).

† p<0.01, * p<0.05, Mann-Whitney U test
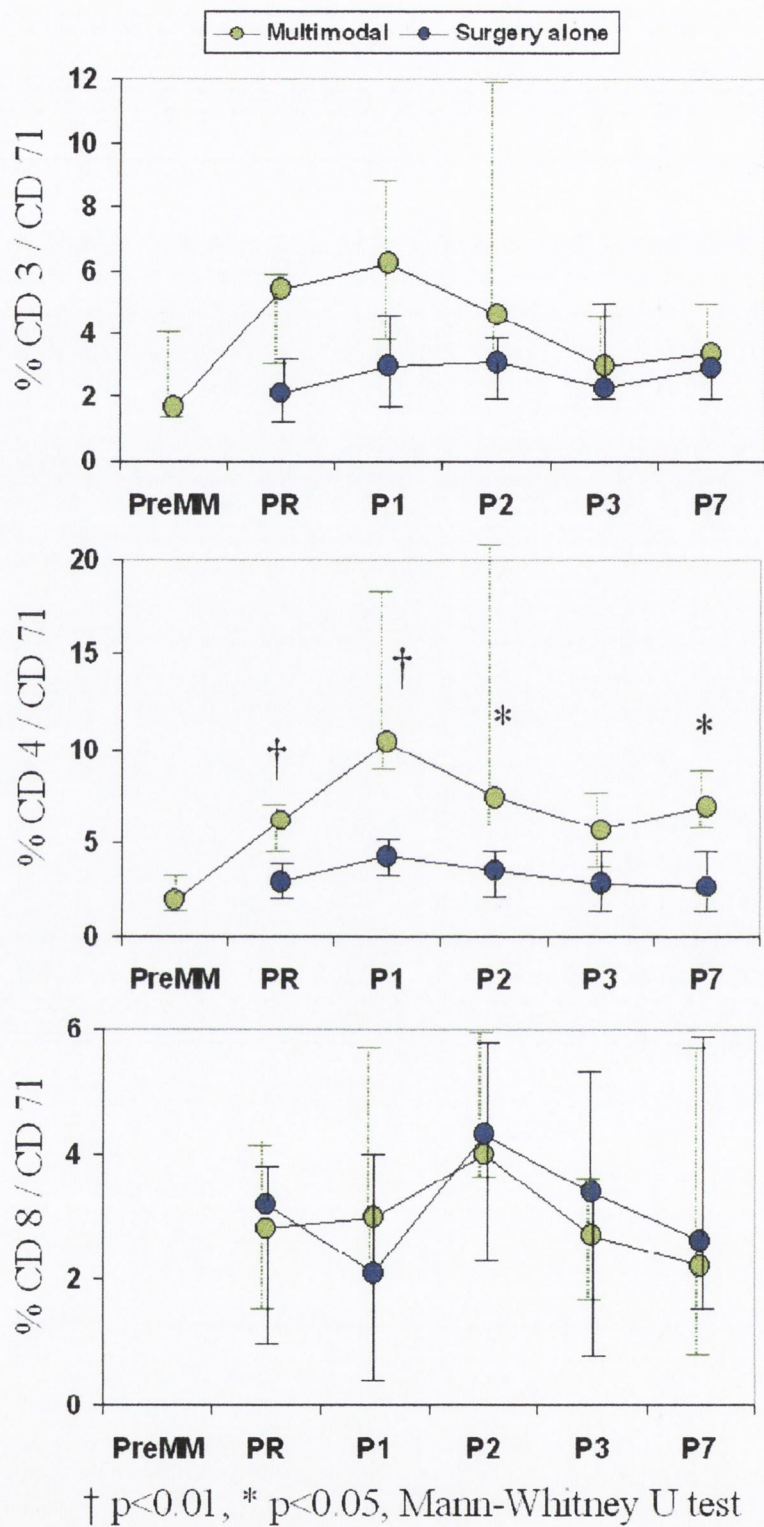
Graph 6.5   Multimodal therapy and T-cell CD71 expression
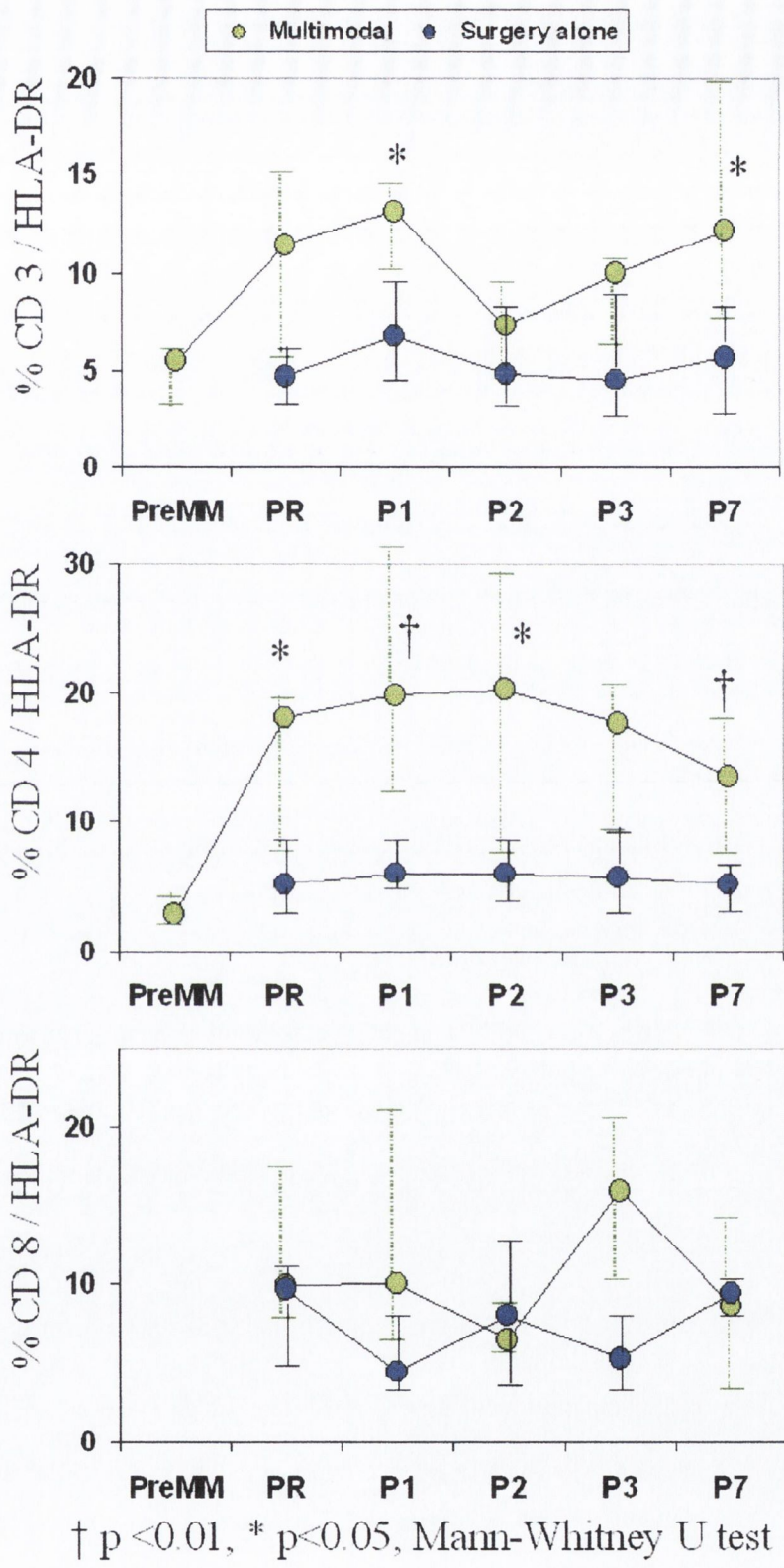
Graph 6.6   Multimodal therapy and T-cell HLA-DR expression

