The Human Response to Endotoxin

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Declaration

I, Robert Charles Meredith Stephens confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Many patients undergoing *high-risk* major surgery suffer complications. Previous studies have shown that patients with low IgM Endotoxin Core Antibodies (EndoCAb) are more likely to have a complication, for relatively unknown reasons. Studying EndoCAb directly is difficult as it is a polyclonal group of antibodies. This thesis explored the relationship between EndoCAb and different measures of outcome using samples from previously conducted clinical trials and a series of basic laboratory studies. A pilot human volunteer study was conducted to try to improve ways of assessing the response to an endotoxin challenge.

An observational study on adults undergoing 1st time Coronary Artery Bypass Grafting was conducted using samples from a previous trial to see if the association between outcome and low EndoCAb IgM extended to this comparatively *low-risk* group and to examine whether this was clearly mediated by an exaggerated inflammatory response. Patients with low EndoCAb IgM had more complications, but had *less* interleukin-6 at 6 hours.

To assess the relationship between low EndoCAb IgM and other antibodies a series of studies was conducted to examine the hypothesis that EndoCAb IgM is part of a wider 'natural IgM antibody group'. Blood donors with low EndoCAb IgM had lower concentrations of the 'natural IgM antibodies'. Furthermore, EndoCAb IgM and the 'natural IgM antibody group' had similar temporal patterns and were present at low concentrations in some umbilical cord blood.

To further understand the clinical relevance of EndoCAb, the association between EndoCAb and the systemic inflammatory response syndrome was retrospectively measured in critically ill children undergoing another clinical trial. Low EndoCAb IgG was associated with early development of the systemic inflammatory response syndrome. The difficulty in determining which factors affect the response to an inflammatory challenge led me to develop a new low-dose endotoxin human volunteer model: one in which there is the largest *range* of change, variability or dispersion in inflammatory markers.

Chapter 1 Introduction

Chapter 2 Methods

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Commonly used Symbols and Abbreviations

bpm	Beats per minute
B cell	B lymphocyte
rBPI	Recombinant Bactericidal/Permeability-Increasing protein
^{o}C	Degrees Centigrade (Celsius)
CABG	Coronary artery bypass graft
CD5, CD19 etc	Cluster of Differentiation antigen 5, 19 etc
CpG	Cytosine Phosphate Guanine
CRP	C reactive Protein
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EndoCAb	Endotoxin core antibody
EuroSCORE	European System for Cardiac Operative Risk Evaluation
FACS	Fluorescent-activated cell sorting
$Fi0_2$	Fraction of inspired Oxygen
^{o}F	Degrees Fahrenheit
gal	Galactose alpha(1,3) Galactose
HDL-C	High-Density Lipoprotein Cholesterol
IABP	Intra-aortic balloon counterpulsation pump
IgA, IgG, IgM	Immunoglobulin A, G or M Isotype
IL-6, 10	Interleukin 6, 10
kg	Kilogramme
LPS	Lipopolysaccharide
LBP	Lipopolysaccharide Binding Protein
L	Litre
μg, mg	Microgramme, Milligramme
mmHg	Millimetres of Mercury
mAb	Monoclonal Antibody
$Pa0_2$	Partial pressure of arterial Oxygen
$PaC0_2$	Partial pressure of arterial Carbon Dioxide
PE	Phycoerythrin
pg	Picogramme
sIgM	Secreted Immunoglobulin M Isotype
SIRS	Systemic Inflammatory Response Syndrome
TLR-4	Toll-like receptor 4
TNFα	Tumour Necrosis Factor alpha
WCC	White cell count

The following publications form part of the work presented in this thesis

Baxendale HE, Johnson M, Stephens RC, Yuste J, Klein N, Brown JS and Goldblatt D (2008). Natural human antibodies to pneumococcus have distinctive molecular characteristics and protect against pneumococcal disease. *Clinical and Experimental Immunology* **151**, 51

Stephens RCM, Fidler K, Wilson P, Barclay GR, Mythen M, Dixon GLJ, Turner MW, Klein NJ and Peters MJ (2006). Endotoxin immunity and the development of the systemic inflammatory response syndrome in critically ill children. *Intensive Care Medicine* **32**, 286

Stephens RCM, O'Malley CMN, Frumento RJ, Mythen MG and Bennett-Guerrero E (2005). Low Dose Endotoxin Elicits Variability In The Inflammatory Response In Healthy Volunteers. *Journal of Endotoxin Research* **11**, 207 *Withdrawn at the request of Columbia Presbyterian Hospital, New York, USA*

Stephens RCM, Dixon G, Rashid M and Mythen MG (2005). Anti-endotoxin therapies: where are we now? *Netherlands Journal of Critical Care* **9**, 20

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Work

A thesis involving over 845 subjects necessarily needed collaborations. In addition to the previous acknowledgments I wish to record my gratitude to patients, relatives, volunteers and colleagues for allowing me to collaborate, use their data or samples and to be transparent about my own role.

Chapter 2

Myself: Assay optimisation

Chapter 3

Myself: EndoCAb measurement, data analysis
Clinical Data: Clinical staff at The Heart Hospital, UCLH and Mr Mark Curtis
Blood samples: Dr David Brull, Prof Hugh Montgomery and Dr Jules Sanders
EndoCAb assay assistance: Mr Mohammed Rashid

Chapter 4

Myself: Antibody measurement, blood donor sample collection and analysis
Hybridoma Monoclonal Antibodies: Dr Helen Baxendale
EndoCAb assay assistance and blood donor collection: Mr Mohammed Rashid and Dr Mala Siva
Mother and Child blood samples: Dr Jilly Lloyd

Chapter 5

Myself: EndoCAb measurement, analysis
MBL levels: Dr Katy Fidler
Clinical data: pICU nurses, Dr Mark Peters and Dr Katy Fidler

Chapter 6

Myself: Cytokine measurement, analysis and study design (USA and UK)
Clinical data: Dr Catherine O'Malley, Mr Rob Frumento
Blood Samples: Dr Catherine O'Malley, Mr Rob Frumento
Study design and conduct: Dr Elliott Bennett-Guerreo

Aims of the thesis

Studies since the 1990's have found that low EndoCAb IgM levels were associated with poor postoperative outcomes in patients having major surgery.
On reading the original studies I had a few doubts. How could an antibody be responsible for altering outcome after surgery? Was there a systematic error not yet considered by the previous authors or perhaps other factors were responsible– maybe EndoCAb was a marker of general antibody levels?
I felt it was important therefore to re-confirm the original associations in an observational study. Then I *intended* to attempt to look at the association to see if the hypothesised mechanism - an exaggerated inflammatory response in those patients with low EndoCAb IgM - was present. In the end I have had to be flexible in response to new different experiments being required and (chapter 6) research fraud by a co-worker. In each chapter I describe to what degree I was able to achieve my original aims. I chapter 7 I set out a summary of the thesis including how successfully I was able to meet my original objectives.

Chapter 1

Introduction

1.1 Endotoxin

The first use of the term endotoxin was reportedly used by Richard Pfeiffer in 1904, referring to 'bacterial products, not secreted, firmly fixed to the bacterium, but potentially set free if the bacteria undergoes lysis' (Rietschel and Cavaillon 2003). Today, after 100 years of research, we now use the word endotoxin to mean a specific chemical, lipopolysaccharide (LPS). Endotoxin is a vital part of the structure and function of the outer cell membrane of all natural gram-negative bacteria. Luderitz and Westphal first elucidated of the it's structure in the 1950's by using acid treatment to give the first carbohydrate-free portion of endotoxin, called lipid A (Neter *et al* 1956). Subsequently, as mutants lacking the major part of the O-polysaccharide portion of the molecule (called 'rough mutants' as the colony morphology has a rough appearance) were found to retain biological activity, it was clear that the lipid portion contained the majority of endotoxin's effects (Tanamoto *et al* 1984).

1.1.1 Endotoxin structure

Endotoxin has 3 structural parts: a hydrophobic lipid-A region, inner and outer core sugars which share broadly similar structures in most Gram-negative bacteria and a variable O-polysaccharide chain specific to the particular bacterial species, summarised in table 1.1 and figure 1.1 (Erridge *et al* 2002a). It is possible (though unproven) that endotoxin released into the human circulation may comprise both smooth and rough forms because of incomplete synthesis, organise into micelle-like formations or be associated with other membrane components (Erridge *et al* 2002a; Poxton 1995). The chemical method of endotoxin purification may also alter the structure.

	Structure	Basic Chemical units	Conserved	'Function'
O polysaccharide	0-20 repeating units. May be absent	1-8 sugars per unit	No: almost limitless. Varying sugars, sequences, chemical linkage and substitutions eg over 160 *E coli* o-serotypes	Antigenic; prevents membrane attack complex, fixes complement
Outer core	0-7 sugars	More common sugars such as hexoses and hexosamines	Relatively conserved e.g. only 5 *E coli* core structures (R1–R4 and K12)	Unknown
Inner core	1-7 sugars; Kdo almost always present	Unusual sugars such as 'Kdo' and 'Hep'	Relatively conserved e.g. only 5 *E coli* core structures (R1–R4 and K12)	Unknown
Lipid A	Disaccharide attached to acyl groups	Number and lengths of acyl chains and the state of the disaccharide's phosphate both major determinants of toxicity	Acyl groups conserved in species, but vary between species in nature, number, length, order and saturation. E.g. *E coli* has only one lipid A structure	Majority of toxicity

Table 1.1: Summary of the sub-structures of endotoxin

Abbreviations: Kdo, 3-deoxy-D-manno-octulosonic acid; Hep, L-glycero-D-manno heptose. Hexoses include glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine

1.1.2 Endotoxin: a potent stimulant of inflammation

Both *in-vitro and in-vivo,* endotoxin is a potent stimulant of inflammation. With co-factors including CD14 and lipopolysaccharide binding protein (LBP) endotoxin binds to Toll-like receptor 4 (TLR-4), one of a family of transmembrane proteins expressed on key cells including macrophages, endothelial cells and neutrophils which recognise 'pathogen-associated molecular patterns' as part of the innate immune response (Medzhitov *et al* 1997). TLR-4 ligation initiates a complex series of intra-cellular signalling events (involving MyD88, IRAK, TRAF6, and NF-κB) resulting in the production of pro-inflammatory mediators including cytokines and adhesion molecules (McGettrick and O'Neill 2010).

When injected into animals, or in small quantities (less than 4 ng/kg) into human volunteers, endotoxin causes an inflammatory reaction similar to the systemic inflammatory response syndrome (Suffredini *et al* 1999). Human volunteers experience symptoms such as headache, chills, myalgia, nausea, fatigue, and general malaise as well as cardiovascular, respiratory, gastrointestinal, immune and haematological effects (Suffredini *et al* 1999). For ethical reasons, because of the larger harmful doses required, there is no good direct evidence (apart from a case report and studies using very subtle measures) that endotoxin administration results in overt organ dysfunction in humans (Taveira da Silva *et al* 1993; Kumar *et al* 2004). Animals administered sufficient endotoxin however suffer significant cardiac, respiratory, renal and hepatic dysfunction (Albertini *et al* 2002; Astiz *et al* 1991; Collin *et al* 2004; Fink *et al* 1987; Gullichsen *et al* 1989; Pittet *et al* 2000; Stitt and DuBois 2001; Tsao *et al* 2004).

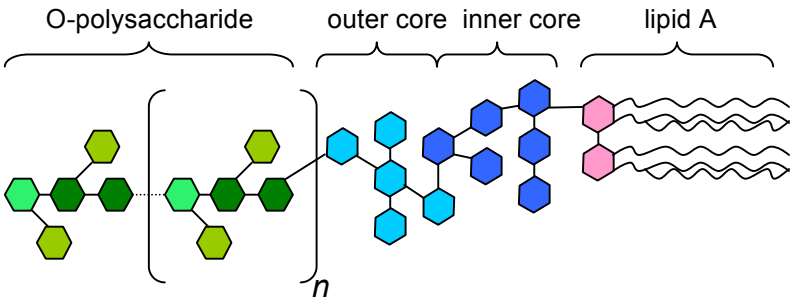

Figure 1.1 Diagrammatic representation of the structure of endotoxin
Different colours represent the different sugars that comprise endotoxin's structure.

1.1.3 Endotoxin and outcome

Overall, endotoxin concentrations in blood are only weakly associated with outcome in septic patients, but endotoxin studies in sepsis are difficult for several reasons (Hurley 2003; Opal 2002). Firstly, endotoxin is not easy to quantify, has a short half-life and sample contamination can be a problem. A newer test for endotoxin activity that detects neutrophils' respiratory burst activity after priming with endotoxin and an anti-endotoxin antibody (the 'endotoxin activity assay') may clarify this (Marshall *et al* 2002; Valenza *et al* 2009). Secondly, the process of sepsis often starts hours or even days before signs become manifest, so the inflammatory cascade has amplified greatly by the time the investigator has access to the patient. Lastly, many different factors are likely to influence outcome, and studies do not always measure all substances thought to comprise the natural anti-endotoxin defences (see later), or other modifying factors such as host genotype. In certain circumstances (such as Meningococcal disease) there is evidence of the link between endotoxin concentrations and severity of illness and even (in Meningococcal disease) outcome (Brandtzaeg *et al* 1989; Marshall *et al* 2004; Valenza *et al* 2009).

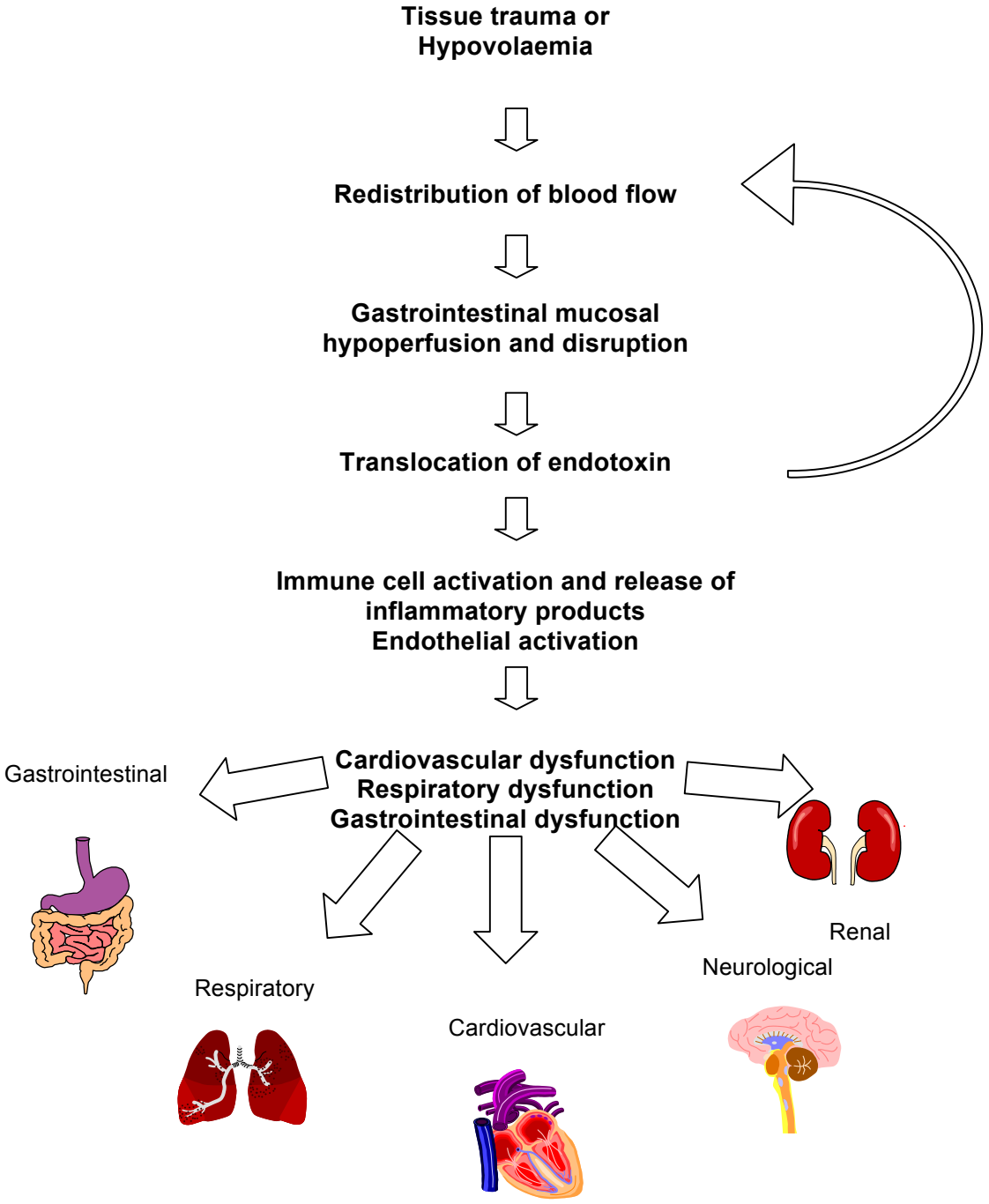

Figure 1.2 Summary hypothesis model of how hypovolaemia may result in organ dysfunction.

Besides sepsis, as mentioned above, there is merit in the hypothesis that endotoxin from the gastrointestinal tract is one of the triggers of the systemic inflammatory response syndrome, which may lead to organ dysfunction after surgery, trauma and burns. This is based on several observations, which at best amount to circumstantial evidence:

- Firstly, the gastrointestinal tract contains a large amount of endotoxin (as part of natural commensal gram negative bacteria) and is therefore an important source of endotoxaemia. These bacteria place them at potential risk of exposure through intestinal leakage of either viable gram-negative cells, dead gram-negative cells, or shed free endotoxin. Endotoxin may enter the bloodstream through an intestinal mucosa damaged by hypoperfusion during periods of hypovolaemia, trauma or physiological stress (Andersen *et al* 1993; Fink and Delude 2005; Lau *et al* 2000; Martinez-Pellus *et al* 1997; Oudemans-van Straaten *et al* 1996; Soong *et al* 1994).

- Secondly, systemic endotoxaemia occurs in many patients undergoing major surgery (Andersen *et al* 1993; Aydin *et al* 2003; Berger *et al* 1995b; Berger *et al* 1995a; Boelke *et al* 2000; Bolke *et al* 2001a; Bouter *et al* 2002; Foulds *et al* 1997; Lau *et al* 2000; Lequier *et al* 2000; Martinez-Pellus *et al* 1993; Myles *et al* 1996; Neuhof *et al* 2001; Oudemans-van Straaten *et al* 1996; Riddington *et al* 1996; Rothenburger *et al* 2001; Soong *et al* 1994). The endotoxin concentrations are also greater if the trauma is greater, for example, more invasive surgery or surgery involving cardiopulmonary bypass for reasons not fully understood (Aydin *et al* 2003; Bolke *et al* 2001a; Lau *et al* 2000).

- Complications following surgery are usually remote from the surgical site (e.g. cardiac, respiratory, renal etc) and are not necessarily directly related to a surgical ‘mistake’ or to the presence of preoperative organ dysfunction (Bennett-Guerrero *et al* 1999; Grocott

et al 2007). This is consistent with the results of endotoxin on animals (Albertini *et al* 2002; Astiz *et al* 1991; Carpati *et al* 1992; Collin *et al* 2004; Fink *et al* 1987; Gullichsen *et al* 1989; Pittet *et al* 2000; Tang *et al* 1998; Tsao *et al* 2004; Yao *et al* 1992).

- Intravenous endotoxin can trigger inflammation in volunteers, of a similar nature and timing (but not degree) to that occurring following surgery in both humans and animal models (Ayala *et al* 1991; Elin *et al* 1981; Hammond and Potgieter 1996; Plebani *et al* 1999; Rothenburger *et al* 2001; Suffredini *et al* 1999; van Berge Henegouwen *et al* 1998).

- Several studies have reported positive associations between peak endotoxin concentrations during major surgery or pancreatitis and outcome although these have not been consistent findings (Andersen *et al* 1987; Aydin *et al* 2003; Berger *et al* 1995b; Bolke *et al* 2001b; Donnelly *et al* 1994; Foulds *et al* 1997; Hoch *et al* 1993; O'Malley *et al* 2004; Rothenburger *et al* 2001; Soong *et al* 1994; Valenza *et al* 2009).

- Reducing the bacterial load (i.e. reducing the pool of bacteria available to generate endotoxaemia, Selective Digestive Decontamination) with oral antibiotics that have a low tendency to systemic absorption has been associated with a reduction in postoperative complications in some studies (Silvestri *et al* 2007; Stoutenbeek *et al* 2007; Zandstra *et al* 2010).

1.2 Natural defences against endotoxin

As there is evidence that endotoxin is one of the causes of postoperative organ dysfunction, it makes sense to review the substances comprising our natural defences against endotoxin. Endogenous factors normally found in healthy human serum comprise the body's natural defence against exposure to endotoxin. These factors may bind to and neutralise endotoxin thereby

minimising endotoxin mediated systemic inflammation. They include antibodies to endotoxin, high-density lipoprotein cholesterol (HDL-C), bactericidal/permeability-increasing protein (BPI) and some antimicrobial peptides (Beamer *et al* 1998; Poxton 1995; Ulevitch *et al* 1979; Warren *et al* 2003). There is some less convincing data that other factors such as albumin, complement components and lysozyme may also play a role in the neutralisation of endotoxin. Of course other *synthetic* theraputic approaches using endotoxin binding polymyxin columns, lipid A analogues such as E5564 and specific targets in the endotoxin signaling pathway such as CD14 have been tried (Cruz *et al* 2009; Iwagaki *et al* 2000; Tidswell *et al* 2010).

1.2.1 High Density Lipoprotein-Cholesterol

Lipoproteins in plasma can reduce the activity of endotoxin (Kitchens *et al* 2003). Intravenous recombinant High Density Lipoprotein Cholesterol (HDL-C) improves survival in animal models of sepsis (Goldfarb *et al* 2003; Levine *et al* 1993). Surgical patients with low HDL-C concentrations have more interleukin 6 (IL-6) release and more surgical infections, mainly attributable to gram-negative organisms (Bonville *et al* 2004; Delgado-Rodriguez *et al* 1997; Fujita *et al* 2001). When volunteers are given intravenous endotoxin, reconstituted HDL-C greatly reduces the release of tumour necrosis factor alpha (TNFα) IL-6, and interleukin 8 (IL-8), while only modestly attenuating the secretion of 'anti-inflammatory' cytokines interleukin 1 receptor antagonist (IL-1ra), soluble TNF receptors and interleukin 10 (IL-10) (Pajkrt *et al* 1996). Several lipid emulsions (such as GR270773) made from plant lipids were in clinical stages of development after animal studies showed improved survival in sepsis but this did not translate into benefit in humans (Dellinger *et al* 2009; Goldfarb *et al* 2003; Gordon *et al* 2003; Winchell *et al* 2002).

1.2.2 Bactericidal/Permeability-Increasing Protein

Bactericidal/permeability-increasing protein (BPI) is naturally released from activated neutrophils, can reduce the binding activity of endotoxin and is cytotoxic for gram-negative bacteria (Elsbach and Weiss 1998; Giroir *et al*

1997). It is protective in animal models of sepsis whilst in human volunteers it reduces the cardiovascular changes and cytokine concentrations associated with endotoxin administration (De Winter *et al* 1995; Von der Mohlen *et al* 1995). Two large interventional studies used a recombinant fragment of BPI, $rBPI_{21}$ but were judged by a variety of criteria not to be successful (Demetriades *et al* 1999; Levin *et al* 2000; Wiezer *et al* 2001; Wu *et al* 2003). This may have been due to an unexpected reduction in mortality in the placebo control group, underpowering the study, or because a significant proportion of the deaths occurred before the complete course of $rBPI_{21}$ had time to be given.

1.2.3 Lactoferrin

Lactoferrin, a non-haeme iron-binding transferrin glycoprotein is found in neutrophil granules and most mucosae. Whilst it's exact mechanisms are yet unknown, it reduces endotoxins effects (such as oxidative damage) in animal models, is bactericidal and bacteriostatic and inhibits the progression of the systemic inflammatory response syndrome (SIRS) into sepsis in endotoxaemic mice (Bellamy *et al* 1992; Kruzel *et al* 2002; Kruzel *et al* 2010; Zagulski *et al* 1989).

1.3 Antibodies

Antibodies (immunoglobulins) are gamma globulin glycoproteins that are found in vertebrate body fluids. Traditionally considered part of the 'adaptive' immune system (in contrast to the 'innate' that also includes macrophages, natural killer, dendritic and mast cells) they identify and neutralize foreign antigens, such as bacteria and viruses (table 1.2) (Delves and Roitt 2000).

1.3.1 Antibody Structure

Antibody monomers consist of two large heavy chains and two small light chains (figure 1.3). The whole structure can be combined to form dimers with two units or pentamers with five units. Each 'tip' of the antibody (Fragment antibody binding, Fab) has one constant and one variable region: the variable

regions from the heavy and light chains together are sometimes called Fv regions or domains as their variety gives rise to the huge (variable) repertoire in paratopes – the area at the end of the Fab domain that binds an antigen's epitope (see fig 1.3) (Jerne 1955). Structures in the paratope end of each variable heavy and light chain domains that bind to a specific antigen are referred to as complementary determining regions.

'Innate'	'Adaptive'
Non specific response	Specific response to antigen
Often triggered by repeated patterns	Triggered by specific antigen structure
Quick maximal response	Delay between exposure and maximum response
Primitive- some aspects are found across most of life	Found only in vertebrates
No memory response	Exposure results in memory

Table 1.2 Summary of the differences between the innate and adaptive aspects of the immune system.

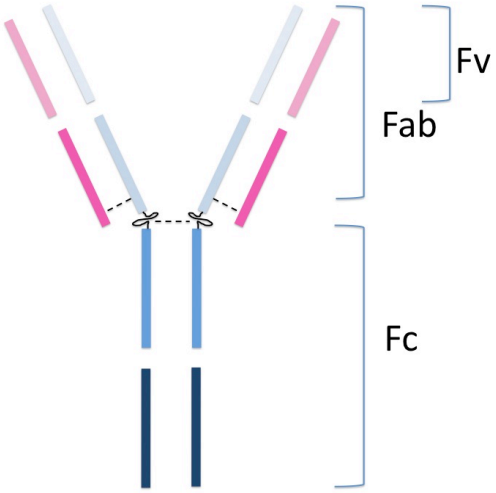

Figure 1.3 Schematic structure of an antibody. Fab: fragment antigen binding; Fc: fragment crystallizable, Fv: fragment variable. The heavy chains are represented in blue and consist of 4 regions (VH, CH1, CH2, CH3), whilst the light chain is represented in pink and consists of 2 regions (VL and CL). The variable parts of an antibody referred to above are its V regions, and the constant part is its C region. Papain digestion yields two Fab fragments and one Fc fragment (Delves and Roitt 2000).

Structural variety in the heavy and light chains' variable domains comes from several mechanisms: combining different genes (VDJ for heavy chains or VJ for light chain combinations), somatic hypermutation in variable domain genes and selection of those combinations that result in antibodies with high affinity (affinity maturation) (Jerne 1955).

Mammals have five different antibody isotypes or classes differing in the constant (C) regions of the immunoglobulin heavy chain (see table 1.3). Each isotype has a different structure, biological property, physical location and ability to direct the appropriate immune response for the types of foreign object encountered. The classic immune response to a foreign antigen (for example following immunization or infection) involves 'recognition' by an antibody that triggers clonal B cell expansion, class switching from IgM to IgG, memory, and

Isotypes	Structure	Summary of function
IgA1 **IgA2**	Heavy chain α1 or α2 Light chain λ or κ MW 150 – 600 Monomer to tetramer	Found in mucosa (oral, gastrointestinal, respiratory and urogenital tract) preventing their colonisation by pathogens. Secreted in milk and is resistant to digestion.
IgD	Heavy chain δ Light chain λ or κ MW 150 Monomer	Uncertain function but mostly bound to B cells. Involved in B-cell development with IgM.
IgE	Heavy chain ε Light chain λ or κ MW 190 Monomer	When bound to allergens triggers mast cell degranulation (histamine and tryptase). Protects against parasitic worms.
IgG1 **IgG2a and b** **IgG3** **IgG4**	Heavy chain γ1, γ2, γ3, γ4 Light chain λ or κ MW 150 Monomer	The main antibody based immunity against pathogens. Can cross placenta.
IgM	Heavy chain μ Light chain λ or κ MW 900 Pentamer	Primary response antibody, eliminating pathogens in the early stages of B cell mediated immunity before there is sufficient IgG. Present both on B cells and in secreted form (sIgM) with high avidity.

Table 1.3: A summary of the structure and function of mammalian antibodies. MW, Molecular weight in kilodaltons.

a large increase in specific antibody production (Stavnezer 1996). Traditionally, interest has mainly focused on the change in IgG antibody concentrations after immunization, which, after all, is its aim, and is often associated with specific protection from disease (Khatami and Pollard 2010; Sinclair *et al* 2011; Zhang *et al* 2011).

1.3.2 Natural Antibody

This approach however ignores the nature of antibodies present prior to exposure to a specific antigen. There has been recent interest in the role of these pre-existing antibodies, called 'Natural antibodies' (as opposed to 'immune'): in particular where they come from and what their function is (Ehrenstein and Notley 2010; Boes 2000). Present in 'germ-free' and 'antigen-free' mice, there are various characteristics commonly attributed to natural antibodies:

IgM

IgM is the first immunoglobulin in the immune response, first in ontogeny, and is present in germ and antigen free mice at *much higher concentrations* than IgG (Haury *et al* 1997). Because of this natural antibodies are generally considered to be IgM isotype. IgM can be either a pentamer secreted (sIgM) or membrane bound (as a B cell receptor) and is the only immunoglobulin present in all vertebrates (Fellah *et al* 1992). Classically IgM is described as having low affinity (the strength of a single bond) but because it is usually in a pentameric structure, high avidity, the combined strength of all the bond interactions (Delves and Roitt 2000).

Measurable in umbilical cord blood

IgM is measurable in umbilical cord blood: in contrast to the smaller IgG, IgM cannot cross the placenta from the maternal circulation and so must be usually of fetal origin (Brasher and Hartley 1969; Wuttke *et al* 1997).
As (in a traditional sense anyway) the fetus is not exposed to external antigens prior to birth this is taken as evidence that natural IgM may be important in the initial protection of the newborn prior to developing an adult immune system and that external antigens (for example infectious) have not stimulated it's production (Brasher and Hartley 1969).

Reactive against self-antigens

Although natural IgM appears to be directed at a range of antigens, most

studies have found reactivity against endogenous structures. These target structures can be substances that become exposed during processes such as apoptosis (e.g. phosphatidylserine) or ischaemia (e.g. annexin IV), have been 'lost' in evolution (e.g. gal) but remain present in other mammals, occur in some individuals and not others (e.g. specific ABO antigens), are nuclear structures or even other antibodies themselves (Chellingworth *et al* 1984; Galili *et al* 1987a; Halpern *et al* 1991; Kremmer *et al* 2001; Kulik *et al* 2009; Wellmann *et al* 2005). Whether these antigens have originally driven their production or the antibodies arrive by chance as cross-reactive to other antigens (for example on bacteria) is not certain (Allen and Kabat 1959; Haury *et al* 1997). However, concentrations of some antibodies considered to be 'natural IgM' such as the A and B antigens of the ABO blood group antigens can be raised by exposure to substances that share parts of their structure (Springer and Horton 1969).

1.3.3 The role and functions of natural antibody

Natural antibodies have many diverse functions, but it is not clear if they are all clinically relevant. However it does appear that natural IgM has a role in:

Cell clearance during apoptosis

Natural IgM can bind antigens newly exposed on the cell surface during apoptosis (Kim *et al* 2002). With complement, this enhances the apoptotic cell opsonization and accelerates its clearance by phagocytes. One of the key targets for natural IgM in this context is the phosphorylcholine epitope on lysophosphatidylcholine, formed during apoptosis when fatty acids are hydrolyzed by phospholipase A2 (Peng *et al* 2005). This mechanism is often thought to be behind the observation that humans with low serum IgM antibodies to phosphorylcholine and oxidized low-density lipoprotein have more cardiovascular events (Sjoberg *et al* 2009; Tsimikas *et al* 2007).

Defence against infectious organisms

There is a large body of evidence that natural IgM is involved in many processes that defend against microbiological organisms including bacteria, viruses, fungi and parasites, in particular *Streptococcus pneumoniae* and the Influenza virus (Baumgarth *et al* 2000; Boes *et al* 1998b; Ghumra *et al* 2008; Ochsenbein *et al* 1999; Rajan *et al* 2005; Reid *et al* 1997; Subramaniam *et al* 2010). One well characterized antibody, phosphorylcholine IgM is part of the initial defence against *Streptococcus pneumoniae* and other gram-positive organisms that have phosphorylcholine as part of their outer cell wall (Baxendale *et al* 2008; Briles *et al* 1981; Brown *et al* 2002).

Of particular relevance to surgery is the fact that mice deficient in secreted IgM (sIgM) are more likely to die (70% vs 20% at 32 hours) than wild type mice after experimental caecal ligation and puncture. The sIgM deficient mice in this study had decreased neutrophil recruitment, more bacteria in the peritoneum, and elevated concentrations of circulating endotoxin and TNFα. Addition of polyclonal IgM from normal mouse serum restored survival to caecal ligation and puncture in the sIgM-deficient mice. Interestingly, circulating IL-6 concentrations were similar between the two types of mice (Boes *et al* 1998b). This result should be interpreted with caution as these sIgM mice also have impaired IgG antibody responses (Boes *et al* 1998a). There is some evidence that IgM enriched immunoglobulin preparations reduce the severity of human sepsis (Norrby-Teglund *et al* 2006).

Immune maintenance and development

Although relatively putative, natural IgM may have a role in the development of the immune system (Ehrenstein and Notley 2010). One way this could happen is through natural IgM binding to the B cell receptor (a non-pentameric, membrane bound IgM) in turn influencing it's differentiation and maintenance.

Cancer

Organisms are continuously exposed to transformed (genetically altered) cells, which arise spontaneously or by inducing factors, some of which result in malignancy. There is evidence (with potential clinical benefit) that natural IgM is part of the recognition and elimination of precancerous and cancerous cells (Vollmers and Brandlein 2009). This surveillance may be mediated by natural IgM's ability to bind tumour-specific cell surface carbohydrate epitopes, which in turn can induce cellular stress and apoptosis (Brandlein *et al* 2003; Vollmers and Brandlein 2005).

1.4 Antibodies to endotoxin

Along with lipids and bactericidal/permeability-increasing protein, naturally produced antibodies are thought to provide protection against endotoxin in the circulation (Poxton 1995). Endotoxin antibodies are directed at the various parts of the endotoxin molecule: lipid A, core or o-polysaccharide. In theory, antibodies against the core or lipid A might be expected to bind to a greater range of endotoxins because these structures are relatively conserved between gram negative species compared to the o-polysaccharide region, which vary greatly (Poxton 1995). The trigger and timing for the production of endotoxin antibodies is unknown, but they may be the result of an overt clinical infection deliberate vaccination (for example with a conjugate vaccine such as *E coli* 0111:B4 J5 mutant, a rough endotoxin complexed to group B meningococcal outer membrane protein) presumed sub-clinical infection or colonisation or they may represent 'natural antibody' from the preimmune antibody repertoire (Berczi *et al* 2000; Boes *et al* 1998b; Cross *et al* 2004; Cryz, Jr. *et al* 1997; Ehrenstein and Notley 2010; Hendry *et al* 2000; Maury *et al* 2003; Pollard and Frasch 2001; Stager *et al* 2003).

Because of the role of endotoxin in initiating inflammation during gram-negative sepsis, many investigators have attempted to detect 'cross reactive' antibodies to endotoxin to use as a potential immunotherapy. These have depended on

finding an antigen or a combination of antigens that are sufficiently cross-reactive i.e. antibodies directed against it reacts with a range of other antigens. Because of it's relatively conserved structure, many investigators have used a rough endotoxin, but even these techniques have differed in the method of antigen fixation, size of the endotoxin (i.e. how much of the core), which species and whether they have used more than one antigen (Cross *et al* 2004; DeMaria *et al* 1988; Di Padova *et al* 1993; Erridge *et al* 2002b; Fomsgaard *et al* 1989; Freeman and Gould 1985b; Gould *et al* 1989; Hardy *et al* 1994; Hiki *et al* 1995; Mattsby-Baltzer *et al* 1994; Takahashi *et al* 1992; Ward *et al* 1988; Weintraub *et al* 1994).

1.4.1 Antibodies to endotoxin core, EndoCAb

The Endotoxin Core Antibody Enzyme-Linked Immunosorbent Assay (EndoCAb ELISA) is one of many assays developed to attempt to detect cross-reactive antibodies to the core region of endotoxin which is much more conserved than the O-polysaccharide (Andersen *et al* 1993; Angus *et al* 2000; Appelmelk *et al* 1985; Baumgartner *et al* 1985; Burd *et al* 1993b; Gould *et al* 1989; Hiki *et al* 1995; Olds and Miller, III 1991; Pollack *et al* 1983; Young and Stevens 1977). This specific assay was developed in order to screen volunteer donors for plasma high in cross-reactive antibodies to use as an anti-endotoxin treatment in sepsis. It is a sandwich ELISA, with the solid (bound) phase consisting of an equimolar mixture of incomplete core endotoxins (i.e. Rc or Rb- an intact inner core and Lipid-A region) of Pseudomonas aeruginosa PAC-611, *Salmonella typhimurium* 878 (Rc), *Escherichia coli* K12 and *Klebsiella aerogenes* M10b (Rb) on polystyrene plates. This particular combination of endotoxins was arrived at by screening donor plasma samples with rough and smooth examples of 13 species of *Salmonella*, 14 species of *E coli*, 2 *Pseudomonas* and one *Klebsiella* alone and in combinations (Scott *et al* 1990; Scott and Barclay 1990). Prior to coating, these incomplete core mutant endotoxins are reacted with polymyxin B, an antibiotic cationic polypeptide that binds to the inner core region of endotoxin, after which excess polymyxin is removed. The addition of polymyxin greatly improves inter-plate variability,

increases the optical density and yet still allows better binding with monoclonal antibodies to the inner core/lipid A compared to other methods such as Magnesium, Albumin and a pH 4.5 method (Appelmelk *et al* 1993; Scott and Barclay 1987). This polymyxin method has been used by other investigators although binding of rough endotoxin to other types of ELISA plates has been found not to need polymyxin (Bantroch *et al* 1994; Mertsola *et al* 1989; Takahashi *et al* 1992).

As mentioned previously there have been several studies in patients undergoing surgery, with pancreatitis or sepsis that has shown these antibodies to be clinically relevant (Ammori *et al* 2003; Braun *et al* 2007; Foulds *et al* 1997; Goldie *et al* 1995; Hamilton-Davies *et al* 1997; Mathew *et al* 2003; Penalva *et al* 2004; Rahman *et al* 2003; Soong *et al* 1999; Strutz *et al* 1999; Windsor *et al* 1998). What is not known for certain is whether they are a marker for some other factor, or if they directly reduce endotoxin-mediated toxicity of if their mechanism depends on cross-reactivity with an unknown antigen. One study that supports the role of IgM EndoCAb in directly neutralizing endotoxin comes from an 'endotoxin recovery study' which demonstrated a strong association between the amount of endotoxin that could be recovered from a 'spiked' sample and IgM (and IgG) EndoCAb concentrations (Bennett-Guerrero *et al* 2001a). Thus of course it could be that EndoCAb concentrations rise in parallel with an unknown factor also capable of neutralizing the LAL test (e.g. HDL cholesterol). There are several studies that have investigated the functional activity of purified IgG from serum high in IgG EndoCAb (Scott *et al* 1990; Scott and Barclay 1987; Scott and Barclay 1990). These examined various functional aspects (such as neutrophils chemiluminescence and bacterial phagocytosis) of 'high IgG EndoCAb immunoglobulins' from 3 different individuals compared with 2 from low IgG EndoCAb donors. In general the purified IgG from sera high in antibodies to endotoxin core were found to increase the opsonic activity against smooth and rough bacteria (Scott and Barclay 1990).

There is a large 80-fold range in EndoCAb antibodies in healthy blood donor populations and both IgG and IgM EndoCAb are detectable in the first few

months of life (Barclay 1995; Oppenheim *et al* 1994). The concentrations of both IgG and IgM EndoCAb inversely reflect the amount of endotoxin that can be recovered by the conventional endotoxin assay, the Limulus Amebocyte Lysate (LAL), suggesting that, at least, it neutralises endotoxin as measured by this assay (Bennett-Guerrero *et al* 2001a).