The surface expression of HLA-DR on the 3 T-cell subtypes in multimodal patients and those who received surgery alone is shown in graph 6.6. In the CD3+ subtype, multimodal patients showed evidence of a marked rise in expression following neoadjuvant chemoradiotherapy (p=0.1, wilcoxin rank test). Comparing the two groups of patients, those who received multimodal therapy had higher levels of HLA-DR expression throughout the study period. The difference in expression between these 2 groups was very marked preoperatively, but just failed to reach significance (p=0.06). The difference between groups did, however, reach significance on the first and seventh postoperative days (p<0.05, Mann-Whitney U test). In the CD4+ subtype, multimodal patients again showed evidence of a marked rise in expression following neoadjuvant chemoradiotherapy (p=0.1, wilcoxin rank test). The difference in expression in this subtype was even more marked than that seen previously within the CD3+ population of T-cells. The multimodal group had a significantly higher preoperative level of HLA-DR expression as compared to the surgery alone group (% cells expressing HLA-DR: 18.2 [7.8 – 20] vs. 5.1 [2.8 – 8.8] respectively, p <0.05 Mann-Whitney U test). This difference between groups persisted in the postoperative period. Expression was higher in the multimodal group at all time periods, significantly so on day 1, day 2 and day 7 postoperatively (p<0.01, <0.05 and <0.01 respectively Mann-Whitney U test), while just failing to reach significance on day 3 (p=0.06, Mann-Whitney U test). There was no difference in expression preoperatively in the CD8+ subtype. Levels of expression were slightly higher in the multimodal group on day 1 and day 3 postoperatively, but these were mild in comparison to the differences seen in the other T-cell subtypes and failed to reach significance on analysis.