The majority of studies investigating the role of endotoxin antibodies on clinical outcome have been in septic patients. However, because of the evidence that endotoxin is a mediator of postoperative morbidity, especially in cardiac surgery involving cardiopulmonary bypass, several trials have examined the association between antibodies to endotoxin core and postoperative complications. The first of these used a qualitative assay (the presence or absence of antibodies to an endotoxin core from an *E coli*) to show that postoperative fever and/or gram negative infection was more common in those patients lacking the antibodies to endotoxin core (Freeman and Gould 1985a). This was followed by another similar study in patients undergoing gynaecological surgery with similar results (see table 1.4) (Gould *et al* 1989). Then, as part of a long term project to source plasma high in antibodies to endotoxin core, several studies appeared that investigated the role of the 'EndoCAb' assay in predicting complications following surgery. These studies involving 2537 patients undergoing surgery are outlined in table 1.4.

First author	Year	n=	Antibodies	Patient group	Main outcomes	Significant Findings
Freeman	1985	86	antibodies to *E coli* endotoxin	'Open heart surgery'	Postoperative fever & gram negative infection	Patients with no antibodies (65%) had more postoperative fevers and postoperative Gram negative infection.
Gould	1989	86	antibodies to endotoxin	Major gynaecology surgery	Postoperative and urinary tract infections	Patients with no antibodies (76%) had more postoperative infections ($p<0.05$) and urinary tract infections ($p<0.02$)
Mythen	1993	26	EndoCAb	Major non-cardiac surgery	GI mucosal perfusion + complications	Patients maintaining good Gastrointestinal mucosal perfusion had higher pre and postoperative IgG EndoCAb concentrations ($p=0.005$)
Bennett-Guerrero	1997	301	EndoCAb	Cardiac surgery*	Death in hospital or pLOS>10 days	Higher IgM EndoCAb associated with good outcome ($p=0.002$)
Hamilton-Davies	1987	59	EndoCAb	Valve surgery*	Composite endpoint++	Poor outcome associated with IgM EndoCAb < 100 MU/mL ($p<0.025$)
Foulds	1997	21	EndoCAb IgG	tAAA repair	Postoperative renal or respiratory failure	Patients with complications had a greater rise in endotoxin ($p=0.031$) and a greater fall in IgG EndoCAb ($p=0.001$)
Bennett-Guerrero	2001	1056	EndoCAb	Major non-cardiac surgery	Death in hospital or pLOS>10 days	Higher IgM EndoCAb associated with good outcome ($p=0.006$)
Rothenburger	2001	100	IgG and IgM against lipid A and endotoxin	CABG	Postoperative ventilation time, endotoxin, IL-6 and IL-8.	All antibodies decreased + endotoxin increased after CPB ($p < 0.001$). In patients with prolonged postoperative ventilation, preoperative endotoxin IgM IgG antibodies were lower, the fall in IgM greater and more sustained and the rise in endotoxin concentrations greater.
Mathew	2003	460	EndoCAb	CABG	Cognitive function at 6 weeks postoperatively	Low preoperative IgM EndoCAb associated with cognitive deficit at 6 weeks ($p=0.03$)
Moretti	2006	474	EndoCAb	CABG	5 year all cause mortality	Low preoperative IgM EndoCAb (<80 MU/mL) significantly associated with mortality up to 5 years ($p=.01$)
Braun	2007	140	EndoCAb	Cardiac surgery*	Organ dysfunction	IgM, G + A EndoCAb fell after surgery, whilst IgG EndoCAb fell more in those developing organ dysfunction.

Table 1.4 Previous adult surgical studies examining the association between preoperative endotoxin antibody concentration and the postoperative course *= includes valve and coronary artery bypass graft surgery or double valve surgery ++=Death or IABP or chest reopening other than gross surgical bleeding or major organ failure (Knaus *et al* 1985) or delay in ICU discharge (>24 hr) or delay in hospital discharge (>48hr). *Abbreviations*: CABG=Coronary artery bypass graft, CPB= cardiopulmonary bypass, EndoCAb©= Endotoxin core antibodies, GI= gastrointestinal, IABP = intra-aortic balloon counterpulsation pump, IL-6 = Interleukin 6, IL-8= Interleukin 8, pLOS= postoperative length of stay, tAAA= Thoracoabdominal aortic aneurysm

1.4.2 EndoCAb and postoperative outcome

In the first of the surgical studies to use the 'EndoCAb' assay, Mythen et al examined the relationship between gastrointestinal mucosal ischaemia, IgM and IgG EndoCAb concentrations, markers of neutrophil activation and postoperative organ dysfunction in 26 patients undergoing routine major non-cardiac surgery (Mythen *et al* 1993). They found that all patients had a significant fall in EndoCAb from baseline to 24 hours following surgery and that those patients with good gastrointestinal perfusion following surgery had higher IgG EndoCAb at baseline and 24 hours and had evidence of less neutrophil activation. This study was not powered to look at the predictive power of EndoCAb concentration for postoperative complications. Bennett-Guerrero and colleagues in 301 patients undergoing cardiac surgery found that IgM EndoCAb was able to predict a postoperative complication, (defined as death in hospital or a postoperative hospital stay exceeding 10 days) above other perioperative risk factors such as duration of cardiopulmonary bypass, Parsonnet score (a risk prediction score in cardiac surgery), age and preoperative renal failure ($p=0.002$) (Bennett-Guerrero *et al* 1997). In particular, 3.7% of patients in the highest IgM EndoCAb quartile compared to 23% of patients in the lowest IgM EndoCAb quartile experienced a complication. Interestingly IgG EndoCAb and total IgM were not related to outcome ($p=0.7$ and 0.1 respectively) and there was no relationship between Parsonnet score and EndoCAb (see appendix 3). Subsequent studies (see table 1.4) have found preoperative IgM EndoCAb to be related to better postoperative outcome (as judged by a variety of measures) but independent of total IgG and IgM, measures of risk in general surgery, height, weight, age and several indices of cardiovascular function (Bennett-Guerrero *et al* 2001b; Hamilton-Davies *et al* 1997). Using another measure of endotoxin antibodies (Lipid A or Re core from *Salmonella minnesota* R595) in 100 CABG patients Rothenburger and colleagues found that patients with complications had lower preoperative endotoxin antibody concentrations, more endotoxaemia and IL-8 following surgery (Rothenburger *et al* 2001). The most recent published study to include EndoCAb, of only 140 patients undergoing cardiac surgery found EndoCAb IgM, G and A to fall following surgery, but only preoperative EndoCAb IgG was associated with an outcome difference (Braun *et al* 2007).

Several *in-vitro* studies have shown that monoclonal IgG antibodies to endotoxin core can increase the uptake of endotoxin to macrophages and in certain circumstances this was accompanied by an inhibition of TNF secretion (Burd *et al* 1993a; Scott and Barclay 1990). The relevance of monoclonal endotoxin antibodies to likely polyclonal groups such as EndoCAb is of uncertain relevance although it does point to at least one potential mechanism.

1.5 Uncertainties, successes and failures

Studying EndoCAb is and has been challenging. Low EndoCAb IgM seems to be associated with a poorer outcome- but is this link causal, and what is the mechanism? The current idea is that EndoCAb IgM neutralises endotoxin (released from the GI tract), resulting in less inflammation around the time of surgery. One clinical approach is to try to examine if the measures of inflammation are higher in those with less EndoCAb. This I did in chapter 3 using samples and data collected from patients enrolled in another study on IL-6 levels after first time CABG, with IL-6 and CRP as the measures of inflammation. In chapter 5 I used the opportunity of another trial (examining the role of MBL in children) to see if patient with low EndoCAb had more SIRS- a clinical measure of inflammation.

Another approach to studying EndoCAb's effects may be to make a 'pure' concentrate and to examine its effects in an *in-vitro* system. To manufacture (for example by affinity purification) an 'EndoCAb' concentrate is technically difficult and was outside the scope of expertise I had available for this thesis. By its nature, EndoCAb is a mixture of antibodies (as the ELISA uses an equimolar mixture of 4 endotoxin cores), so it is not strictly possible to make a 'monoclonal EndoCAb'. As an alternative during my research time I tried to fractionate a serum from volunteers with high or low EndoCAb IgM to use in *in-vitro* studies. These *in-vitro* studies involved both whole blood endotoxin stimulation (with serial cytokines as the outcome) and Flow Cytometric techniques ('FACS' using Neutrophil CD11b as the measure of cell activation). Unfortunately, although I mastered the *in-vitro* techniques and was able to make an IgM fraction from serum it was not possible for me to do this in a sterile environment**.** Because of these difficulties I decided to look at the

similarities between EndoCAb IgM and a panel of natural IgM's and to try to see how much EndoCAb IgM had the characteristic of a natural IgM. I reasoned that, if EndoCAb and natural IgM levels were linked, that reduced the likelyhood that the association between EndoCAb IgM and postoperative outcome was causal or specific to EndoCAb IgM. It also points to a new mechanism.

Aside from clinical (chapters 3,5) and basic science (chapter 4) techniques, volunteer studies (chapter 6) may help elucidate the mechanism of EndoCAb's association with outcome. However like all trials, endotoxin volunteer studies have to be conducted correctly, using appropriate doses and dose regimens depending on the outcome being measured. Most human studies use a large endotoxin 4ng/kg bolus that causes robust clinical and immune markers of inflammation in all volunteers. I wondered if the *range of responses* could be increased if the dose of inflammatory stimulus was reduced. We wanted an endotoxin does that caused inflammation in some but not in all. Then we could use this in a later study to examine what the predictors of a large (or absent) inflammatory response are.

Chapter 2

Methods

2.1 Introduction

The experimental work in this thesis required the modification and subsequent validation of existing techniques. This chapter will describe the methods used in the thesis. I have not included the methods I learnt but did not use in the final thesis such as Flow Activated Cell Sorting, cell density centrifuge separation, Magnetic Activated Cell Separation, Cell culture, cell stimulation with endotoxin and molecular weight chromatography.

2.2 Reagents and Equipment

Reagent	Supplier	Address
NaCl	Sigma-Aldrich	London, UK
KCl	Sigma-Aldrich	London, UK
Na_2HPO_4	Sigma-Aldrich	London, UK
$KH2PO_4$	Sigma-Aldrich	London, UK
NaN_3	Sigma-Aldrich	London, UK
Na_2CO_3	Sigma-Aldrich	London, UK
$NaHCO_3$	Sigma-Aldrich	London, UK
Diethanolamine	Sigma-Aldrich	London, UK
4-Nitrophenyl phosphate powder	Sigma-Aldrich	London, UK
Tetramethylbenzidine Dihydrochloride	Sigma-Aldrich	London, UK
Bovine Serum Albumin	Sigma-Aldrich	London, UK
$MgCl_2$	Sigma-Aldrich	London, UK
$NaHPO_4$	Sigma-Aldrich	London, UK
Low Endotoxin Fetal Calf Serum	Cambrex	Charles City, USA
Heparin Sulphate	Celcus	Cincinnati, USA

Table 2.1: General Chemical Laboratory reagents

Reagent	Supplier	Address
E coli Endotoxin 0111:b4	Sigma-Aldrich	London, UK
E coli Endotoxin K12	Prof I Poxton	Edinburgh, UK
E coli Endotoxin 0113	National Institutes of Health, USA	Bethesda, USA
Salmonella typhimurium 878 Endotoxin	Prof I Poxton	Edinburgh, UK
Klebsiella aerogenes M10b Endotoxin	Prof I Poxton	Edinburgh, UK
Pseudomonas aeruginosa PAC-611 Endotoxin	Prof I Poxton	Edinburgh, UK
'EndoCAb' ELISA plates	Dr R Barclay	Edinburgh UK
Phosphatidylserine	Sigma-Aldrich	London, UK
Phosphatidylcholine	Sigma-Aldrich	London, UK
Phosphorylcholine	Biosearch Technologies	Novato, USA
Phosphorylcholine-Albumin	Biosearch Technologies	Novato, USA
α*1-3 Gal* antigen (PAA-Bdi)	Lectinity Holdings	Moscow, Russia
Blood Group A antigen (PAA-Atri)	Lectinity Holdings	Moscow, Russia
Blood Group B antigen (PAA-Btri)	Lectinity Holdings	Moscow, Russia
Tetanus toxoid	List Biologicals	Campbell, USA
Rabbit anti-human IgM	Dako UK Ltd	Ely, UK
Recombinant Human TNF	Invitrogen Biosource	Paisley, UK
Recombinant Human IL-6	Invitrogen Biosource	Paisley, UK
Recombinant Human IL-10	Invitrogen Biosource	Paisley, UK

Table 2.2: Antigens and Endotoxins

Reagent	Manufacturer	Address
TNFα anti-human capture antibody	Invitrogen Biosource	Paisley, UK
TNFα anti-human detection antibody; biotinylated	Invitrogen Biosource	Paisley, UK
IL-6 anti-human capture antibody	Invitrogen Biosource	Paisley, UK
IL-6 anti-human detection antibody; biotinylated	Invitrogen Biosource	Paisley, UK
IL-10 anti-human capture antibody	Invitrogen Biosource	Paisley, UK
IL-10 anti-human detection antibody; biotinylated	Invitrogen Biosource	Paisley, UK
anti-human IgM; alkaline phosphatase conjugated	Sigma-Aldrich	London, UK
anti-human IgG; alkaline phosphatase conjugated	Sigma-Aldrich	London, UK
anti-human IgA; alkaline phosphatase conjugated	Sigma-Aldrich	London, UK

Table 2.3: Antibodies

Equipment	Manufacturer	Address
ELISA reader: Dynatech MRX	Dynatech Laboratories	Billingshurst, UK
ELISA Plate washer LP4	Adil Instruments	Schiltigheim, France
pH meter: HI-2211-02	Hanna instruments	Leighton Buzzard, UK
Weighing scales: Sartorius Basic	Sartorius	Gottingen Germany
Biological safety cabinet Class 2 BIOHIT NCB-D	The Baker Company	Maine USA
Acetate ELISA plate adhesive	MP Biomedicals	Solon, USA
Nunc Maxisorb 96 well ELISA plate	Fisher scientific	Loughborough, UK
Nunc Polysorb 96 well ELISA plate	Fisher scientific	Loughborough, UK
Linbro/Titertek ELISA plate	ICN Biomedicals	Aurora, USA
MilliQ Water purification system	Millipore UK LTD	Watford, UK
Centrifuge ROTANTA 460 R	Hettich GmbH & Co	Tuttlingen, Germany
Falcon polypropylene 50 mL tubes	BD biosciences	Oxford, UK
Eppendorf 1.5mL tubes	Eppendorf UK	Cambridge, UK

Table 2.4: Equipment and disposables

2.3 Endotoxin Core Antibody Enzyme-Linked Immunosorbent Assay

2.3.1 Reagents and solutions

Name	Constituent	Amount
Phosphate Buffered Saline	NaCl Na_2 $HPO_4.H_20$ KH_2P0_4 KCl Distilled Water	8g 1.42g 0.2g 0.2g 1L MilliQ pH whole solution to 7.4
Wash buffer	0.1% 'Tween20' Sodium Azide 0.05% Phosphate Buffered Saline	1mL 0.5 g Make up to 1L
Diluent	Phosphate Buffered Saline Tablet 0.1% 'Tween20' Sodium Azide 0.05% BSA 1% Polyethylene Glycol 8000 4% Distilled H_20	5 per 1L 1mL in 1L 0.5 g in 1L 10g in 1L 40g in 1L Make up to 1L
Substrate Buffer	Diethanolamine $MgCl_2$ Sodium Azide Distilled H_20	97mL 0.1g 0.2g Make volume up to 1L; Adjust to pH 9.8 with 10 M HCL
Conjugated Fc-specific antibodies	Alkaline phosphatase–conjugated IgG, IgM or IgA	Diluted 1:500

Table 2.5: Reagents for EndoCAb ELISA.

2.3.2 Method of the EndoCAb ELISA

Blood samples were collected from patients or volunteers by venipuncture into nonadditive glass tubes, allowed to clot, centrifuged and the serum stored at -80°C until assayed. For some studies plasma was used.

Antigen

Serum IgM, IgG and IgA EndoCAb were measured with a sandwich ELISA. Equimolar amounts of an endotoxin from each of a selected *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa* rough mutant strain were complexed with polymyxin B in a carbonate bicarbonate buffer (pH 9.6), and coated on 96 well-polystyrene microplates by the manufacturer, Dr Robin Barclay PhD, Scottish National Blood Transfusion Service Cell Therapy Group, Edinburgh, UK. These 4 mutant strains lacked the endotoxin O-polysaccharide and part of the endotoxin outer core, but retained an intact inner core structure.

Assay method

All reagents were made up in glassware that has been baked at 170^0 centigrade for 2 hours. Following one 250µL plate wash in wash buffer, an eight-point standard curve was constructed using doubling dilutions in diluent of a calibrated serum that has an undiluted value of EndoCAb IgM of 165 MU/mL, an EndoCAb IgG of 784 MU/mL and an IgA EndoCAb of 226 MU/mL. Following the addition of 100 µL of the standards and samples, the plate was covered and incubated for 1 h at 37 °C. The samples are diluted 1 in 200, and the quality controls in 2 serial dilutions of 1 in 100 to 1 in 200. All standards, quality controls, blanks (diluent only) and samples were run in duplicate. The top standard concentration was 1 in 50, if the concentration of EndoCAb in the sample was high, the standard or sample concentration was altered and repeated. The ELISA plates were then washed three times with wash buffer and finally blotted dry. Alkaline phosphatase conjugated goat antihuman IgM, IgG or IgA antibody (Sigma-Aldrich Chemical Co; Poole, UK) was diluted 1 in 1000 with dilution buffer, and 100 µL of conjugated antibody was added to each well; the plates were covered and incubated for 1 h at 37 °C. After incubation at

37 °C, the plates were washed three times with wash buffer, rinsed twice with distilled water, and blotted dry on absorbent tissue.
P-Nitrophenyl Phosphate Substrate (Sigma-Aldrich Chemical Co; Poole, UK) (5mg p-Nitrophenyl Phosphate per 5mL Diethanolamine substrate buffer; 180 µL per well) was added to each plate and incubated at room temperature in the dark for approximately 20 minutes for IgM, 10 min for IgG and 30 minutes for IgA. The reaction was stopped with 2M NaOH. The plate was read at 405 nm wavelength using an automated plate reader (Dynatech MRX, Virginia, USA). A standard curve was constructed at 405 nm optical density against EndoCAb concentration and the result of test samples was deduced from the curve.

Results

Results were expressed as median units (MU) per milliliter, where 100 MU/mL is the median value of the original population of 1,000 healthy volunteers as determined by Dr Robin Barclay (Barclay 1990). The assay was standardized using a calibrated pooled-serum standard of a predetermined EndoCAb IgM, IgG and IgA concentration and by the use of a quality control serum included on each plate. Results from the whole plate were rejected if the concentration of the control sample varied by more than 15% from the known value or the correlation coefficient (r^2) for the standard curve was less than 0.98. Individual sample results were rejected and repeated if the coefficient of variation of the samples were greater than 15% or the optical density of the sample is outside the range of the standard curve.
Assay consistency (intra-assay and inter-assay variation) was measured by running 5 duplicate samples on each of two plates and calculating the coefficient of variation (standard deviation/mean expressed as a percentage). The intra-assay coefficient of variation was 9.0, 8.7 and 8.3%, whilst inter-assay variation was 11.3, 10.3 and 8.2**%** for EndoCAb IgM, IgG and IgA respectively.

Because of the relatively high EndoCAb values seen in other surgical studies, and the fact that there is no published data on IgA EndoCAb concentrations, a new standard for the study on EndoCAb and outcome after first time Coronary Artery Bypass Graft surgery was manufactured. After screening 24 consenting healthy volunteers four with the highest IgA EndoCAb values were selected.

15mL blood was taken from each allowed to clot and spun separately, then the serum mixed together. The resulting mixture was then filtered with a 30 kilodalton filter (to remove excess solvent) to 2/3 it's original value. The resulting new standard had an IgA EndoCAb value of 306 MU/mL compared to the original of 226 MU/mL.

Blinding

All laboratory measurements were performed on coded samples so as to blind the investigators to the patients' identity and outcome.

Statistical Analysis

The distribution of EndoCAb is non-Gaussian with positive skew: nonparametric methods were therefore used, or the data was log transformed, to permit parametric analysis.

2.4 Cytokine Enzyme-Linked Immunosorbent Assay

2.4.1 Reagents

Name	Constituent	Amount
Phosphate Buffered Saline	NaCl Na_2 $HPO_4.H_20$ KH_2P0_4 KCl distilled water	8 g 1.42 g 0.2 g 0.2 g 1 L distilled water pH whole solution to 7.4
Wash Buffer	Phosphate Buffered Saline 0.1% 'Tween20'	1 L 1 mL
'Blocking Solution'	Bovine Serum Albumin Phosphate Buffered Saline	5 g 1 L
Diluent	Phosphate Buffered Saline 0.1% 'Tween20' BSA 1%	100 mL 0.1 mL 0.1 g
Phosphate Citrate Buffer	0.2 M Na_2HPO_4 0.1 M $C_6H_8O_7$ (Citric Acid)	14.196 g in 500mL distilled water 10.51g per 500mL distilled water 5.14 mL of 0.2 M Na_2HPO_4 and 4.86 mL of 0.1 M Citric Acid above and 10 mL distilled water to make Phosphate Citrate Buffer
Substrate Buffer	Tetramethylbenzidine H_2O_2 Phosphate Citrate Buffer	1 mg Tablet 2 µL 10 mL
Streptavidin-Horse Radish Peroxidase	4 µLStreptavidin-Horse Radish Peroxidase	10 mL of Phosphate Buffered Saline
Stop Solution	H_2SO_4 distilled H_20	55 mL 445 mL

Table 2.6: Reagents for Cytokine ELISA

2.4.2 Conduct of the Cytokine ELISA

Cytokine Enzyme linked Immunosorbent Assays (ELISA) to Tumour Necrosis Factorα (TNFα), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) were performed according to previously published methods using paired commercial capture and detection antibody sets (Allen 2006).

ELISA Plate coating with capture antibodies

Capture antibodies were reconstituted according to the manufacturer's instructions (Cytoset, Invitrogen Biosource, Paisley, UK). 11 µL of the capture antibody concentrate was added to 11 mL of Phosphate Buffered Saline on the day of use: this dilute solution is then at 1 μg/mL, except Tumour Necrosis Factor α (TNF-α) at 5 μg/mL. Using a multichannel pipette, 100 μl of this dilute capture antibody solution was added to each well of a 12 x 8 format Nunc Maxisorb flat bottomed ELISA plate. These were covered with plate adhesive and put in 4^{o}c refrigerator overnight for 12-18 hours.

Blocking

The next day, the coating antibody was aspirated from the wells, which were washed once with wash buffer. 300 μL of blocking solution was added to each well and the plate gently agitated at room temperature for a minimum of 2 hours. The blocking solution was aspirated and the plate washed 4 times.

Standards, Quality Control and Samples

Standards were reconstituted from powder according to the manufacturer's instructions (Cytoset, Invitrogen Biosource, Paisley, UK). The powdered antibody was diluted to a concentration of 10,000 pg/mL in blocking solution and diluted as appropriate. The top concentration for the standards was 5,000 pg/mL, apart from Tumour Necrosis Factorα (TNFα) that was started at 10,000 pg/mL. The standards were added to the plate in doubling dilutions, 12 dilutions in all, giving a concentration range 10,000 pg/mL to 4.88 pg/mL (TNFα) and 5000 pg/mL (IL-6 and IL-10) to 2.44 pg/mL. Plates were covered, put on a plate shaker (2 Hz) for 1 hour and refrigerated at 4 ^{o}c overnight.

Blood samples were collected from patients or volunteers by venipuncture into plain glass tubes, allowed to clot, centrifuged, and the serum stored at -80 °c

until assayed. Defrosted serum samples were mixed, then centrifuged (5 minutes at 2400 revolutions per minute) to pellet any particulate matter. 100 μL of the samples, diluent alone (for 'negative controls', blanks) or the quality controls were added in duplicate to each well as appropriate. Samples were added to wells neat. 100 μL of diluent as a 'negative control' or 'blank' were added in duplicate to two wells.

Secondary antibody and Substrate

Plates were then washed 4 times. The respective appropriate biotinylated detection antibody solution was diluted 1 in 1250 in diluent; 50 μL of this dilute solution added to each well and the plate incubated at room temp for 2 hours on a rotational shaker. Plates were again washed 4 times. 100 μL of diluted Streptavidin-Horse Radish Peroxidase was added to each well, the plate was put on a rotational shaker for 45 minutes and washed 4 times. 2μL Hydrogen peroxide was mixed with 10mL of the Phosphate Citrate Buffer and 1mg Tetramethylbenzidine (TMB) tablet was added. 100 μL of this TMB substrate solution was added to each well. The plates were protected from light with foil, and placed on a rotational shaker for 45 to 60 min. The solution was stopped by 100μL4M sulfuric acid per well when the highest concentration standard developed a blue- green appearance and some colour development started in the most dilute standard. Plates were read at 450 nm, aiming for the range 0 to 2 Optical Density Units.

Validation and Quality Control

A quality control serum was generated to ensure plate to plate consistency. 20mL of volunteer anticoagulated blood was mixed in a 1:1 ratio with RPMI with *L-glutamine* culture media, and 10 ng/mL *E. coli* 0111:b4 in a 50 mL Falcon tube. This was gently mixed and incubated at 37 ^{0}C with 5%CO_2 for 1.5 hours. This was spun (at 4 ^{0}C and at 2000 rpm for 15 minutes) after which the supernatant was removed into sterile tubes and frozen at -80 ^{0}C. This quality control serum was used on each ELISA plate. Assay consistency (intra-assay and inter-assay variation) was determined by running 5 identical duplicate samples on each of two plates on one day and calculating the coefficient of

variation (standard deviation/mean value expressed as a percentage). The intra-assay coefficient of variation was 3.6, 7.5 and 3, whilst inter-assay variation was 8.1, 12.5 and 10.8 for TNFα, IL-6 and IL-10 respectively. If optical density readings were greater than 2.0 at the highest dilution used in the assay, the sample was reassayed after additional dilution. Results from the whole plate were rejected and repeated if the concentration of the control sample varied by more than 15% from the known value or the correlation coefficient (r^2) for the standard curve was less than 0.98. Individual sample results were rejected and repeated if the correlation of variance of the samples were greater than 15% or the optical density of the sample is outside the range of the standard curve.

2.5 Gal, A, B and phosphorylcholine IgM Enzyme-Linked Immunosorbent Assay

2.5.1 Buffers and Reagents

Name	Constituent	Amount
Phosphate Buffered Saline	NaCl Na_2 $HPO_4.H_20$ KH_2P0_4 KCl distilled water	8 g 1.42 g 0.2 g 0.2 g 1 L distilled water pH whole solution to 7.4
Wash Buffer	Phosphate Buffered Saline 0.01% 'Tween20'	1 L 0.1 mL
'Blocking Solution'	Bovine Serum Albumin Phosphate Buffered Saline	20 g 1 L
Diluent Buffer	Phosphate Buffered Saline 0.1% 'Tween20' BSA 1%	100 mL 0.1 mL 0.1 g
Coating buffer	0.1 M Carbonate buffer	Na_2CO_3 3.11 g $NaHCO_3$ 5.94 g distilled water to 1 L pH 9.6
Substrate Buffer	Diethanolamine $MgCl_2$ Sodium Azide Distilled H_20	97 mL 0.1 g 0.2 g Make volume up to 1 L; Adjust to pH 9.8 with 10 M HCL
Conjugated Fc-specific antibodies	Alkaline phosphatase–conjugated IgM	Diluted 1:1000

Table 2.7: Reagents for Gal, A, B and phosphorylcholine ELISA

2.5.2 Conduct of the ELISA

The conduct of the ELISA follows previously published methods (Fan *et al* 2004; Padilla *et al* 2004; Rieben *et al* 1997; Rieben *et al* 2000). Nunc Maxisorb 96 ELISA well plates were coated with 50 µL of antigen, A (4 µg/mL), B (4 µg/mL), gal (5 µg/mL) or phosphorylcholine (2.5 µg/mL) diluted in coating buffer, covered and incubated overnight at 4 °C. The next day after washing 4 times with wash buffer, phosphorylcholine plates alone were blocked for 1.5 hours at room temperature with blocking solution.

Standards

As there are no agreed units for antibodies to the blood group antigens A and B, to α*1-3 Gal* or Phosphorylcholine, I developed my own standard from pooled serum. Blood from 4 volunteers with high concentrations of IgM antibodies to these antigens after screening was allowed to clot and the serum removed. After centrifuging (2000 rpm for 15 minutes) to remove larger particles it was concentrated by running though a 30 kilodalton filter. The subsequent pooled serum was then run as a standard for the four ELISAs. The units are expressed as arbitrary units/mL in all the studies, where the top standard (at 1 in 50) has arbitrarily been allocated a value of 128. Thus any sample diluted 1 in 50 with the same optical density as the top standard (which was diluted 1 in 50) would be given a value of 128 arbitrary units/mL.

Samples

Blood was collected from volunteers by venipuncture into plain glass tubes, allowed to clot, centrifuged, and the serum stored at -80 °C until assayed. Samples were mixed, then centrifuged for 5 minutes at 3000 revolutions per minute to bring down any particulate matter. 50 µL of the samples, diluent alone (for 'negative controls', blanks) or the quality controls were added in duplicate to each well as appropriate. Samples and quality controls (50 µL diluted 1 in 50 in diluent buffer) were added to wells whilst diluent buffer as a 'negative control' or 'blank' was added in duplicate. Standards (see below) had a top concentration of 1 in 50 in diluent buffer from the neat serum, and were diluted downwards 1 in 2 to construct a 'standard concentration curve'.

Samples, standards, blanks and quality control samples were incubated for 90 minutes at 37 °C, after which they were washed 4 times. Goat Anti-Human IgM (50 µL/well) diluted 1 in 1000 was added and the plates incubated for 60 minutes at 37 °C, then washed 4 times.

Colour was developed by adding substrate (100 µL per well) and the plates incubated for 30 min at 37 °C. Plates were then read at 405 nm, aiming for the range 0 to 2 Optical Density Units. Sample and quality control concentrations were calculated from the standard curve.

ELISA consistency

Assay consistency (intra-assay and inter-assay variation) was determined by running 5 identical duplicate samples on each of two plates on one day and calculating the coefficient of variation (standard deviation/mean value expressed as a percentage). The intra-assay coefficient of variation was 6, 7.8, 4.3 and 5.4 %, whilst inter-assay variation was 5.6, 8, 4.8 and 8.1 **%** respectively for A, B, α*1-3 Gal* and phosphorylcholine IgM.

Chapter 3

Antibodies to Endotoxin core and outcome after first time Coronary Artery Bypass Graft surgery

3.1 Introduction

Whilst the mortality following Coronary Artery Bypass Graft (CABG) is low, under 2% in-hospital after 1st time CABG, complications occur in at least 20% of patients, causing suffering and extending hospital stay (Keogh and Kinsman 2009). Patients may experience a poor outcome for several reasons, but many complications are thought to result from an exaggerated systemic inflammatory response. Endotoxin is a potent trigger of systemic inflammation (see introduction chapter 1) and is thought to be one of the causes of inflammation after CABG surgery. Endotoxin is detectable in the blood of majority of patients undergoing cardiac surgery involving cardiopulmonary bypass although the origin of the endotoxin is controversial (Aydin *et al* 2003). The cardiopulmonary bypass circuit, contaminated intravenous fluids, and an underperfused gastrointestinal tract leading to increased translocation of bacterial products have all been suggested as potential causes (Andersen *et al* 1987).

Previous studies (see table 1.4, introduction, chapter 1) involving a total of 2537 patients have shown that low preoperative IgM EndoCAb is associated with a poor outcome following both cardiac and non-cardiac surgery (Bennett-Guerrero *et al* 1997; Bennett-Guerrero *et al* 2001b; Hamilton-Davies *et al* 1997; Mathew *et al* 2003; Mythen *et al* 1993). These 5 studies have all used different definitions of a complication or a poor outcome and have involved cardiac surgical patients (including high risk groups such as repeat surgery, cardiac valve *and* Coronary Artery Bypass Grafts or two simultaneous cardiac valve replacements) or general surgical patients undergoing major surgery (e.g. radical prostatectomy, hip surgery revision or colon surgery). Only one of these, the smallest, attempted to elucidate any connection between EndoCAb and inflammation. In this study involving patients undergoing major general surgery, Mythen (n=26) found an association between higher preoperative and 24 hour IgG EndoCAb, better gut perfusion, reduced activation of the contact coagulation system, evidence of less neutrophil activation and organ failure. One study investigated the link between endotoxin antibodies and inflammation after CABG but comparisons with UK cardiac surgical practice are difficult because a different antibody assay to EndoCAb was used and a different risk stratification system not used in the UK (Higgins *et al* 1992; Rothenburger *et al*

2001). A further study measured EndoCAb in cardiac surgical patients but appeared not to be designed to examine its predictive power on postoperative outcome (Braun *et al* 2007).

There are several reasons why patients undergoing *elective 1st time* CABG are an appropriate group to study. Firstly, it is not known if the associations between IgM and outcome seen in the other studies, which generally involved higher risk patients (such as valve surgery or revision surgery) would extend to lower risk groups such as patients undergoing elective 1st time CABG. Compared to other cardiac surgical patients, this group would be expected to have a relatively low rate of complications (Keogh and Kinsman 2009). In theory it might be much harder to detect the consequences of endotoxin-mediated inflammation in these individuals because they have fewer co-morbidities, better cardiac function, are younger and have lower 'risk scores' (Keogh and Kinsman 2009). Surgery and cardiopulmonary bypass times should be shorter, and as the patients are in better health they should be better able to withstand the effects of tissue trauma, cardiopulmonary bypass, hypovolaemia and endotoxaemia compared to patients undergoing longer, more complex or repeat procedures (Keogh and Kinsman 2009). Secondly, the previous studies in UK cardiac surgical patients were published over 14 years ago since when practice has changed (Hamilton-Davies *et al* 1997; Mythen *et al* 1993). The relevance of comparisons with cardiac surgical today practice is unknown. Thirdly whilst two of these surgical studies (the smallest two; involving just 75 of the 1902 patients studied) were performed in the UK, the vast majority of the patients were from the USA. Along with differences in clinical practice, the population in the USA may also have different EndoCAb concentrations making comparisons with the UK even harder (Down *et al* 2004). Lastly since patients undergoing 1st time CABG are the largest cardiac surgical group, they are the largest potential group in which one could use a theraputic intervention (Keogh and Kinsman 2009).

To investigate the hypothesis that the relationship between low preoperative EndoCAb IgM and poor postoperative outcome may be explained by increased postoperative inflammation, I used interleukin-6 (IL-6) and C-reactive protein

(CRP) as general markers of postoperative inflammation, already being measured as part of the larger 'CASIS' (Coronary Artery Surgery Inflammation Study) trial. IL-6 and CRP concentrations are associated with mortality and complications after a variety of traumatic events although this has not been a universal finding (Fernandez-Serrano *et al* 2003; Giannoudis *et al* 2008; Kilciler *et al* 2008; Marti *et al* 2007; Mei *et al* 2007; Nieman 1997; Pape *et al* 2008; Simmons *et al* 2004).

Other secondary outcomes were measured either because of previous reported associations between them and EndoCAb (i.e. progression of heart failure) or because they are potentially confounding variables (Bolger *et al* 2001).

3.2 Hypothesis

The primary hypothesis was that low concentrations of EndoCAb are associated with a poorer clinical outcome and raised concentrations of postoperative inflammatory markers.

3.3 Aims

Primary Aims

To examine the relationship between IgM, IgG and IgA EndoCAb and clinical outcome ('complications') after 1st time elective CABG.

To examine the relationship between EndoCAb and markers of postoperative inflammation (IL-6 and CRP) after 1st time elective CABG.

Secondary Aims

To examine the relationship between IgM, IgG and IgA EndoCAb and

- Surrogate markers of preoperative cardiovascular dysfunction (New York Heart Association functional class, ejection fraction)
- Post operative haemorrhage
- High Density Lipoprotein-cholesterol (HDL-C)
- Age, Gender and ABO blood group

3.4 Methods

Approval for this study was obtained from our institution's ethics committee (REC ref. number 99/0099 The Joint UCL/UCLH Committees on the Ethics of Human Research: Committee α and A). Patient data was stored according to the requirements of the Data Protection Act. The clinical data, samples and blood for EndoCAb in this chapter were obtained from a double blind, observational cohort study ('CASIS' Coronary Artery Surgery Inflammation Study) part of a wider prospective investigation into the effects of key genotypes on concentrations of inflammatory mediators. CASIS was conducted by the Centre for Cardiovascular Genetics Department of Medicine, Royal Free and University College London Medical School, UK. The Centre for Anaesthesia and the Centre for Cardiovascular Genetics, both at University College London, jointly ran this part of the study. The idea for this chapter, analysis and EndoCAb measurement were mine.

Subject selection

All adult patients undergoing elective first-time Coronary Artery Bypass Grafting at University College London Hospitals (London, UK) were invited to take part in the CASIS study. Exclusion criteria included evidence of a pre-existing inflammatory state (infection, active arthritis, malignancy); systemic use of anti-inflammatory agents other than aspirin (e.g. steroids); surgery other than bypass grafting during the procedure (e.g. surgery on a ventricular aneurysm discovered at the time of operation); the requirement for chronic renal replacement therapy; surgery performed off cardio-pulmonary bypass and refusal to consent for the study.

Study Course

After written consent, citrated blood samples (4.5 mL) were drawn before surgery and then again at 6 and 24 hours after cardiopulmonary bypass. These were centrifuged immediately (3000 rpm, 10 minutes) and the plasma separated and frozen at -20°C until analysis. Surgery was performed by 4 experienced senior cardiac surgeons via a midline sternotomy and using standard surgical procedures. Hypothermic cardiopulmonary bypass was instituted after cannulation of the right atrium and ascending aorta. Myocardial

protection was maintained by cross-clamp fibrillation. Perioperative anticoagulation with heparin was reversed after cardiopulmonary bypass with protamine sulfate.

Clinical Measurements

Clinical and demographic information was prospectively collected for outcome measurement, risk scoring and to measure other potential confounding variables. The primary outcome ('complications') was death in hospital (after a continuous period of hospitalization between surgery and death) or a length of stay more than 10 days after surgery. If a patient both died in hospital following surgery *and* stayed longer than 10 days, they were only counted once as a 'complication'. To investigate the hypothesis that low IgM EndoCAb is associated with raised inflammatory markers we used IL-6 and CRP at baseline, 6 and at 24 hours, *already* being measured in a subset of patients in the CASIS study. Markers of preoperative cardiovascular dysfunction (cardiac ejection fraction and the preoperative New York Heart Association classification, NYHA) and evidence of postoperative haemorrhage (defined as a need to return to theatre for excessive bleeding or a decision by the clinician that excessive bleeding had occurred) were also measured. Investigators collecting clinical data were blinded to the laboratory measurements and vice-versa. Total mortality was obtained by 'tracking' patients with the UK Office of National Statistics who gave us data (including the date of death) each time a patient died. The median length of follow up was 990 days after surgery.

Antibodies to endotoxin core, EndoCAb

Preoperative antibodies to endotoxin core (EndoCAb IgM, IgG and IgA) were measured according to methods previously described in 'Methods', chapter 2.

IL-6 and CRP

IL-6 concentrations were measured preoperatively and at 6 and 24 hours after bypass in a subset of the CASIS study using a standard commercial assay (R&D Systems, Minneapolis, USA) whilst CRP was measured at the same timepoints on a BN Prospec machine (Dade Behring, Illinois, USA). Inter- and intra-assay coefficients of variation and assay sensitivity were 5%, 3% and 0.70

pg/mL respectively for IL-6 and 4%, 2% and 0.20 mg/L respectively for CRP. For this study both IL-6 and CRP were measured in a commercial contract laboratory.

Statistics

As EndoCAb *concentrations* are not normally distributed, medians and interquartile *ranges have been reported.* EndoCAb quartiles were used firstly to enable comparison with the previous studies (Bennett-Guerrero *et al* 2001, Hamilton-Davies *et al* 1997) that had reported quartiles. Secondly I chose to compare the lowest EndoCAb IgM quartiles in this cohort with other quartiles as previous authors had found this quartile to have the *highest complication rate*. I therefore reasoned it would be a sensible threshold to choose as a way of detecting the effects of low EndoCAb IgM on outcome and markers of inflammation.