(ii)   Monocyte activation and surface marker expression

Monocyte expression of HLA-DR in multimodal patients and those who received surgery alone is shown in graph 6.7. Here, patients within the multimodal group had a reduction in monocyte HLA-DR expression following chemoradiotherapy, but this did not reach statistical significance. Both groups of patients had the expected postoperative depression in HLA-DR expression (as previously described in section 3.4.3 [ii]). This depression was slightly more pronounced in patients who had received multimodal therapy on day 3 and at one week, but did not reach significance (p=0.5 and p=0.9 respectively).
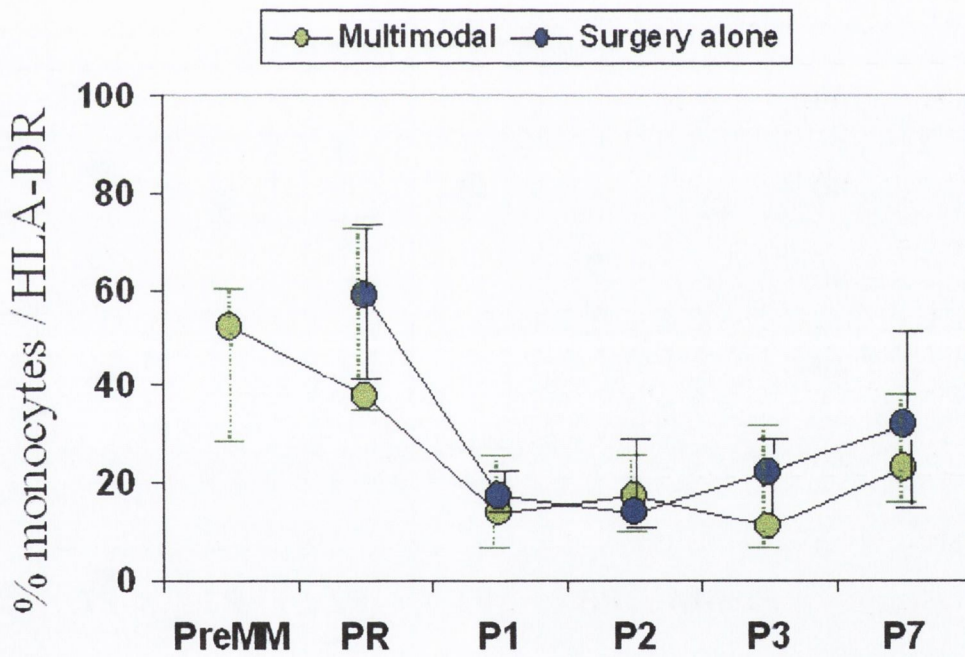
Graph 6.7   Multimodal therapy and monocyte HLA-DR expression

Monocyte expression of CD62-L in multimodal patients and those who received surgery alone is shown in graph 6.8. Again, there appears to be a reduction in CD62-L expression following chemoradiotherapy within the multimodal groups of patients, but this was not significant. Postoperatively, all patients experienced a rise in CD62-L expression, as would be normally expected (section 3.4.3.[ii]). This rise was more pronounced in patients who received surgical treatment alone particularly at one week postoperatively, but the difference in expression between the 2 groups failed to reach significance (% cells expressing CD62-L at one week: 84 [59 – 89] vs. 65 [55 – 69], p=0.09, Mann-Whitney U test).
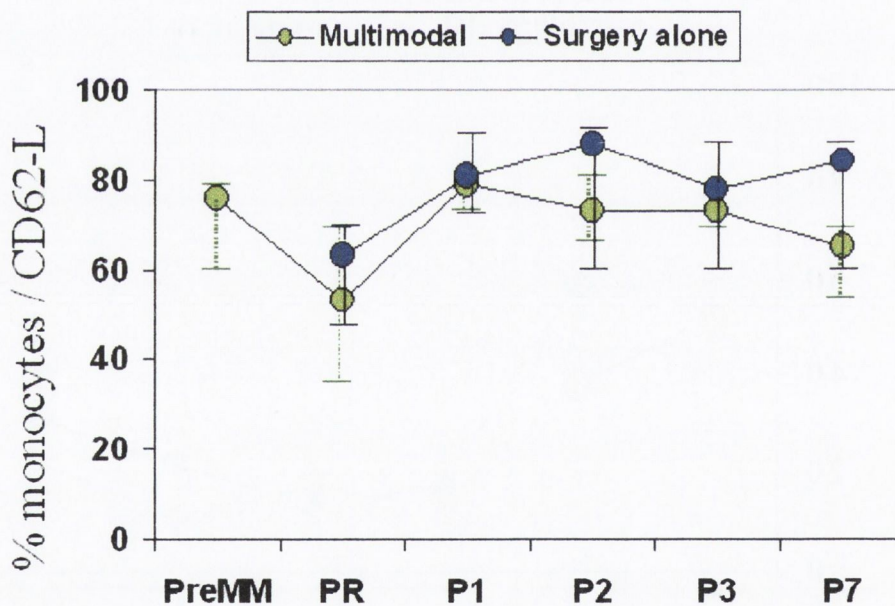
Graph 6.8   Multimodal therapy and monocyte CD62-L expression

6.4.4   POST-OPERATIVE INTESTINAL PERMEABILITY

Intestinal permeability preoperatively and on postoperative days 1 and 7 is shown in graph 6.9, comparing patients who received multimodal therapy and those who received surgical treatment alone. There was no significance alteration in intestinal permeability following chemoradiotherapy in the multimodal group (LMER: 0.028 [0.026 – 0.073] vs. 0.031 [0.022 - -0.066]). In the postoperative period, patients who underwent surgery alone had a more marked elevation in their lactulose:mannitol ratio, which reached significance on statistical analysis when compared to their preoperative value (LMER: 0.018 [0.014 – 0.031] vs. 0.331 [0.119 – 0.335], $p<0.05$, wilcoxin rank test). Patients in the multimodal group also had an elevation in their lactulose:manitol ratio on the first post-operative day, but this was not as high and failed to reach significance ($p=0.06$, wilcoxin rank test). Direct comparison was then made between the two groups; although median permeability was higher in those who had surgery alone on the first postoperative day as compared to those who had multimodal therapy, this was not significant ($p=0.8$, Mann-Whitney U test).