Non-parametric analytical statistics (Mann-Whitney U test, Chi squared test, Kruskal-Wallis test and Spearman's Rank Correlation Coefficient) were used apart from linear regression where data were log transformed. Normal distribution of the transformed data was confirmed using the Kolmogorov-Smirnov test. Statistical analyses were conducted using SPSS (SPSS 15, Chicago, IL, USA).

3.5 Results

Of 448 consented patients we were able to obtain full plasma, demographic and clinical measurements with confounding variables from 337 patients for EndoCAb IgM and IgG and 272 for EndoCAb IgA. IL-6 and CRP measurements were taken from a subset of these patients (for numbers and results see Table 3.4)

3.5.1 EndoCAb IgM and clinical outcome

A full dataset of preoperative IgM and potential confounding variables (EuroSCORE, Cardiopulmonary bypass times, NYHA scores) was measured in 337 patients. Their characteristics are shown in table 3.1. Those patients with a low preoperative EndoCAb IgM (the lowest quartile, <32.3 MU/mL) had more complications (27.4% vs 13.8%; p= 0.007; Chi squared test) than those with higher EndoCAb IgM. They were also older (69 vs 64 years; p<0.01), had higher EuroSCORE values (3 vs 2; p = 0.03) but included fewer patients with hypertension (34.5% vs 52.6%; p = 0.004, table 3.2).

Characteristic n=337		Result
Age (years)		65 (59-71)
Gender (%, number of males)		81.6 (275)
EuroSCORE		2 (1-4)
Hypertensive (%, number)		48.1 (162)
Family History (%, number)	yes	54.6 (184)
	unknown	3.3 (11)
Preoperative HDL cholesterol (mmol)		1.2 (1-1.5)
Diabetes (%, number)	yes	18.7 (63)
	unknown	0.3 (1)
Smokers (%, number)	never smoked	22.6 (76)
	current smokers	15.7 (53)
	ex-smoker	61.4 (207)
	unknown	0.3 (1)
Preoperative NYHA grade (%, no with grade)	1	40.1 (135)
	2	43.3 (146)
	3	15.7 (53)
	4	0.9 (3)
Preoperative Ejection fraction (%, number)	>50%	71.5 (241)
	30-49%	21.7 (73)
	<30%	6.8 (23)
Preoperative EndoCAb (MU/mL)	IgM	57 (32-99)
	IgG	196 (101-381)
Cardiopulmonary bypass time (minutes)		66 (56-82)
Postoperative bleeding (%, number)		5.6 (19)
Length of ITU stay (days)*		2 (2-3)
Length of hospital stay (days)*		6 (5-8)
Length of hospital stay > 10 days (%, number)*		14.2 (48)
Mortality: total (%, number)		5.6 (19)
Mortality after hospital discharge (%, number)		2.3 (8)
Mortality in this hospital admission (%, number)		3.3 (11)
Complication^{+} (%, number)		17.2 (58)

Table 3.1 Characteristics of the 337 patients.

Data presented are median and interquartile ranges unless specified. Complications^{+} were defined as an in hospital death or a postoperative stay exceeding 10 days. *In those that survived to discharge i.e. survivors only. Abbreviations: CRP = C reactive protein; IL-6 = Interleukin 6; EndoCAb= Endotoxin core antibody; HDL cholesterol = High density Lipid Cholesterol; Preoperative NYHA class= Preoperative New York Heart Association Functional class (1 to 4, see appendix 1). For EuroSCORE see appendix 2.

Characteristic n=337		EndoCAb IgM <32.3 MU/mL	EndoCAb IgM >32.4 MU/mL	p value
Number of patients		84	253	
Age (years)		69 (63-74)	64 (57-71)	**<0.010**
Gender %, (number of males)		81.0 (68)	81.8 (207)	0.859
EuroSCORE		3 (1-4)	2 (1-4)	**0.030**
Hypertensive (%, number)		34.5 (29)	52.6 (133)	**0.004**
Family History (%, number)	yes unknown	51.2 (43) 2.4 (2)	55.7 (141) 3.6 (9)	0.344
Preoperative HDL cholesterol (mmol)		1.3 (1.1-1.5)	1.2 (1-1.5)	0.383
Diabetes (%, number)	yes unknown	19 (16) 0 (0)	18.6(47) 0.4 (1)	0.936
Smokers (%, number)	never smoked current smokers ex-smoker unknown	21.4(18) 10.7 (9) 67.9 (57) 0	22.9 (58) 17.4 (44) 59.3 (150) 0.4 (1)	0.538·
Preoperative NYHA grade (%, no with grade)	1 2 3 4	33.3 (28) 44 (37) 20.2 (17) 2.4 (2)	42.3 (107) 43.1 (109) 14.2 (36) 0.4 (1)	0.062·
Preoperative Ejection fraction (%, number)	>50% 30-49% <30%	69 (58) 23.8 (20) 7.1 (6)	72.3 (183) 20.9 (53) 6.7 (17)	0.580
Cardiopulmonary bypass time (minutes)		66 (55-78)	66 (56-83)	0.359
Postoperative bleeding (%, number)		9.5 (8)	4.3 (11)	0.075
Length of ITU stay (days)*		2 (2-3)	2 (2-3)	0.713
Length of hospital stay (days)*		7 (6-9)	6 (5-8)	0.081
Length of hospital stay > 10 days (%, number)*		22.6 (19)	11.5 (29)	**<0.010**
Mortality: total (%, number)		11.9 (10)	3.6 (9)	**<0.010**
Mortality after hospital discharge (%, number)		4.8 (4)	1.6 (4)	0.098
Mortality in this hospital admission (%, number)		7.1 (6)	2 (5)	**<0.010**
Complication⁺ (%, number)		27.4 (23)	13.8 (35)	**0.007**

Table 3.2 Patient characteristics by EndoCAb IgM 'threshold'.

Patients are classified according to whether the patient had a preoperative EndoCAb IgM more or less than 33.2 MU/mL. Data presented are the median and interquartile ranges unless specified. Complications⁺ were defined as an in hospital death or a postoperative stay exceeding 10 days. Continuous, bivariate variables and categorical variables were compared to IgM EndoCAb with the Mann-Whitney U, chi-squared tests and Kruskal-Wallis test· respectively. For abbreviations see the text of Table 3.1. *In those that survived to discharge i.e. survivors only.

Only low EndoCAb IgM (<32.3 MU/mL), EuroSCORE, Diabetes, age, New York Heart Association grade and preoperative cardiac ejection fraction were individually associated with development of a complication in a univariable binary logistic regression. New York Heart Association grade, age and cardiac ejection fraction were not included in the final model as they are components of the EuroSCORE.

The individual odds ratios and those in the final model of low EndoCAb IgM, EuroSCORE and Diabetes are shown in table 3.3. A low EndoCAb IgM remained independently associated with the development of a complication in a binary logistic regression analysis after adjusting for the effects of EuroSCORE and Diabetes; patients with a low EndoCAb IgM were 1.89 (95% confidence intervals 0.99 to 3.6) times as likely to have a complication as those with higher EndoCAb IgM (p=0.052).

Factor	unadjusted			adjusted		
	*OR	+95% CI	p=	* OR	+95% CI	p=
EuroSCORE	1.42	1.22- 1.64	<0.001	1.41	1.21- 1.64	<0.001
IgM EndoCAb	2.32	1.29- 4.35	0.005	1.89	0.99-3.57	0.052
Diabetes	2.33	1.23- 4.42	0.01	2.64	1.34- 5.24	0.005

Table 3.3: **Binary logistic regression of the effects of EuroSCORE, low EndoCAb IgM and Diabetes on complications after first time CABG.** The individual univariate unadjusted relationships (and their confidence intervals) between EuroSCORE, low EndoCAb IgM or Diabetes and complications are presented as odds ratios in the left hand columns. The odds ratios of complications in the final mutually adjusted model including all three variables are presented to the right ('adjusted'). For example, a patient with low EndoCAb IgM has 2.3 times more likely to have a complication than a patient with higher EndoCAb IgM. After taking into account the independent association of EuroSCORE and Diabetes, this likelihood is reduced to 1.89 (95% confidence intervals 0.99 to 3.6). Abbreviations: OR, Odds Ratio; +95% CI, 95% confidence interval of the odds ratio.

3.5.2 EndoCAb IgM and markers of postoperative inflammation

IL-6 and CRP was available in 212 and 192 patients respectively. In those patients, preoperative EndoCAb IgM overall as a continuous variable was positively albeit weakly associated with preoperative IL-6 (0.161, p=0.019 Spearman's correlation coefficient) but not at any other time.

The lowest preoperative EndoCAb IgM quartile (less than 32.3 MU/mL, which was associated with a poorer clinical outcome, above) was associated with a lower IL-6 at 6 hours (158 vs 209 pg/mL; p=0.015 Mann Whitney U test) shown in table 3.4 and figure 3.1. Patients with a low preoperative EndoCAb IgM (less than 32.3 MU/mL) had a lower IL-6 rise from 0 to 6 hours (153 vs 205 pg/mL; p=0.016) but a larger rise between 6 and 24 hours (34 vs -39 mg/mL; p=0.001).

A low preoperative EndoCAb IgM (less than 32.3 MU/mL) was not associated with a CRP difference at any timepoint (table 3.3). Patients with a low preoperative EndoCAb IgM (less than 32.3 MU/mL) had trends to a smaller rise in CRP from 0 to 24 hours (68 vs 80.5 mg/mL; p=0.058) and between 6 and 24 hours (68.9 vs 80.7 mg/mL; p=0.096) compared to patients with normal or high EndoCAb IgM.

	EndoCAb IgM		p value[+]
	<32.3 MU/mL	>32.4 MU/mL	
IL-6 (pg/mL)	n=51	n=161	
Preoperative	4.4 (2.7- 5.9)	4.7 (3.0- 6.4)	0.271
6 hour	158 (117- 238)	209 (135- 307)	**0.015**
24 hour	203 (108- 301)	165 (119- 246)	0.195
Δ 0 -6 hours	153 (112- 237)	205 (127- 304)	**0.016**
Δ 0 -24 hours	199 (104- 298)	160 (111- 242)	0.179
Δ 6 -24 hours	34 (-56- 120)	-39 (-150- 44)	**0.001**
CRP (mg/mL)	n=45	n=147	
Preoperative	1.79 (1.22- 4.14)	2.07 (0.85- 4.43)	0.893
6 hour	2.3 (1.34- 5.72)	2.62 (1.21- 5.56)	0.974
24 hour	72.4 (52.9- 95.9)	84 (67.1- 98.2)	0.104
Δ 0 -6 hours	-0.13 (-1.35-1.91)	0.04 (-0.75- 1.61)	0.604
Δ 0 -24 hours	68 (50.4- 92)	80.5 (64.5- 94.8)	0.058
Δ 6 -24 hours	68.9 (48.9- 93.3)	80.7 (65.3- 92.3)	0.096

Table 3.4 IL-6 and CRP classified by EndoCAb IgM in a subset of patients. IL-6 and CRP concentrations at baseline, 6 and 24 hours following cardiopulmonary bypass according to whether the patient's preoperative EndoCAb IgM was more or less than 32.3 MU/mL. Patients were only included that had IL-6 or CRP measured at all 3 timepoints. Data presented are the median and interquartile range. [+]Mann-Whitney U test. For *abbreviations* see the text of Table 3.1

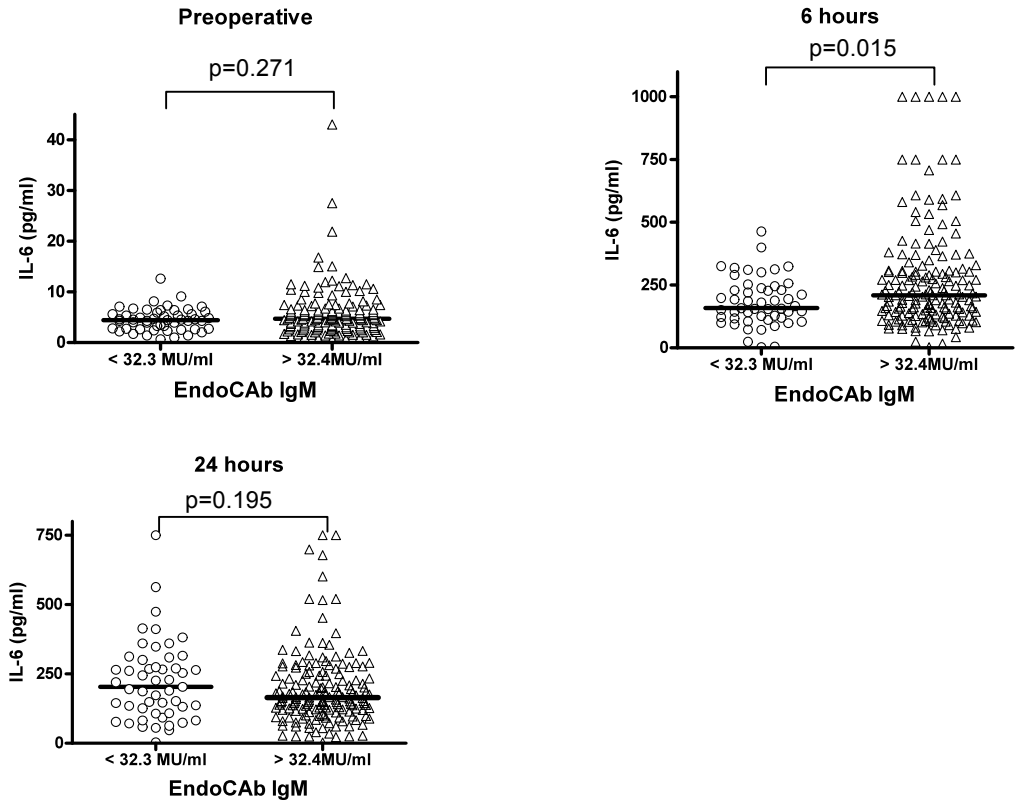

Figure 3.1 IL-6 classified by EndoCAb IgM in a subset of patients undergoing CABG. IL-6 concentrations at baseline, 6 and 24 hours following cardiopulmonary bypass according to whether the patient's preoperative IgM EndoCAb was more or less than 32.3 MU/mL. Patients were only included that had IL-6 measured at all 3 timepoints. Solid lines are the median. P values are calculated by the Mann-Whitney U test. For *abbreviations* see the text of table 3.1

3.5.3 EndoCAb IgM and secondary outcomes

No EndoCAb IgM measurement was significantly associated with any of the other secondary study outcomes: surrogate markers of preoperative cardiovascular dysfunction, postoperative haemorrhage or High Density Lipoprotein- cholesterol (table 3.2).

Increasing age was associated with a decreasing EndoCAb IgM (-0.18 p=0.001; Spearman's correlation coefficient): the median age of those patients with a low EndoCAb IgM (below 32.3 MU/mL) was greater than those with a higher EndoCAb IgM (69 vs 64 years p<0.01; Mann Witney U test).
Preoperative EndoCAb IgM was positively associated with IgA but not IgG EndoCAb (0.321, p<0.001 and 0.086, p=0.105 respectively with Spearman's correlation coefficient).

3.5.4 EndoCAb IgG and primary outcome

A full dataset of preoperative EndoCAb IgG and potential confounding variables (EuroSCORE, Cardiopulmonary bypass times, NYHA scores) was measured in 337 patients (table 3.1).

Overall, IgG EndoCAb was not associated with a subsequent complication (p=0.26; Mann Whitney U test**).** Patients with a low EndoCAb IgG (below 101 MU/mL; the lowest quarter) did not have more complications than those with normal or high EndoCAb IgG (16.7% vs 17.4% p=0.879 Mann Whitney U test), nor different IL-6 or CRP concentrations at any timepoint (tables 3.5 and 3.6). Between EndoCAb IgG values of 10 to 200 MU/mL there was no detectable threshold below which an increased complication rate could be detected.

3.5.5 EndoCAb IgG and secondary outcomes

EndoCAb IgG was not associated with any secondary outcome aside from there being more postoperative bleeding events in patients with low EndoCAb IgG (10.7 % vs 4% p= 0.02). Patients with low EndoCAb IgG were more likely to have a lower EuroSCORE but were more likely to give a family history of ischaemic heart disease (table 3.5).

Increasing age was associated with an increasing EndoCAb IgG (0.106 p=0.051; Spearman's correlation coefficient), but the ages of patients with a low EndoCAb IgG (below 101 MU/mL) was no different to those with a higher EndoCAb IgG (63 vs 65 years p<0.12; Mann Witney U test).

Preoperative EndoCAb IgG was positively associated with IgA but not IgM EndoCAb (0.197, p=0.001 and 0.092, p=0.092 respectively with Spearman's correlation coefficient).

Characteristic n=337		EndoCAb IgG <101 MU/mL	EndoCAb IgG >101.1 MU/mL	p value
Number of patients		84	253	
Age (years)		63 (58-71)	65 (59-72)	0.120
Gender (%, number of males)		84.5 (71)	08.4 (204)	0.426
EuroSCORE		2 (1-4)	3 (1-4)	**0.046**
Hypertensive (%, number)		46.4 (39)	48.6 (123)	0.706
Family History (%, number)	yes unknown	64.3 (54) 4.8 (4)	51.4 (130) 2.8 (7)	**0.022**
Preoperative HDL cholesterol (mmol)		1.2 (1-1.5)	1.2 (1-1.5)	0.419
Diabetes (%, number)	yes unknown	15.5 (13) 0	19.8 (50) 0.4 (1)	0.375
Smokers (%, number)	never smoked current smokers ex-smoker unknown	16.7 (14) 19 (16) 64.3 (54) 0	24.5 (62) 14.6 (37) 60.5 (153) 0.4 (1)	0.110
Preoperative NYHA grade (%, no with grade)	1 2 3 4	34.5 (29) 46.4 (39) 17.9 (15) 1.2 (1)	41.9 (106) 42.3 (107) 15 (38) 0.8 (2)	0.226
Preoperative Ejection fraction (%, number)	>50% 30-49% <30%	76.2 (64) 15.5 (13) 8.3 (7)	70 (177) 23.7 (60) 6.3 (16)	0.363
Cardiopulmonary bypass time (minutes)		64 (50 -81.8)	67 (58-82)	0.105
Postoperative bleeding (%, number)		10.7 (9)	4 (10)	**0.020**
Length of ITU stay (days)*		2 (2-4)	2 (2-2)	0.064
Length of hospital stay (days)*		6 (5-8)	6 (5-8)	0.238
Length of hospital stay > 10 days (%, number)*		14.3 (12)	14.2 (36)	0.991
Mortality: total (%, number)		6 (5)	5.5 (14)	0.886
Mortality after hospital discharge (%, number)		3.6 (3)	2 (5)	1.000
Mortality in this hospital admission (%, number)		2.4 (2)	3.6 (9)	0.886
Complication^{+} (%, number)		16.7 (14)	17.4 (44)	0.879

Table 3.5 Patient characteristics by EndoCAb IgG 'threshold'.

Patients are classified according to whether the patient had a preoperative EndoCAb IgG more or less than 101 MU/mL. The median and interquartile ranges are presented unless specified. Complications^{+} were defined as an in hospital death or a postoperative stay exceeding 10 days. Continuous, bivariate variables and categorical variables were compared to IgG EndoCAb with the Mann-Whitney U, chi-squared tests and Kruskal-Wallis test respectively. For abbreviations see the text of table 3.1. *In those that survived to discharge i.e. survivors only.