*- significant rise compared to pre-operative level.
p < 0.05, Wilcoxin Rank Test
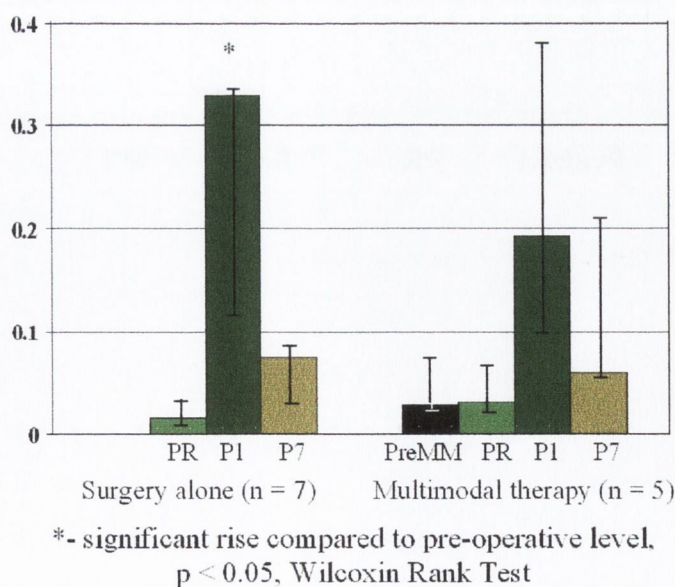
Graph 6.9   Multimodal therapy and intestinal permeability

6.4.5 ALBUMIN

In order to assess albumin levels, laboratory results were gathered retrospectively from the second group of 100 patients as described in section 2.1. As can be seen in graph 6.10, there was no difference evident in serum albumin levels in the multimodal modal group when their pre-multimodal samples were compared to preoperative levels. In addition, the serum albumin levels between the two groups (multimodal therapy and surgery alone) were identical at all time periods.
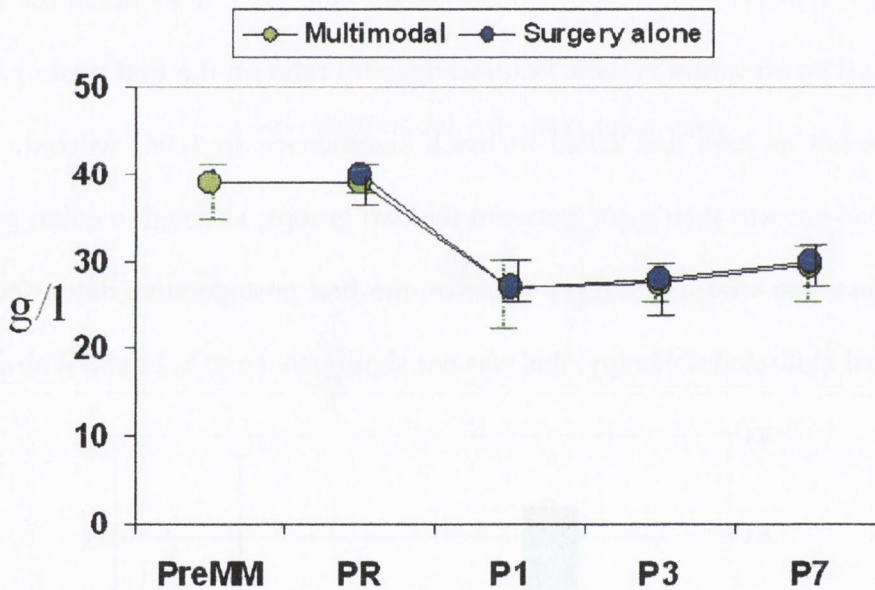
Graph 6.10   Multimodal therapy and serum albumin

6.5   DISCUSSION

There were no significant differences found between the two groups of patients with regard to serum albumin levels. As previously discussed in section 6.1, multiple studies have found evidence of an increase in intestinal permeability following chemotherapy, in both paediatric and adult populations. Paradoxically, this thesis demonstrated a greater increase in postoperative intestinal permeability in patients who did *not* undergo preoperative chemoradiotherapy. While this failed to reach statistical significance on analysis, the trend towards higher permeability in those patients who underwent surgery alone is contrary to what one would have anticipated from previous research. It is possible that the data have resulted in a type 1 error, due to the small number of patients included in this section of the study, but this is a result that certainly warrants further investigation.

This study confirmed, however, that neo-adjuvant chemoradiotherapy had a notable impact on leucocyte proliferation and function in patients who received multimodal therapy. Chemoradiotherapy resulted a significant reduction of absolute white cell counts following treatment; this was evident in samples taken from the multimodal patients prior to their subsequent surgery postoperative specimens showed persistently lower white cell counts when compared to patients who underwent surgical therapy alone.