Cytokine	EndoCAb IgG <101 MU/mL	EndoCAb IgG >101.1 MU/mL	p value[+]
IL-6 (pg/mL)	n=49	n=163	
Preoperative	4.6 (2.7-6.3)	4.6 (2.9-6.3)	0.835
6 hour	161 (118-281)	204 (134-306)	0.100
24 hour	184 (133-268)	166 (112-264)	0.554
Δ 0 -6 hours	157 (112-272)	200 (126-303)	0.097
Δ 0 -24 hours	179 (129-263)	160 (106-260)	0.555
Δ 6 -24 hours	-2.3 (-95-102)	-32 (-132- 56)	0.185
CRP (mg/mL)	n=44	n=148	
Preoperative	1.8 (1.0-4.5)	1.9 (1.2-5.6)	0.742
6 hour	3.2 (1.3-5.6)	2.6 (1.2-5.6)	0.698
24 hour	79.2 (59.6-97.7)	83.1 (66.6-97.5)	0.663
Δ 0 -6 hours	0.1 (-1.1-2.3)	-0.1 (-1 (1.7)	0.736
Δ 0 -24 hours	75.2 (58.1-95)	80 (64.8-92.5)	0.702
Δ 6 -24 hours	74.4 (57.9-94.7)	77.3 (63.5-92.7)	0.647

Table 3.6 IL-6 and CRP classified by EndoCAb IgG in a subset of patients. IL-6 and CRP concentrations at baseline, 6 and 24 hours following cardiopulmonary bypass according to whether the patient's preoperative EndoCAb IgG was more or less than 32.3 MU/mL. Patients were only included that had IL-6 or CRP measured at all 3 timepoints. Data presented are the median and interquartile range. [+]Mann-Whitney U test. For *abbreviations* see the text of table 3.1

3.5.6 EndoCAb IgA

Preoperative IgA was measured in 318 patients. Overall as a continuous variable EndoCAb IgA was not associated with a subsequent complication (p=0.298; Mann Whitney U test). However those patients with a very low preoperative EndoCAb IgA (<34.3 MU/mL; n=31) had significantly more complications (32% vs 20%; p= 0.035; chi-squared test) than those with normal or high EndoCAb IgA. This effect did not persist however once the 46 patients who had *not* had a full dataset of potential confounding variables (EuroSCORE, Cardiopulmonary bypass times, NYHA scores) were excluded (28.1% vs 18.3%; p=0.155, n=272, tables 3.7 and 3.8). IgA was not associated with any of the potential confounding variables, any marker of cardiovascular dysfunction, with IL-6 or CRP at any timepoint (tables 3.8 and 3.9).

Preoperative EndoCAb IgA was positively associated with IgM but not IgG EndoCAb (0.321, p<0.001 and 0.086, p=0.105 respectively with Spearman's correlation coefficient). Increasing age was not associated with IgA EndoCAb (-0.167 p =0.234; Spearman's correlation coefficient).

Characteristics n=272		Result
Age (years)		65.4 (58.5-71)
Gender (%, number of males)		82.4 (224)
EuroSCORE		2 (1-4)
Hypertensive (%, number)		48.5 (132)
Family History (%, number)	yes	55.9 (152)
	unknown	3.3 (9)
Preoperative HDL cholesterol (mmol)		1.2 (1-1.4)
Diabetes (%, number)	yes	17.8 (48)
	unknown	0.7 (2)
Smokers (%, number)	never smoked	22.4 (61)
	current smokers	15.1 (41)
	ex-smoker	61.8 (168)
	unknown	0.7 (2)
Preoperative NYHA grade (%, no with grade)	1	39 (106)
	2	43.4 (118)
	3	16.5 (45)
	4	1.1 (3)
Preoperative Ejection fraction (%, number)	>50%	69.9 (190)
	30-49%	22.8 (62)
	<30%	7.4 (20)
Preoperative EndoCAb (MU/mL)	IgM	57.7 (32.4 (94.6)
	IgG	198.8 (99.6-380)
	IgA	74.7 (48.5 -115.4)
Cardiopulmonary bypass time (minutes)		57 (67-81)
Postoperative bleeding (%, number)		17.6 (48)
Length of ITU stay (days)*		2 (2-3)
Length of hospital stay (days)*		6 (5-8)
Length of hospital stay > 10 days (%, number)*		15.4 (42)
Mortality: total (%, number)		6.3 (17)
Mortality after hospital discharge (%, number)		2.9 (8)
Mortality in this hospital admission (%, number)		3.3 (9)
Complication^{+} (%, number)		19.5 (53)

Table 3.7 Characteristics of the 272 patients in whom EndoCAb IgA and confounders were measured. The median and interquartile ranges are presented unless specified. Complications^{+} were defined as an in hospital death or a postoperative stay exceeding 10 days. *In those that survived to discharge i.e. survivors only. Abbreviations: CRP = C reactive protein; IL-6 = Interleukin 6; EndoCAb= Endotoxin core antibody; HDL cholesterol = High density Lipid Cholesterol; Preoperative NYHA class= Preoperative New York Heart Association Functional class (1 to 4).

Characteristics n=272		EndoCAb IgA <34.3 MU/mL	EndoCAb IgA >34.4 MU/mL	p value
Number of patients		31	241	
Age (years)		64.9 (59.7 -70.8)	65.8 (59 -70.7)	0.119
Gender (%, number of males)		87.1 (27)	81.7 (197)	0.487
EuroSCORE		3 (1- 4)	2 (1- 4)	0.421
Hypertensive (%, number)		22.5 (7)	51.9 (125)	**0.050**
Family History (%,number)	yes unknown	67.7 (21) 6.5 (2)	51.4 (131) 2.9 (7)	0.092
Preoperative HDL cholesterol (mmol)		1.2 (1- 1.4)	1.2 (1- 1.4)	0.635
Diabetes (%, number)	yes unknown	6.5 (2) 3.2 (1)	19.1 (46) 0 (0)	0.105
Smokers (%, number)	never smoked current smokers ex-smoker or unknown	5.9 (2) 73.5 (25) 20.6 (7)	16.7 (46) 60.4 (166) 22.9 (63)	0.370
Preoperative NYHA grade (%, no with grade)	1 2 3 4	32.3 (10) 48.4 (15) 16.1 (5) 3.2 (1)	39.8 (96) 42.7 (103) 16.6 (40) 0.8 (2)	0.440
Preoperative Ejection fraction (%, number)	>50% 30-49% <30%	64.5 (20) 22.6 (7) 4 (12.9)	70.5 (170) 22.8 (55) 6.6 (16)	0.400
Cardiopulmonary bypass time (minutes)		68 (60- 75)	68 (56- 83)	0.925
Postoperative bleeding (%, number)		16.1 (5)	5.4 (13)	**0.020**
Length of ITU stay (days)*		2 (2- 4.5)	2 (2- 2)	**0.028**
Length of hospital stay (days)*		7 (6- 11)	6 (5- 8)	0.495
Length of hospital stay > 10 days (%, number)*		22.6 (7)	14.1 (34)	0.126
Mortality: total (%, number)		9.7 (3)	5.8 (14)	0.403
Mortality after hospital discharge (%, number)		3.2 (1)	1.7 (4)	0.088
Mortality in this hospital admission (%, number)		6.5 (2)	4.1 (10)	0.558
Complication^{+} (%, number)		29 (9)	18.3 (44)	0.155

Table 3.8 Patient characteristics by EndoCAb IgA 'threshold'.

Patients are classified according to whether the patient had a preoperative EndoCAb IgA more or less than 34.3 MU/mL. Data presented are the median and interquartile ranges unless specified. Complications^{+} were defined as an in hospital death or a postoperative stay exceeding 10 days. Continuous, bivariate variables and categorical variables were compared to IgM EndoCAb with the Mann-Whitney U, chi-squared tests and Kruskal-Wallis test respectively. For abbreviations see the text of Table 3.1. *In those that survived to discharge i.e. survivors only.

	EndoCAb IgA		p value[+]
	<34.3 MU/mL	>34.3 MU/mL	
IL-6 (pg/mL)	n=23	n=147	
Preoperative	3.9 (2.7- 7.3)	4.7 (2.5- 6.6)	0.146
6 hour	186 (129- 249)	179 (113- 290)	0.76
24 hour	175 (108- 229)	173 (118- 282)	0.57
CRP (mg/mL)	n=18	n=128	
Preoperative	2.7 (0.67- 6.7)	1.8 (1- 4.6)	0.93
6 hour	4.1 (1.29- 6.3)	2.6 (1.2- 5.5)	0.37
24 hour	79.8 (65.2- 101)	78.1 (56.2- 93)	0.63

Table 3.9 IL-6 and CRP classified by EndoCAb IgA in a subset of patients. IL-6 and CRP concentrations at baseline, 6 and 24 hours following cardiopulmonary bypass according to whether the patient's preoperative EndoCAb IgA was more or less than 34.3 MU/mL. Patients were only included that had IL-6 or CRP measured at all 3 timepoints. Data presented are the median and interquartile range. [+]Mann-Whitney U test. For *abbreviations* see the text of Table 3.1

3.6 Discussion

This study has shown that for patients undergoing elective coronary artery bypass grafting for the first time, a low IgM EndoCAb is associated with a poorer outcome. This is consistent with the work of the previous studies showing an association between EndoCAb and outcome after surgery. Furthermore, contrary to my expectation, we found a low IgM EndoCAb to be associated with a *reduced* rise in interleukin-6 in the initial postoperative period compared to patients with higher IgM EndoCAb, six hours after cardiopulmonary bypass (figure 3.1 and table 3.4).

There are several interesting aspects to this study. This is one of the first studies to investigate a possible link between IgA EndoCAb and postoperative outcome. Secreted IgA is thought to be in the first line of defence against enterobacteriae, and whilst the load of secreted IgA may not relate to the plasma level, there have been very few studies examining the effect of serum IgA concentrations on outcome in non-oral conditions (Chia *et al* 2000; Engstrom *et al* 2002; Fagarasan and Honjo 2003). The association between low IgA EndoCAb (<34.3 MU/mL) and more complications (p=0.035) was reduced once individuals without a full dataset were excluded to reduce potential bias and examine for confounding variables (table 3.7).

Inflammatory Markers

We found that patients with a low EndoCAb IgM had *lower* IL-6 concentrations at 6 hours (p=0.015) but a larger rise in IL-6 (p=0.001) between 6 and 24 hours compared to those with higher EndoCAb IgM (table 3.4). This group of patients also had more complications. This suggests that low concentrations of EndoCAb IgM are associated with a *reduced* initial inflammatory response which has subsequently increased by 24 hours (as judged by IL-6 concentrations) whilst the opposite is true of those with normal-high EndoCAb IgM: their IL-6 peaks at 6 hours and had fallen by 24 hours (figure 3.1 and table 3.4). As this was not the original intent of the study any retrospective analysis must be interpreted with caution especially as IL-6 and CRP were measured in a subset of patients only.

Many investigators have measured CRP or IL-6 as manifestations of systemic inflammation. Preoperative CRP concentrations relate to short term outcome and long term survival in patients with ischaemic heart disease whilst intra or post-operative IL-6 concentrations relate to clinical manifestations of SIRS, complications or death after a variety of insults including surgery, pneumonia and acute renal failure and appears to be related to the degree of trauma (Biancari *et al* 2003; Kolsuz *et al* 2003; Lenderink *et al* 2003; Mythen *et al* 1993; Schwenk *et al* 2000; Simmons *et al* 2004).

Age

Increasing age was associated with decreasing IgM EndoCAb: this is a new finding not commented on in the previous studies (table 3.2). There is very little data on IgM antibodies to specific antigens. Most of the literature is concerned with IgG responses to Pneumococcal vaccination as IgG concentrations are IgG levels are thought to relate to protection against invasive Pneumococcal disease (Moberley *et al* 2008). However, some studies have found the elderly to have similar total IgM concentrations to younger adults whilst others have found them to be lower (Challacombe *et al* 1995; De Nardin *et al* 1991), so the reduction in IgM EndoCAb may just be a reflection of a reduced total IgM. Most studies indicate that older adults have similar pre-vaccine IgG concentrations to those of younger cohorts. Interestingly older volunteers have a different response when challenged *in-vivo* with endotoxin: they had a longer fever, more hypotension and larger rises in CRP (Artz *et al* 2003; Krabbe *et al* 2001a; Krabbe *et al* 2001b). Whilst some of this variation appears in serum-free *in-vitro* systems it is possible that part of the altered response of elderly people may be due lower EndoCAb IgM concentrations in older volunteers (Von Haehling *et al* 2003).

Study limitations

There are several potential limitations to this retrospective study; a number of factors need to be considered when interpreting this data. The CASIS study was originally designed to examine the effects of IL-6 gene polymorphisms on IL-6 levels, so any retrospective analysis must be regarded cautiously.

To limit the amount of blood in the original parent study, samples were taken prior to surgery and 6 and 24 hours after the start of cardiopulmonary bypass, similar to other published studies (Damgaard *et al* 2010; Diegeler *et al* 2000; Weis *et al* 2009). Rothenburger at al sampled at 11 perioperative timepoints and found that both IL-6 and IL-8 both peaked within 2 hours of our 6 hour sample. (Rothenburger 2001). However it is possible that, as fewer samples of inflammatory markers were taken, we may have missed significant differences in timing and concentrations between those patients with low or normal/high EndoCAb IgM concentrations.

A possible concern in all studies is selection bias. The design of the parent study ('CASIS') specifically excluded patients from 6 and 24 hour samples if one of a set of pre-defined intra or postoperative complications occurred. Therefore some blood tests in patients that experienced complications (and were therefore excluded from the main analysis) are missing (i.e. 6 or 24 hour IL-6 and CRP), leading to smaller numbers. However, *all patients that gave consent* and had initial blood samples taken for EndoCAb were followed up to death, complication or discharge from hospital (including our primary outcome), even though they may not have had a full range of 6 and 24 hour samples taken. For this reason the CRP and IL-6 results were considered separately as a subgroup and the results regarded as hypothesis generating. The majority of patients that were excluded from the parent study still had the full range of potential confounding variables measured (see table 3.1).

One potential limitation is that fact that as no control IgM (i.e. an IgM to another antigen) and total IgM were not measured we do not know if the relationship between EndoCAb IgM and outcome is specific and unique or present with other IgM antibodies.

Some investigators have criticised the choice of length of stay as a marker of postoperative morbidity (Grocott *et al* 2007). Currently approximately 20% of patients undergoing CABG in the UK experience a postoperative hospital stay more then 10 days (Keogh and Kinsman 2009). Our primary outcome (death in hospital-after a continuous period of hospitalisation following surgery -or a

postoperative stay exceeding 10 days) was used for several reasons. Two previous studies involving EndoCAb (from the USA) have used these endpoints, allowing comparisons with them (Bennett-Guerrero *et al* 1997; Bennett-Guerrero *et al* 2001b). Secondly clinicians at our institution regard 10 days as a length of stay generally associated with a clinically important complication rather than a social issue related to suitability for discharge from hospital. There is evidence that the majority of patients with a length of stay of greater than 10 days suffered complications that could be consistent with an exaggerated mediated inflammatory response to endotoxin (Welsby *et al* 2002). We included death in our assessment of morbidity because it is an important patient-centred endpoint, may preclude the diagnosis of other serious morbidities and may shorten the length of stay (Higgins *et al* 1997).

3.7 Conclusion

This study has shown that, even in the lowest-risk group undergoing adult cardiac surgery, a low EndoCAb IgM is adversely associated with outcome. A low preoperative EndoCAb IgM was also associated with a lower IL-6 level 6 hours following cardiopulmonary bypass in this dataset. EndoCAb IgA is associated with outcome following surgery though to a lesser extent than EndoCAb IgM. This study has not answered the question as to *why* EndoCAb IgM is associated with outcome, or if it is a causal relationship. These questions will be investigated in chapter 4.

3.8 Further Work

This chapter, whilst broadly confirming that low EndoCAb IgM is associated with a poorer postoperative outcome, only touches on the possible mechanism.

It is possible that the association between clinical outcome and antibody level may hold for several antibodies, or that EndoCAb IgM may be associated with other antibodies. An initial piece of work to investigate this may be to answer the question ‘is EndoCAb IgM independent of other antibodies?’ Unfortunately in this CABG cohort that was not possible as it would have required new consent from every patient, but it was possible to explore that in chapter 4 in a different cohort.

Another pathway is to investigate whether patients with low EndoCAb are more likely to have the systemic inflammatory response syndrome (SIRS) after an insult. Because cardiac surgery induces SIRS in the many patients (as judged by conventional clinical criteria) cardiac surgical patients are not an ideal group to study.

A third line of enquiry may be to ask: Is there anything different about the group of people with a lower EndoCAb IgM compared to those with normal/high concentrations? Certainly this study has shown they are older, with a higher EuroSCORE, whilst one small study found lower concentrations in those with advanced heart failure (Bolger *et al* 2001). It may be that people with low EndoCAb IgM have poorer exercise tolerance because of worse cardio-respiratory function. This hypothesis could be tested with cardiopulmonary exercise testing (CPET).

Lastly, it would be ideal to repeat this study in a prospective fashion with more data collection points to ensure that the correct cytokine peak was not missed, to exclude bias in selected subpopulations and to measure other 'neutral' antibodies.

Chapter 4

EndoCAb IgM and ‘Natural antibody’

4.1 Introduction

In chapter 3 I showed that, consistent with previous studies, low preoperative EndoCAb IgM was associated with a poor clinical outcome after 1st time cardiac surgery (see table 1.4). It has always been assumed that this association is causative as patients with low EndoCAb IgM have less endotoxin-neutralising antibody and might therefore be expected to experience more of the inflammatory effects of endotoxin, leading to complications (Bennett-Guerrero *et al* 2001a). However, for a variety of reasons this may be too simplistic. Firstly, no study has validated or demonstrated the complete chain of events described in figure 1.2 linking tissue trauma, reduced gastrointestinal blood flow, endotoxaemia and consequent inflammation-induced organ dysfunction. Secondly, as suggested by the results in chapter 3, patients with low EndoCAb IgM do not have higher postoperative inflammatory markers at than those with higher EndoCAb concentrations. This suggests that whilst endotoxin induced-inflammation may be attenuated by EndoCAb IgM, the response is not a simple dose-dependent interaction. Thirdly a synthetic lipopolysaccharide compound E5564 (Eritoran, Eisai Medical Research, New Jersey, USA) that acts as a lipopolysaccharide antagonist at the Toll-4 receptor and attenuates the inflammatory response in endotoxin-challenged volunteers has failed to significantly alter any clinical or inflammatory postoperative marker in patients undergoing cardiac surgery (Bennett-Guerrero *et al* 2007; Hawkins *et al* 2002; Lynn *et al* 2003; Mullarkey *et al* 2003; Rossignol and Lynn 2002; Wong *et al* 2003). Whilst Toll-4 receptors are not the only route through which endotoxin induces inflammation, these studies suggest that endotoxin may not be a major direct cause of the postoperative inflammatory response, and the improved clinical outcome described previously in patients with high EndoCAb IgM may not be directly attributable to endotoxin neutralisation.

One non-clinical approach to studying EndoCAb's effects may be to make a 'pure' concentrate, and to examine its effects in an *in-vitro* system. Manufacturing an 'EndoCAb' concentrate is technically difficult, would not represent a real life scenario and was outside the scope of expertise I had available for this thesis. EndoCAb is a mixture of antibodies (at least 4 as the ELISA uses an equimolar mixture of 4 endotoxin cores), so a 'monoclonal

EndoCAb' strictly cannot be made. A second option is to make an IgM fraction from serum taken from volunteers with a range of IgM EndoCAb levels. I had tried to fractionate a serum from volunteers with high or low EndoCAb IgM to use in *in-vitro* studies. These *in-vitro* studies involved both endotoxin stimulation of whole blood (with serial cytokines as the outcome) and Flow Cytometric techniques ('FACS' using Neutrophil CD11b as the measure of cell activation). Unfortunately, whilst I mastered the *in-vitro* techniques and was able to make an IgM fraction from serum it was not possible for me to do this in a sterile environment- thus rendering any cell stimulation studies pointless.

Because of this I decided to look at the similarities between EndoCAb IgM and a panel of natural IgM's and to try to see how much EndoCAb IgM had the characteristics of a natural IgM. I reasoned that, if EndoCAb and natural IgM levels were linked, that reduced the likelihood that the association between EndoCAb IgM and postoperative outcome was causal or specific to EndoCAb IgM. It also points to a new mechanism. This alternative viewpoint proposes that EndoCAb IgM is just a surrogate marker, simply associated with other factors that are *themselves* the cause of an improved clinical outcome.

These natural antibodies have had a variety of functions ascribed to them (see chapter 1.3.2). They arise from germline DNA, may recognise 'self' antigens, are often 'promiscuous', can be IgG or IgM and have been detected in the absence of specific external antigens. The origin of these human natural antibodies is not well defined, but is most similar to the murine B-1a B-cell populations in the pleural and peritoneal cavities. This population forms the preimmune repertoire of natural antibody with low-grade polyspecificity for both bacterial and self antigens. The natural antibody group includes antibodies directed against A and B red blood cell antigens, antibodies against the terminal disaccharide Gal α1-3 Gal and against cell wall phospholipids such as phosphorylcholine, phosphatidylserine and phosphatidylcholine (Alaniz *et al* 2004; Baumgarth *et al* 2005; Binder and Silverman 2005; Buonomano *et al* 1999; Cabiedes *et al* 2002; Galili *et al* 1987a; Galili *et al* 1988; Hamadeh *et al* 1992; Janczuk *et al* 1999; Parker *et al* 1996; Peng *et al* 2005; Rieben *et al*

1991; Rieben *et al* 1992; Rieben *et al* 1995; Sharma *et al* 2001; Spalter *et al* 1999; Su *et al* 2005; Teranishi *et al* 2002; Weiser *et al* 1998).