In contrast to previous studies, there was little alteration in innate immune function following neoadjuvant chemoradiotherapy, as assessed by monocyte function and activation. However, significant alteration in T cell function was observed, as assessed by expression of activation markers, with visible evidence of phenotypic priming and activation of T lymphocytes following neoadjuvant chemoradiotherapy.

Within the CD3+ T cells, expression of CD25, CD71 and HLA-DR was elevated following chemoradiotherapy. The observed rises were not significant on analysis, but this may be due to the small sample size involved (five multimodal patients were included in this study). These levels were persistently elevated when compared to pre- and postoperative levels in patients who had received no other treatment prior to their surgery.

When expression of these activation markers in the CD4+ subtype was then examined, significant elevation was evident in the expression of CD25, CD71 and HLA-DR on CD4+ T lymphocytes. Expression of each of these activation markers was significantly higher preoperatively in patients who had received neoadjuvant chemoradiotherapy as compared to those who underwent surgical resection alone. In addition, postoperative levels of expression were also significantly elevated for the duration of the study period.

It has been established in clinical studies that patients with advanced carcinomas of the oesophagus exhibit a higher postoperative morbidity and mortality following neoadjuvant chemoradiotherapy. The two-hit hypothesis of SIRS/MODS proposes that an initial insult to the immune system then results in "priming" of resident leucocytes. Any subsequent insult, such as infection, would lead to an amplified or an exaggerated immune response.

This research demonstrates evidence of phenotypic priming and activation of the CD4+ T-cell subpopulation of patients receiving neoadjuvant chemoradiotherapy. This immune dysfunction in theory heightens the consequences of a "second-hit" in the postoperative period and increases the risk of perioperative sepsis or systemic inflammatory response syndrome. Manipulation of T cell responses may provide a novel approach for future therapies in complications that arise following multimodal treatment.

CHAPTER 7: CONCLUSIONS

7.1 THE EXPERIMENTAL PROCESS

This work was initiated in order to further explore the mechanisms by which postoperative or posttraumatic immune responses may predispose to immune dysregulation in the form of the systemic inflammatory response syndrome or multiple organ dysfunction syndrome. Improvements in laboratory techniques and ongoing development of new commercially available antibodies enable us to gain ever more detailed information about specific immune responses. It must be recognized, however, that specific cellular responses within a laboratory setting may not directly reflect clinical responses in human populations.

Additionally, when undertaking human-based studies numbers may be limited, particularly when performing such a large number of specialized immunological investigations. The strict inclusion criteria used in these experiments may have limited the size of the study groups, as did the amount of time which was necessary to invest in each individual patient. The findings outlined here, nonetheless, provide a basis for prospective studies of research directed toward more specific areas, which would allow for larger population recruitment.

7.2   CONCLUSIONS REACHED

Multiple organ dysfunction syndrome (MODS) refers to a condition that has become recognized in the last three decades as a major cause of surgical morbidity and mortality. It is estimated that it is responsible for 50-80% of all surgical intensive care deaths. Although MODS was first described over 20 years ago, treatment for this condition is still supportive and mortality rates have remained relatively unchanged during this time. Despite the high percentage of ICU deaths attributable to this condition, much of our treatment to date remains supportive (Deitch, 1992; Moore et al, 1996). With this research, the aim was to examine two of the main hypotheses in current literature regarding the development of SIRS and MODS in postoperative surgical populations.

As previously stated, gut injury and gut barrier failure have been considered to be likely contributors to the development of SIRS and subsequent distal organ injury; this theory is central to the gut hypothesis. Simply stated, reduced splanchnic perfusion causes ischaemic damage to the mucosa and disrupts the physiological gut barrier; intestinal permeability is increased and translocation of bacteria and endotoxins from the gut can occur, resulting in activation of the systemic immune system. This hypothesis of bacterial translocation can provide an explanation as to why no septic focus can be found in 30% of bacteraemic MODS patients or how patients may develop SIRS in the absence of infection or another recognizable cause (Deitch, 1992; Fiddian-Green, 1988; Reynolds, 1996; Saadia, 1995; Swank et al, 1996; Vatner, 1974).

Assessment of splanchnic perfusion, by tonometric pHi measurement, as a method of predicting clinical outcome has been validated in a number of clinical studies. Under normal conditions, the splanchnic vascular bed receives approximately 25% of cardiac output from the heart. During haemorrhage or shock, a decrease in splanchnic blood flow occurs, resulting in mucosal hypoxia, which is then accompanied by a fall in mucosal pH. While the many ICU studies examining gastric pHi in critically ill patients use a pHi of 7.35 or 7.32 as a predictor of clinical complications, there is some variance as to the cut-off level used in the literature examining pHi levels intraoperatively. This thesis concurs with previous intraoperative studies, demonstrating that patients undergoing major surgical intervention or procedures experience a fall in intestinal blood flow intraoperatively, as reflected by a fall in gastric pHi; only one of our patients maintained their pHi at a 'normal' level throughout the procedure. With regard to the predictive value of intraoperative gastric pHi, a significant correlation was demonstrated between low intraoperative pHi measurements and subsequent development of septic complications in the post-operative period. Other studies have shown that a fall in pHi is also predictive of outcomes such as postoperative complication rate, mortality and anastomotic breakdown following upper gastrointestinal surgery (Björck et al, 1994; Poeze et al, 2000; Roumen et al, 1994; Tarui et al, 1999).

While analysis of clinical outcome measures as described above was included as part of this research, the main focus was on examining the effect of splanchnic hypoperfusion on post-operative gut and cellular immune responses, in order to understand the mechanism by which this hypoperfusion results in adverse clinical outcomes.

It has long been postulated that episodes of hypovolaemic ischaemia and splanchnic vasoconstriction compromise the integrity of the gut barrier function. Ischaemia of the gut may occur from low flow (haemorrhage or shock, as mentioned above), interference in arterial inflow and, less commonly, from an obstruction to venous outflow. Low flow may occur in conditions such as cardiogenic or hypovolaemic shock. Decrease in mucosal perfusion results in tissue hypoxia as oxygen is shunted away from the villous tip by the "counter-current" exchange. This hypoxia is accompanied by anaerobic metabolism and a reduction in intramucosal pH level. The extent of the necrosis is dependant on the degree and duration of the ischaemia, but can develop within minutes of the onset of hypoxia, disrupting the physiological gut barrier. This thesis demontrated that a reduced gastric intramucosal pH was associated with a significant rise in intestinal permeability on the first post-operative day, with levels returning to normal by the seventh post-operative day. Previous research has also demonstrated this relationship between intraoperative intramucosal acidosis and post-operative intestinal permeability, but found that there was some overlap in results between those with a normal gastric pHi and those who did not (Sinclair et al, 1995).
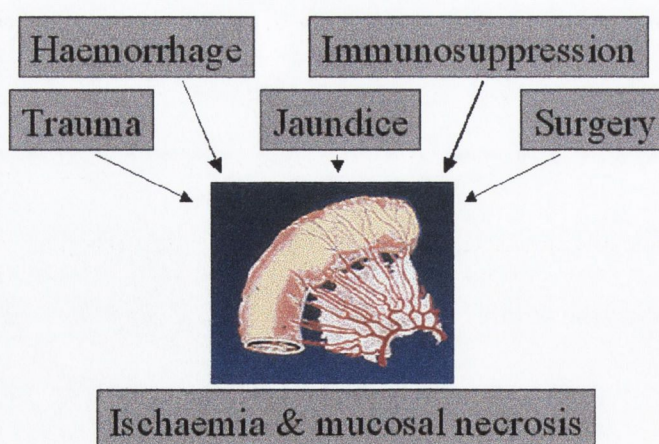
Figure 7.1   Causes of intestinal ischaemia

As previously stated, there is increasing evidence supporting the paradigm that a reduction in blood flow occurs during trauma such as major surgery, causing damage to the mucosal barrier. How does this damage to the gut mucosa then cause systemic immune activation? The original concept of bacterial translocation from the damaged gut has been central to the gut hypothesis for many years; damage to the mucosa disrupts the physiological gut barrier, intestinal permeability is increased and translocation of bacteria and endotoxins from the gut can occur, resulting in activation of the systemic immune system. By what route then, does this translocation occur?

Originally, it was thought that the translocating bacteria and endotoxin reached the systemic circulation via the portal vein and that cytokines produced by stimulated Kupffer cells contributed to the systemic septic state. However, studies examining this theory have not found sufficient supporting evidence to prove this hypothesis. While some animal studies have shown evidence of portal bacteraemia, there is as yet no hard evidence in human clinical studies to support the hypothesis of bacterial translocation via the portal vein. Clinical studies in trauma patients have to date failed to find bacteria in the portal vein of patients undergoing laparotomy, although cytokine studies suggest higher concentrations of IL-6 within the portal circulation, as compared to those in systemic venous samples, following cross-clamping of the aorta during aneurysm surgery (Baigrie et al, 1993; Moore et al, 1991; Pogetti et al, 1992; Swank et al, 1996).

The investigation performed of cellular immune activation in this thesis also found little evidence to support the portal venous system as a route by which systemic immune activation then occurs. While initial analysis of T cell and monocyte function in this study may show a trend towards earlier activation within the portal venous system, there is little statistically significant evidence and some results are contradictory.

The phenomenon of bacterial translocation and the gut hypothesis of MODS have subsequently been reevaluated in the last 5 years, and recent studies have instead examined the role of the intestinal lymphatic system in the development of lung injury and MODS. The gut-associated lymphoid tissue (GALT) is the largest in the body; there is more organized lymphoid tissue in the gut than in the rest of the body as a whole. Translocation of bacteria to the mesenteric lymph nodes has been demonstrated in a number of animal studies. Factors that have been shown to induce translocation include haemorrhagic shock, intestinal obstruction, obstructive jaundice and thermal injury. Evidence regarding the presence of bacteria in human mesenteric lymph nodes following insult is limited, but translocation has been documented in patients with intestinal obstruction, Crohn's disease and those undergoing laparotomy. However, translocating bacteria are rarely found in any other tissues, so how is a systemic response propogated (Ambrose et al, 1984; Deitch et al, 1989 & 1990; Reynolds et al, 1995; Sedman et al, 1994)?