Their functions have been described as widely as clearing apoptotic cells and bacterial antigens, mediating ischaemia-reperfusion injury, participating in immune defence prior to the development of specific antibody, regulating the immune response and confining enteric bacterial infectious injury to prevent systemic spread (Boes *et al* 1998b; Chen *et al* 2009; Melero *et al* 1997; Peng *et al* 2005; Reid *et al* 1997; Stahl and Sibrowski 2003; Zhang *et al* 2008). Many of these functions could contribute to regulating the inflammatory response after surgery and affect outcome.

EndoCAb has been previously assumed to be an 'immune' antibody, specific for endotoxin and generated by the adaptive immune system after exposure to a range of exogenous endotoxins in the environment after birth however it may also be part of the natural or pre-immune antibody pool that facilitates early protection against infection and regulation of the inflammatory and adaptive immune response.

In order to further understand the mechanism of EndoCAb IgM's protective effects after surgery, I wanted to investigate whether EndoCAb IgM was part of a polyreactive natural antibody pool or a specific adaptive one. If EndoCAb IgM had no features in common with natural IgM, this strengthens the idea that it may play a specific role in modulating postoperative outcome. However, if EndoCAb IgM and natural IgM concentrations were strongly associated for example, concerted efforts into raising EndoCAb in order to reduce perioperative morbidity may be flawed (Bennett-Guerrero *et al* 2000; Hamilton-Davies *et al* 1996) as the mechanism of protection may not necessarily involve EndoCAb IgM (Bennett-Guerrero *et al* 2000; Hamilton-Davies *et al* 1996).

Hence I decided to try to evaluate what characteristics EndoCAb IgM shares with the natural IgM antibody pool.

In this series of linked studies described below I investigated the relationship between serum IgM binding to EndoCAb IgM and a panel of natural antigens. I then looked at the EndoCAb binding properties of human monoclonal antibodies that were characterised as natural antibodies (Baxendale *et al* 2008).

Firstly, in study 4A, I measured concentrations of the natural IgM panel, EndoCAb IgM and total IgM in blood donor volunteers. This allowed me to see how closely EndoCAb and natural IgM concentrations are linked, gave me baseline reference concentrations, observe the spread of values, and potentially, compare with a clinical cohort. If EndoCAb IgM concentrations are associated with other 'natural IgM' antibody concentrations I reasoned that it may be the case that natural IgM concentrations might also be associated with postoperative outcome. Secondly in study 4B I examined the binding of germline IgM human hybridomas to endotoxin and a panel of endogenous antigens to which natural antibodies are known to bind (Baxendale *et al* 2008). These hybridomas had variable immunoglobulin genes in germline (i.e. non-mutated) configuration and shared many characteristics with natural antibodies from the pre-immune and early post natal period (Baker and Ehrenstein 2002). Thirdly, as natural IgM antibodies are said to be measurable in human umbilical cord blood, I measured EndoCAb IgM and natural IgM concentrations in the blood of mothers and their newborn (study 4C). Lastly, as it has been hypothesized that natural IgM concentrations are a relative constant throughout life, I measured EndoCAb M and natural IgM concentrations over a 6 month period (study 4D).

4.2 Hypothesis

EndoCAb IgM shares many characteristics with the wider natural IgM antibody group.

4.3 Aims

Study 4A

To measure the relationship between EndoCAb IgM and IgM binding to blood groups antigens A and B, Gal α1-3 Gal, phosphorylcholine and total IgM concentrations in a blood donor population.

Study 4B

To characterise the binding of seven germline IgM human hybridomas to endotoxin core and the natural antigen panel.

Study 4C

To measure EndoCAb IgM and natural IgM concentrations in the blood of mothers and the umbilical cord veins of their newborn.

Study 4D

To measure EndoCAb M and natural IgM concentrations at 0, 2 and 6 months in human volunteers.

4.4 Methods

These studies were performed at the departments of Immunobiology, Infectious Disease and Microbiology and Portex Units, Institute of Child Health, University College London.

Populations

The samples for study 4A were taken from established adult ambulant blood plasmapheresis donors presenting to two London National Blood Service centres as part of a larger study into the development of a plasma product high in antibody activity to endotoxin. After ethical approval (North West MREC 01/8/58) donors who had given informed consent were screened by the NHS blood transfusion screening programme consisting of a focussed history and testing for evidence of exposure to Human Immunodeficiency Virus 1 and 2, Hepatitis B and C, Syphilis, Human T-cell lymphotropic Viruses 1 and 2 and (where indicated) Malaria and Trypanosoma cruzi. Donors were excluded if they had any known autoimmune condition, any haematological malignancy or were taking any drugs to reduce their systemic immune response. Blood for studies 4B and 4E were taken from healthy ambulant male and female

volunteers. Blood from mothers and their newborn (study 4C) was obtained from a 'control' arm of a separate study investigating the role of infection and inflammation in neonatal brain injury (UCL/UCLH Joint Research Committee A' REC ref number 03/0179).

Study course

Study 4A: Concentrations in a blood donor population

After informed consent, a single 5mL clotted serum sample was taken off at the initial stage of plasmapheresis along with other samples collected by the National Blood Service. Demographic data (age and gender) and ABO blood group were supplied by the National Blood Service. Donors were free to eat and drink at any time.

Study 4B: IgM human Hybridomas

The human germ-line IgM monoclonal antibodies described in this study were a kind gift from Dr Helen Baxendale, Institute of Child Health, University College London. They had been generated from a series of human hybridomas derived from Peripheral Blood Mononuclear Cells isolated from four healthy volunteers on day 7 (n=4) and day 28 (n=1) following immunisation with a single dose of pneumococcal 23 valent plain polysaccharide vaccine, 'Pneumovax' (Sanofi Pasteur MSD, Maidenhead, UK) or a 7 valent polysaccharide conjugate vaccine, 'Prevenar' (Wyeth, Maidenhead, UK) and were originally selected on the basis of reactivity to pneumococcal capsular polysaccharides (Baxendale *et al* 2000; Jessup *et al* 2000). Specificity analysis and immunoglobulin variable gene sequencing demonstrated that these antibodies were in germ-line configuration and had the antigen binding characteristics of natural antibodies (Baxendale *et al* 2008). The monoclonal antibodies were prepared from concentrated supernatants of clonal hybridoma cultures and IgM concentration was assessed by IgM ELISA as described previously. Reactivity of the IgM monoclonal antibodies with the endotoxin cores and the natural antigen panel was assessed by ELISA as described for human sera, standardised for IgM concentration based on IgM capture ELISA.

Study 4C: Mothers and their newborn

Mothers were approached at antenatal clinic, given information sheets and had a discussion with an investigator. When they subsequently came in at term either for a planned caesarian section or in labour they were asked if they were willing to give consent. The maternal blood sample was taken, spun and the plasma frozen and stored at this point. Once the mother had delivered (by either elective caesarian section, emergency caesarian section or vaginally) 5 mL of umbilical cord venous blood was taken as soon as practicable. Routinely collected clinical data was recorded.

Study 4D: Longitudinal concentrations

Blood was taken from 21 healthy ambulant volunteers at baseline, 2 and 6 months and the serum frozen for later analysis. Age, gender, vaccinations, travel outside the UK or illnesses 2 months prior to the study and during it were recorded.

Measurement of antibody concentrations

After venepuncture, blood was allowed to clot (unless specified) then spun at 3000rpm for 10 minutes and the serum removed. All samples were stored in 0.5mL in Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and frozen at $^{-}$80°C until subsequent analysis. Concentrations of blood group A, B, Galα1-3 Gal (gal), phosphorylcholine (Pc), total IgM and EndoCAb IgM were measured according to the methods detailed in Chapter 2 from the thawed samples.

Ethics

Ethical approval was obtained from the relevant Research Ethics Committee (North West MREC 01/8/58 and UCL/UCLH Joint Research Committee A' REC ref number 03/0179) and informed consent from each subject.

Statistics

Normal distribution of data was not assumed and was tested for by examining a histogram of the antibody concentrations and with the Kolmogorov–Smirnov test of the naïve and log-normalised data. Correlations between log-normalised antibody concentrations were tested with the Pearson product-moment

correlation coefficient. Antibody concentrations in studies 4A, B and D were tested with the Mann-Whitney U 2-tailed test. Linear regression was performed using the logged test antibody (A, B, gal, phosphorylcholine) as the dependent variable and EndoCAb IgM as the independent, firstly without, then with the addition of total IgM. Serial changes in concentrations between 3 timepoints were compared with the Friedman test. All statistical tests were calculated using SPSS (Statistical Package for the Social Sciences 16, Chicago, IL, USA).

4.5 Results

4.5.1 Study 4A Concentration in a blood donor population

Consent, demographic data and a full set of A, B, gal and phosphorylcholine, total IgM and EndoCAb IgM concentration was obtained from 179 blood donors. Their age, gender and ABO blood group characteristics are shown in table 4.1 and the antibody concentrations in table 4.2. There was a large concentration range in all antibodies measured (table 4.2). The distribution was positively skewed (but log-normalised) for all antibodies apart from total IgM, which was normally distributed. As expected, donors with blood groups O and B (i.e. no A antigen) had more A IgM antibodies than those with groups A and AB ($p<0.01$;t-test, table 4.2). Likewise donors with blood groups O and A (i.e. no B antigen) had higher B IgM antibody concentrations than those with blood groups B and AB. ($p<0.01$; t-test, table 4.2).

Characteristic		Result
Age: median (interquartile range) in years		47 (40-52)
Gender: % males, females		72, 28
ABO Blood group*: number (% of total)	O	84 (46.9)
	A	68 (38.0)
	AB	18 (10.1)
	B	9 (5.0)

Table 4.1: Basic demographics of the 179 blood donors. *This is similar to the overall UK blood group distribution of 44% O, 42% A, 10% AB and 4% B in the population (Blood Transfusion Service 2008).

Antibody		Median	Interquartile range	n =
A IgM (arbitrary units)	All	19.9	4.2-51.9	179
	O and B	50	7.9 – 424.2	93
	A and AB	3.9	2.4- 9.7	86
B IgM (arbitrary units)	All	13.9	6.3 - 23.7	179
	O and A	16.2	8.6-25.9	152
	B and AB	3.8	1.9 - 6.7	27
gal IgM (arbitrary units)		29.4	16.9- 47.8	179
phosphorylcholine IgM (arbitrary units)		28.6	18.1 - 45.9	179
Total IgM (mg/mL)		1.17	0.85- 1.52	179
EndoCAb IgM (MU/mL)		100.6	65.3 – 159.2	179

Table 4.2: IgM antibody concentrations in 179 blood donors. Median and interquartile range for A, B, gal, phosphorylcholine, total IgM and EndoCAb. Concentrations of the blood group antibodies A and B are shown for the whole donor group and according to their appropriate blood group.

To estimate whether patients with low EndoCAb IgM were also likely to have low concentrations of A, B, gal, and phosphorylcholine IgM, the donors were split up into 2 groups: those with EndoCAb IgM below or above 33.2 MU/mL. This EndoCAb IgM threshold was taken from Chapter 3 and represented the *level of EndoCAb IgM below which a poor postoperative outcome was more likely in that CABG cohort*. Analysed in this way (figure 4.1) there were statistically significant differences in the concentrations of A, B, gal, phosphorylcholine and total IgM antibodies in donors with low compared to those with normal-high EndoCAb IgM. Donors with EndoCAb IgM below 33.2 MU/mL had between 1.8 to 4 times lower concentrations of the natural antibody panel than those with higher EndoCAb IgM (figure 4.1).

There were significant associations between both IgM and EndoCAb IgM and the natural antibody panel A, B, gal, and phosphorylcholine. The association strength was greater between EndoCAb and the other antibodies (figures 4.2 and 4.3) than between total IgM and the other antibodies. In a linear regression model EndoCAb IgM concentrations were independently associated with concentrations of the other antibodies, after adjusting for total IgM (table 4.3). As shown in table 4.3, 36.8% to 43.7% of the variability in A, B, gal, and phosphorylcholine IgM is accounted for by this model that includes both EndoCAb and total IgM.

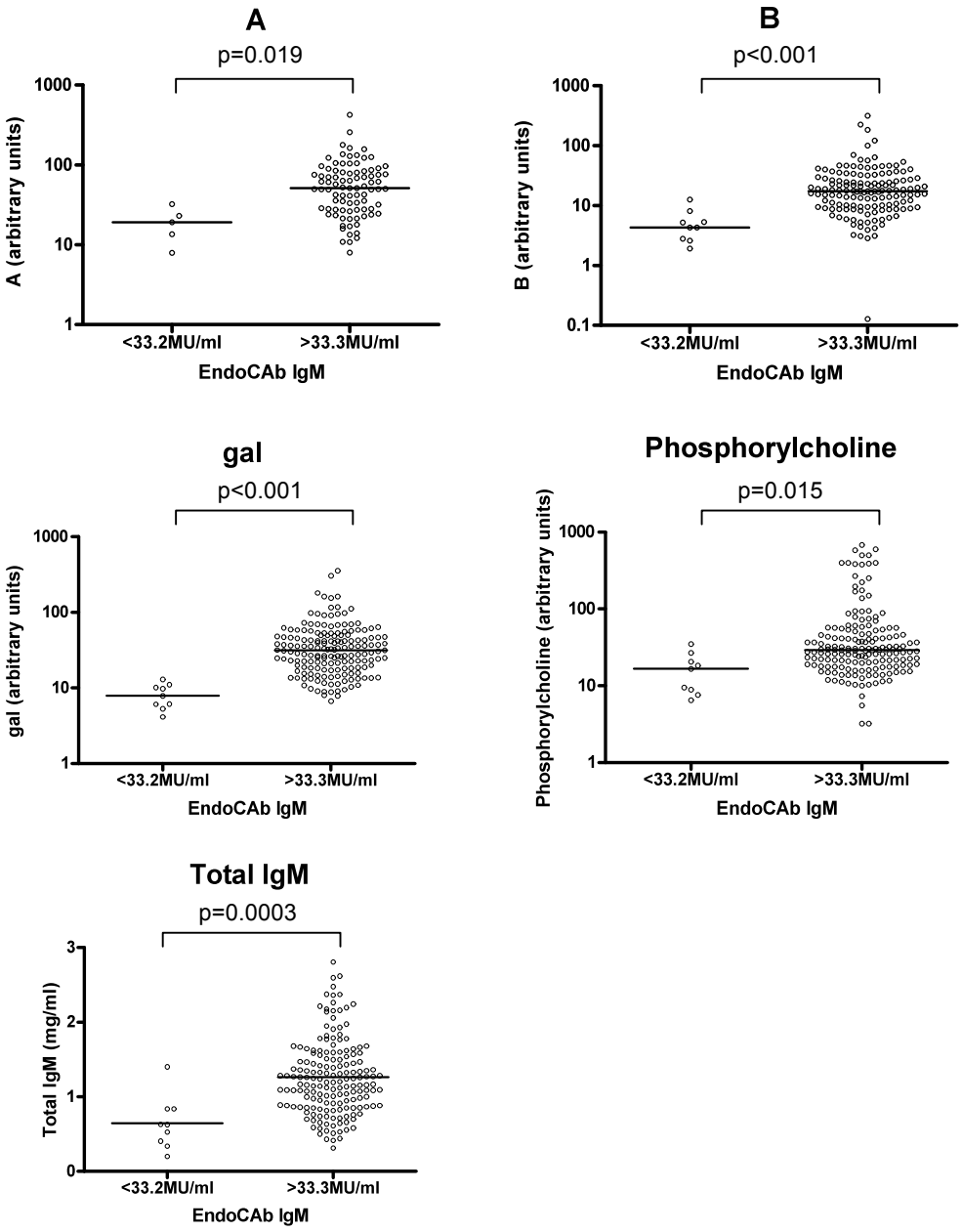

Figure 4.1: A, B, gal, phosphorylcholine or total IgM concentrations according to an EndoCAb IgM threshold. Concentrations of A, B, gal, phosphorylcholine and total IgM are shown according to whether the EndoCAb IgM level of that individual is less or greater than 33.2 MU/mL. All results apart from total IgM are shown on a log scale. Each circle represents the antibody concentrations of one donor. Only results for the relevant blood groups have been shown. i.e. values for donors with blood groups B and O for A IgM and groups A and O for B IgM. Lines are shown for the median values, apart for total IgM, where the mean is shown. 'P values' are shown for a Mann-Whitney U 2-tailed test apart from Total IgM, where an unpaired t-test is used.

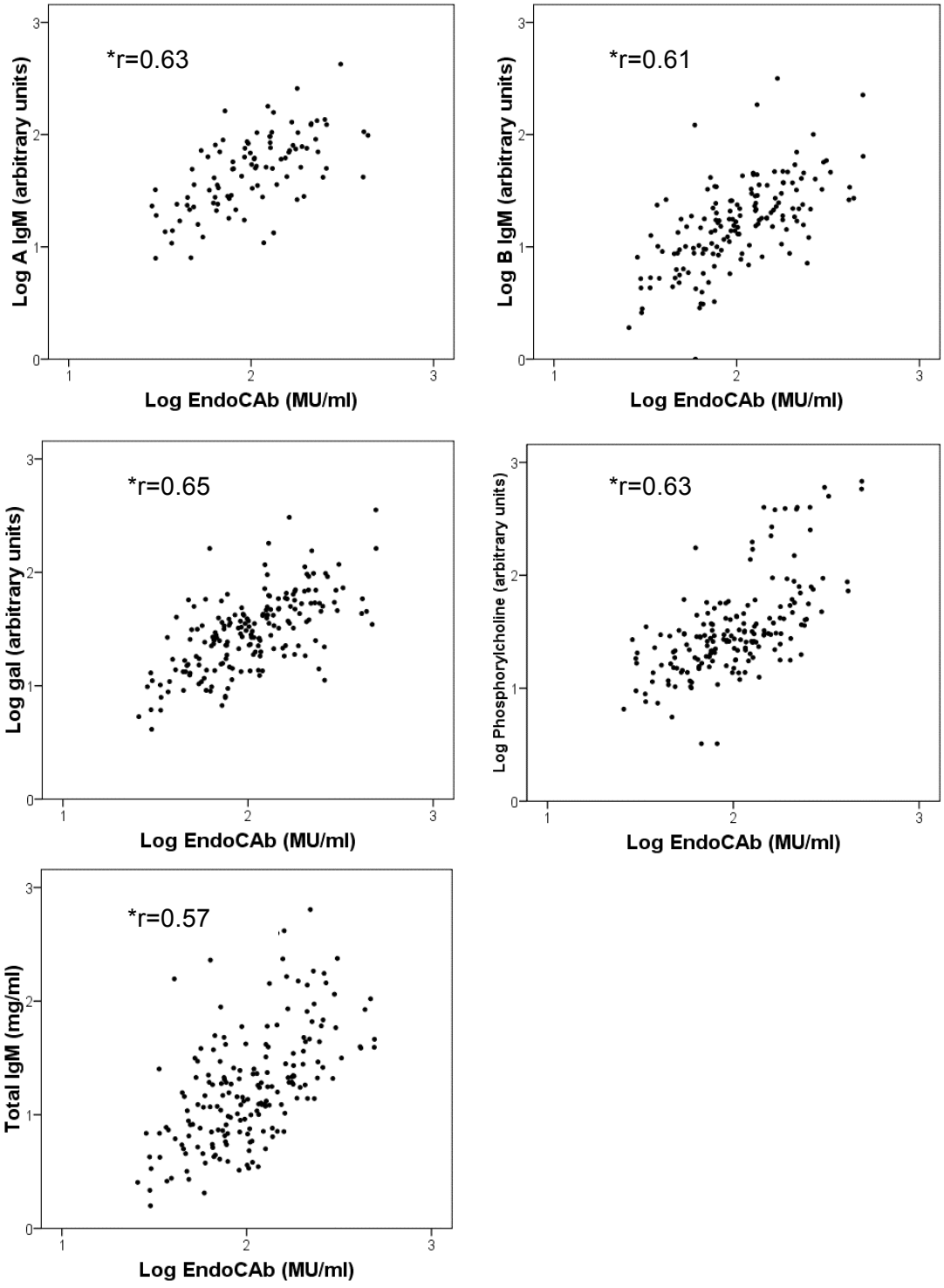

Figure 4.2: Associations and correlation coefficients between concentrations of EndoCAb IgM and A, B, gal, phosphorylcholine or total IgM. Each circle represents the antibody concentrations of one donor. EndoCAb IgM is on the x axis and A, B, gal, phosphorylcholine or total IgM are on the y axis. All results apart from total IgM have been logged. Only results for the relevant blood groups have been shown. i.e. values for donors with blood groups B and O for A IgM and groups A and O for B IgM. *= $p < 0.001$ using the Pearson correlation coefficient, r.