It now appears that the same insults that cause intestinal mucosal injury and promote bacterial translocation, including shock and trauma, are also able to induce the gut and its associated lymphoid tissue to produce cytokines and other inflammatory mediators. Animal studies have shown conclusively that mesenteric lymph obtained following experimentally induced haemorrhagic shock primes circulating neutrophils and contributes

to lung injury. Furthermore, it has been shown that ligation of the mesenteric lymph ducts prior to induction of trauma can prevent or alleviate both this neutrophil activation and lung injury. Early human studies also show evidence that mesenteric lymph activates endothelial cells in vitro. The exact factors contained within the lymph that are responsible for these responses remain to be determined; it seems that bacteria and endotoxin themselves are not the direct mediators as the mesenteric lymph appears to be sterile, with only low levels of endotoxin present (Chen et al, 2004; Gonzalez et al, 2001 & 2003; Magnotti et al, 1998 & 1999).

In summary, the current gut hypothesis supposes that bacteria and endotoxin cross the mucosal barrier following intestinal injury and mucosal necrosis, where they come into contact with enterocytes and immune cells. Most are phagocytosed, with only a small number escaping from the intestine to become subsequently trapped within mesenteric lymph nodes. The bacteria and endotoxin do cause an intestinal immune response, resulting in the production of pro-inflammatory mediators which then enter the mesenteric lymphatics, traveling via the thoracic duct and entering the systemic circulation. Some researchers have postulated that this may be why the respiratory system is generally the first organ to be affected in MODS; the lung is the first vascular bed to be exposed to mesenteric lymph after it enters the left subclavian vein by means of the thoracic duct (Deitch, 2002; Magnotti et al, 1999).

Mucosal necrosis

Bacteria/endotoxin cross
the mucosal barrier

Arteriole

Lymphatic
vessel

Venule

Activation of GALT –
Pro-inflammatory
Cytokine production

Mediators enter thoracic duct,
then the systemic circulation
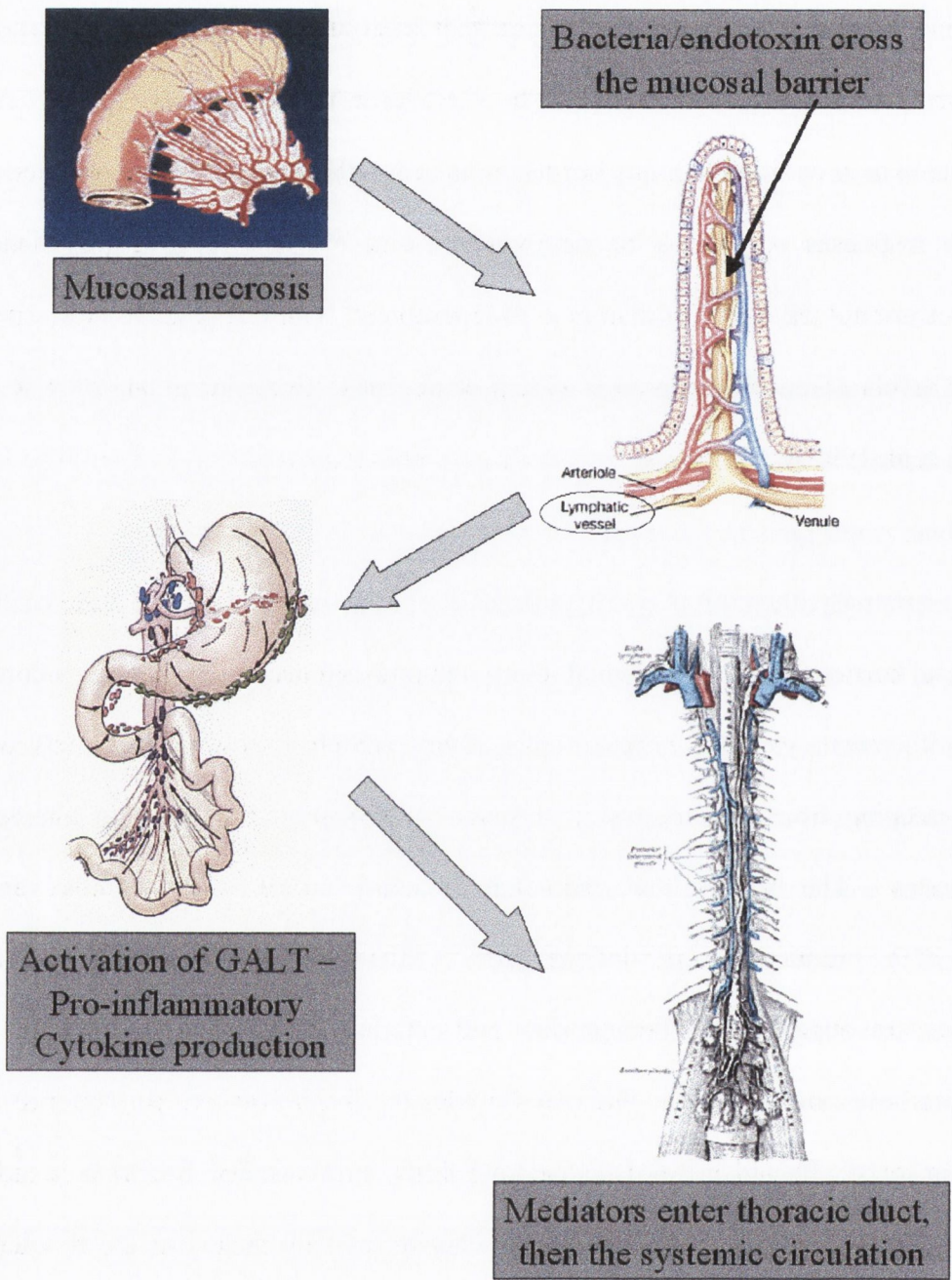
Figure 7.2   The gut hypothesis

In conclusion, this study has found evidence to support the current gut hypothesis of MODS. It has been demonstrated that patients undergoing major surgery have interruption or reduction in intestinal blood flow intraoperatively, reflected by a fall in gastric pHi and that this fall is associated with a subsequent increase in post-operative morbidity. Furthermore, it has been shown that this is also associated with detectable alteration in both local and systemic immune responses; those with reduced intraoperative pHi have increased intestinal permeability, elevation in systemic acute phase markers and alteration in monocyte activation markers both intraoperatively and in the postoperative period.

The second of the hypotheses studied in this research was the 'two-hit' hypothesis. The two-hit hypothesis, also referred to as cellular priming, states that the host's immune system is primed by an initial insult so that its response is amplified or altered by a subsequent insult ('second-hit'). This may result in either a constructive adaptive response, for example improved tolerance to injury in myocytes, or a destructive maladaptive response (i.e. an exaggerated inflammatory response in neutrophils). It has been established in clinical studies that patients with advanced carcinomas of the oesophagus exhibit a higher postoperative morbidity and mortality following neoadjuvant chemoradiotherapy. Little information is available regarding the exact effects of neoadjuvant chemoradiotherapy on the immune system, but is known that chemotherapy itself induces a transient, and possibly permanent, immune deficit in treated patients. Preoperative or induction chemotherapy in patients undergoing resection for lung carcinoma has also been shown to increase the risk of perioperative complications; is this attributable to cellular priming (Endo et al, 2004; Roberts et al, 2001)?

This study found that neo-adjuvant chemoradiotherapy had a notable impact on overall leucocyte counts and activation of specific subsets in patients who received multimodal therapy. Chemoradiotherapy resulted in reduction of absolute white cell counts following treatment, present prior to their subsequent surgery and persisting throughout the postoperative period. In addition, there was evidence of phenotypic priming and activation of the CD4+ T-cell subpopulation following chemoradiotherapy, which again continued throughout the postoperative period studied. This confirms the results of earlier studies, which have also found reduction of overall cell counts of T lymphocytes and T helper cells following chemoradiotherapy, in addition to evidence of increased T cell expression of HLA-DR. With regard to functional studies, it appears that while these T cell populations have unchanged cytokine production profiles, the usual proliferative response following exposure to superantigen is significantly reduced. This immune dysfunction may put patients at an additional risk for sepsis or delayed healing after undergoing subsequent major surgery (Heidecke et al, 2002; Wichmann et al, 2003).

7.3 POTENTIAL CLINICAL APPLICATIONS

This work demonstrates some areas which may be potential targets for improving patient outcome following major surgery. Firstly, as reduction in splanchnic blood flow during surgery has a demonstrable effect on postoperative immune function and clinical outcome measures, pHi monitoring in patients undergoing complex major surgery may enable early prediction of those at risk of postoperative complications. Of even greater interest, some researchers are now investigating the effectiveness of pHi-directed therapy, commencing ionotropic support in order to correct low pHi in the intensive care setting; whether this may benefit surgical patients in response to intraoperative hypoperfusion has yet to be studied.

Secondly, this research demonstrates that in addition to the more easily measurable effects of chemoradiotherapy on the immune system, specific 'priming' of T-cells occurs, particularly of the CD4+ population. Therapeutic strategies to overcome this may be as simple as delaying surgical treatment in at-risk patients until T-cell function has returned to normal. Some authors' have begun to suggest that a 2-stage approach, with oesophagectomy and delayed reconstruction, could be introduced for high risk patients, or those receiving neoadjuvant chemoradiotherapy. The results of these studies will bear close examination, as this would be an alteration of current standards of care. Abrogation of T-cell function has been demonstrated *in vitro* (i.e. by CTLA-4 Ig); there is a danger, however, that this method may not be specific enough for use in a clinical setting as it has the potential for immunosuppression, placing the patient at risk for sepsis.

7.4   FUTURE STUDIES

1. Trial to address pHi-directed maintenance and monitoring in patients undergoing complex major surgery.

2. Modification of the method used for analysis of neutrophil NF-kappa B; while this research was performed, the only appropriate antibody available for flow cytometric analysis was polyclonal, giving rise to some limitations in data analysis. Recently, a monoclonal antibody specific to the p65 subunit of NF-kappa B has become commercially available, which would be more appropriate for use.

3. Investigation of T-cell responses during chemoradiotherapy and monitoring the subsequent restoration of normal function and activation following cessation of treatment.

4. Further investigation of intestinal permeability, both prior to and following chemoradiotherapy in patients receiving multimodal therapy. Comparison of postoperative alterations in intestinal permeability should then be made between this group of patients and those who undergo surgical resection alone.

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