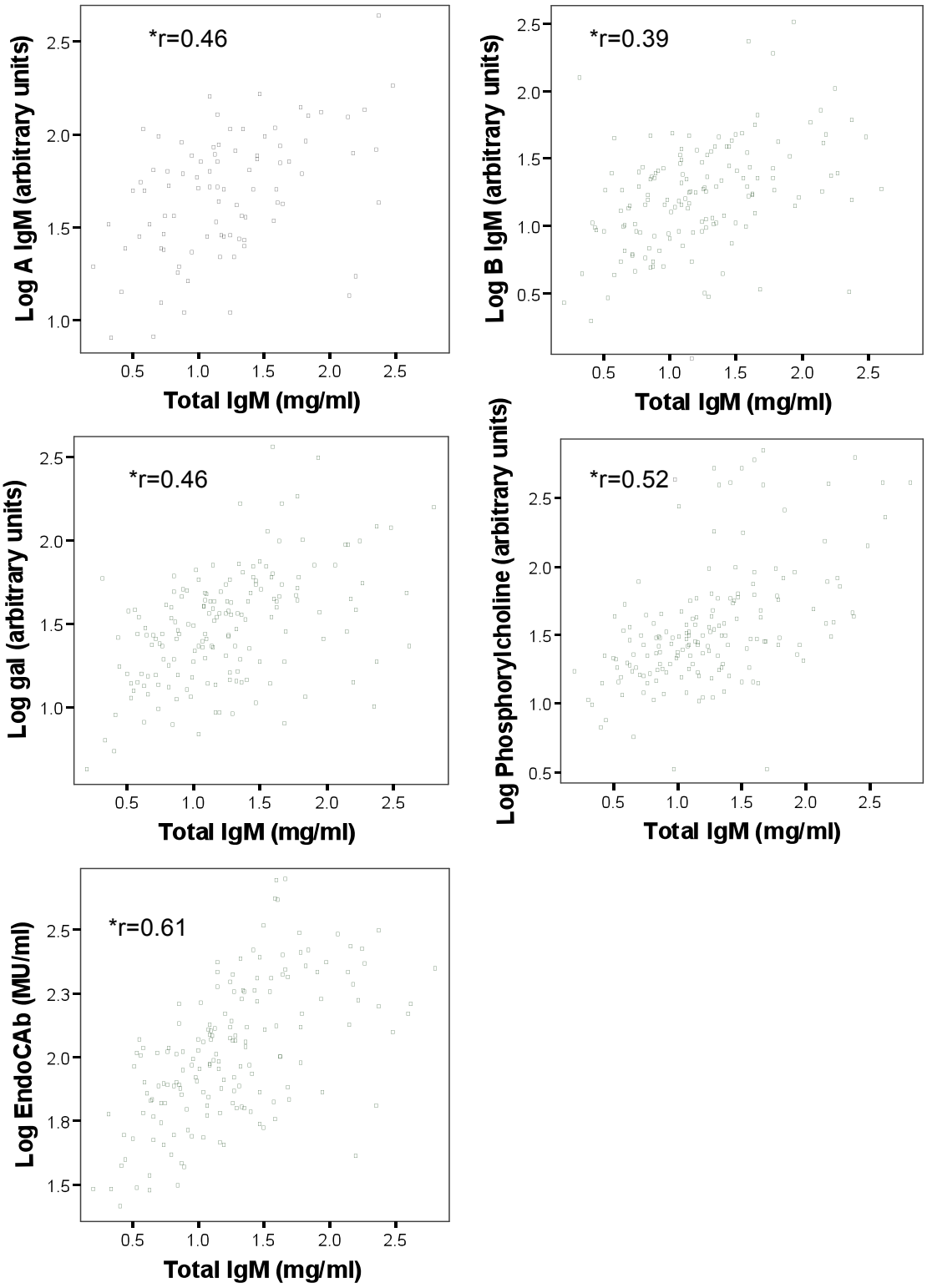

Figure 4.3: Associations and correlation coefficients between concentrations of total IgM and A, B, gal, phosphorylcholine or EndoCAb IgM. Each circle represents the antibody concentrations of one donor. All results apart from total IgM have been logged. Only results for the relevant blood groups have been shown. ie values for donors with blood groups B and O for A IgM and groups A and O for B IgM. *= $p < 0.001$ using the Pearson correlation coefficient, r.

Antibody	Unadjusted with EndoCAb IgM				Adjusted with EndoCAb and total IgM			
	*β	+95% CI	p=	R^2	* β	+95% CI	p=	R^2
A	0.792	0.589- 0.995	<0.001	0.398	0.682	0.44 - 0.924	<0.001	0.415
B	0.97	0.791- 1.139	<0.001	0.367	0.917	0.663-1.171	<0.001	0.368
gal	0.81	0.67 - 0.951	<0.001	0.422	0.721	0.55 - 0.891	<0.001	0.432
Pc	0.998	0.816- 1.118	<0.001	0.398	0.780	0.565-0.995	<0.001	0.437

Table 4.3: Regression between EndoCAb IgM and the natural IgM antibodies without (left) and with (right) adjusting for total IgM. *Unstandardised β regression coefficient. +95% confidence intervals for the B coefficient. R^2: coefficient of determination the proportion of variability in antibody level accounted for by the model. Only results for the relevant blood groups have been shown. i.e. values for donors with blood groups B and O for A IgM and groups A and O for B IgM. For example, for every increase in 1 MU/mL EndoCAb unit there is a 0.792 (β) arbitrary unit increase in A IgM antibody level. EndoCAb IgM alone accounts for 39.8% (R^2) of the variability in A IgM. Once both total IgM and EndoCAb are included, the model accounts for 41.5% of the variability in A IgM antibody level (adjusted R^2). Pc = phosphorylcholine.

4.5.2 Study 4B: IgM Human Hybridomas

The binding of the seven IgM monoclonal antibodies to endotoxin cores and the natural antigen panel was measured standardised for total IgM concentration and is displayed in figure 4.4.

Two of the seven monoclonal antibodies, DM17 and BCL which were derived from the same donor, bound to the endotoxin cores. All of the monoclonal antibodies except 6b5B12 bound to at least one endogenous antigen. DM17, BCL and 6b1A7 bound most strongly to the natural antigens A, B, Gal and phosphorylcholine (figure 4.4).

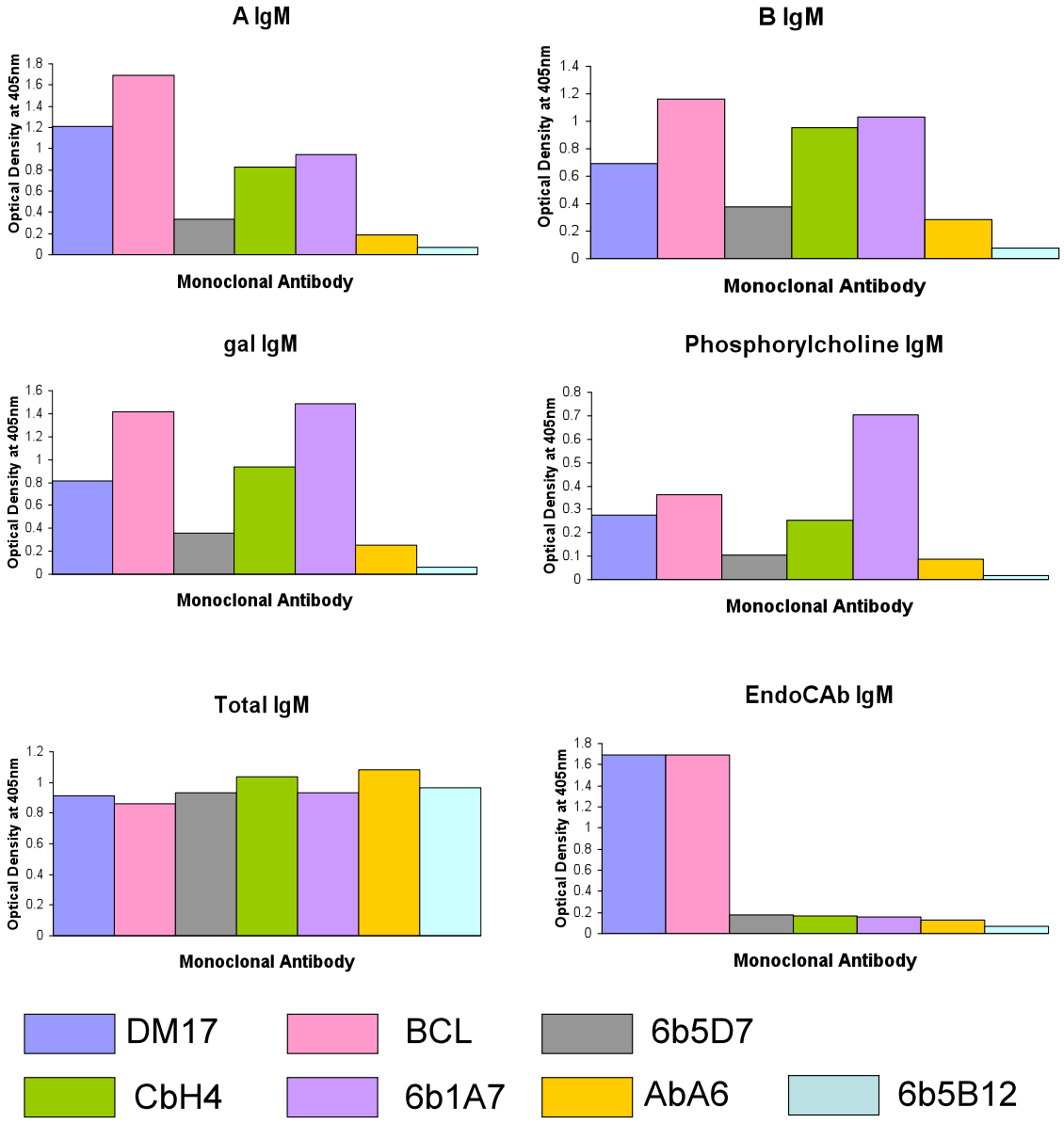

Figure 4.4: IgM binding activity of 7 monoclonal germ-line derived antibodies. The ELISAs were performed at standardised total IgM concentration of the monoclonal (see above total IgM graph) to antigens A, B, gal, phosphorylcholine and EndoCAb IgM. The plates, coated with antigens A, B, gal, phosphorylcholine, EndoCAb IgM or total IgM were exposed to the monoclonal antibodies, washed and a secondary anti-IgM antibody added. The results shown are for the different monoclonal antibodies in optical density units at 405 nm above background. Each colour represents one of the different monoclonal antibodies.

4.5.3 Study 4C: Mothers and their newborn

Blood samples were obtained from 18 mothers and 12 umbilical cords. Nine mothers had elective caesarean sections, whilst 9 were in labour when their blood was taken. A, B, Gal, phosphorylcholine, total IgM and EndoCAb IgM concentrations in the 18 mother's samples were comparable to those from the 179 blood donors in study 4A (figure 4.5 left hand side). Although total IgM was present in cord blood with a median level approximating 1/10 normal adult values concentrations (maternal 1466 μg/mL, fetal 143 μg/mL) concentrations of A, B, Gal, phosphorylcholine and EndoCAb IgM were either undetectable or very low (figure 4.5 right hand side), even accounting for the lower total IgM concentration.

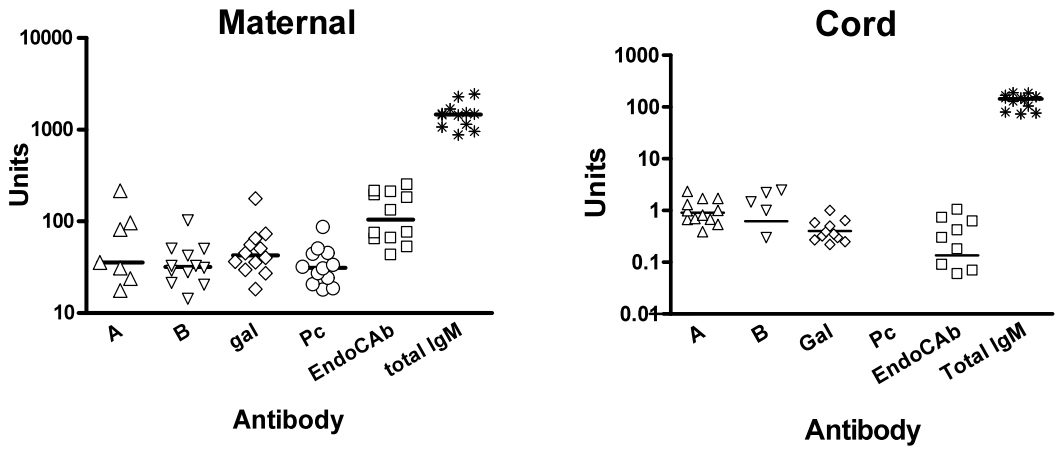

Figure 4.5 Concentrations of Maternal and Umbilical cord IgM antibodies. Individual concentrations (with the median as a solid line) are shown for the 12 paired samples in arbitrary units (for A, B, gal and Pc), median units per mL (EndoCAb) and μg/mL (for display purposes, total IgM) on log scales. Only results for the relevant blood groups have been shown.

4.5.4 Study 4D: Longitudinal concentrations

Samples were collected from 21 volunteers. Only 17 volunteers were available for all 3 timepoints and their results are presented here. The majority were female (13 females, 4 males) and they had a median age of 30 (range, 23 - 45). No volunteer received a vaccination during the study, and there was no obvious relationship between reporting symptoms of mild illnesses ('sort throat' etc) in the study period and antibody level changes. One volunteer went on holiday out of the UK to India.

There was no statistically significant change in any of the antibody concentrations over the 6 month period (table 4.4). Of interest, the one individual who went to India during the study period had a 3 fold rise in EndoCAb IgA, but no rise in EndoCAb IgG or IgM, whilst there was not enough sample to measure the natural antibody panel (data not shown).

Antibody	Baseline	2 Months	6 Months	CV	* p=
A	34.0 (11.8 - 45.6)	39.2 (13.6 - 46.4)	37.6 (14.1 - 52.3)	9 (4 -10)	0.12
B	23.9 (8.8 - 54.1)	24.4 (8.2 - 65.4)	23 (8.0 - 62.2)	8 (6 -11)	0.16
Pc	36.2 (23.6 - 68.0)	41.2 (23.4 - 78.4)	38.8 (23.5 - 98.8)	8 (6 -14)	0.94
Gal	37.6 (28.1 - 88.9)	36.6 (27.3 - 91.1)	33.6 (27.4 - 90.3)	8 (4 -12)	0.33
EndoCAb M	272 (174 - 402)	304 (172 - 416)	260 (164 - 388)	11 (7 -14	0.81
EndoCAb G	344 (250 - 432)	336 (224 - 442)	328 (252 - 438)	8 (6 -13)	0.55
EndoCAb A	78 (37 - 117)	72 (40 - 105)	67 (36 - 114)	9 (7 -13)	0.26

Table 4.4: Volunteers antibody concentrations at baseline, 2 and 6 months. Antibody concentrations (median, interquartile range) in 17 volunteers for A, B, gal, phosphorylcholine and EndoCAb IgM, IgG and IgA. Results for all blood groups have been included as blood groups were not measured. Values for EndoCAb are presented in median units/mL and for all others as arbitrary units/mL. *Friedman test. CV= Coefficient of variation (median, interquartile range).

4.6 Discussion

In this set of studies I have examined the idea that EndoCAb IgM shares some characteristics with the pre-immune B cell population that produces natural antibodies specific for A and B blood group antigens, gal and phosphorylcholine. Overall, EndoCAb IgM and natural IgM were present in similar concentrations, did not change significantly with time and were found in a proportion of cord blood samples. In addition two of the germline monoclonal hybridomas, which bound to the majority of the natural antigen panel, bound to endotoxin core. Taken together this suggests that EndoCAb IgM *may* come from the same pre-immune B cell population of cells that make the IgM natural antibodies to A, B gal and phosphorylcholine.

4.6.1 Study 4A: Concentrations in a blood donor population

Concentrations of EndoCAb IgM were associated with concentrations of A, B, gal and phosphorylcholine IgM antibodies, over and above their association with total IgM. Furthermore, donors with concentrations of EndoCAb IgM identical to the group that had a poor clinical outcome (in chapter 3) had lower concentrations of A, B, gal and phosphorylcholine IgM antibodies to those with higher EndoCAb IgM concentrations.

These findings are interesting for several reasons. Firstly they point to a new potential mechanism linking EndoCAb IgM to postoperative outcome. Were the patients in chapter 3 undergoing cardiac surgery to have had similar concentrations of natural antibodies to this cohort, there would be lower concentrations (of A, B, gal and phosphorylcholine IgM) in the poor outcome group. This raises the possibility that lower concentrations of these natural antibodies themselves may be causing the poor outcome. As mentioned previously, there are several functions ascribed to natural antibodies that might plausibly be linked to mechanisms that could alter postoperative outcome. Secondly, low concentrations of phosphorylcholine IgM has been associated with development of cardiovascular disease possibly by interacting with oxidised low density lipoprotein (Su *et al* 2005). Interestingly low EndoCAb IgM is associated with lower 5 year survival after hospital discharge following cardiac surgery: 12.8% vs 5.4% deaths in the low and high EndoCAb IgM

groups respectively (figure 4.6) (Moretti *et al* 2006). Although the authors did not state the reasons for excess deaths, they do speculate that low EndoCAb IgM 'may have a genetic predisposition to unfavourable outcomes'. As low concentrations of EndoCAb IgM are associated with low phosphorylcholine IgM, as shown in this chapter, then the low phosphorylcholine IgM may a possible cause.

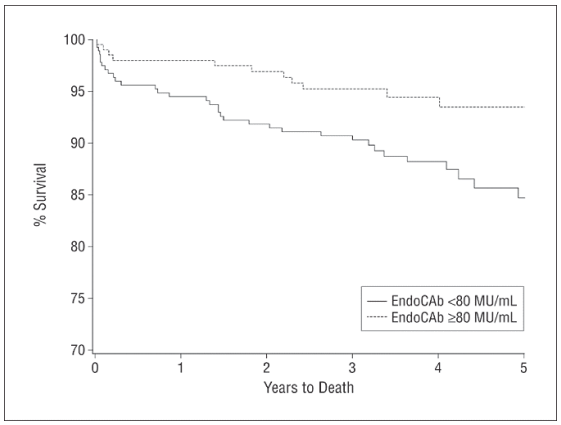

Figure 4.6: Kaplan-Meier estimates of the probability of 5-year survival based on high vs low antiendotoxin core antibody (EndoCAb IgM) concentrations. Taken from Moretti et al (Moretti *et al* 2006). Reproduced with permission.

Thirdly these findings suggest that there is a group of people with low concentrations of a range of natural IgM antibodies that may have altered postoperative outcomes: this area has not been well studied previously. The structural similarity of blood group B and gal antigens (figure 4.7), their role in rejection of transplanted animal organs and the question of the origin of natural antibodies has led others to investigate interactions between antibodies directed at these antigen (Chen *et al* 2009; Galili *et al* 1987a; Gerber *et al* 2001; Kuwaki *et al* 2004; Nordenstam *et al* 1990; Sandrin *et al* 1993). Other investigators have reported an association between concentrations of blood

group B and gal, and between blood group B and phosphorylcholine IgM antibody concentrations (Nordenstam *et al* 1989).

The structural similarity between the blood group B antigen and strains of Escherichia and the link between gal antibodies, Klebsiella, and Salmonella (all similar to components of the EndoCAb ELISA) has been previously reported. However, concentrations of these IgM antibodies have not previously been measured in the same human subjects nor compared with an antibody associated with outcome (i.e. EndoCAb IgM) following non-transplant surgery (Andersson *et al* 1989; Galili *et al* 1988; Kochibe and Iseki 1968).

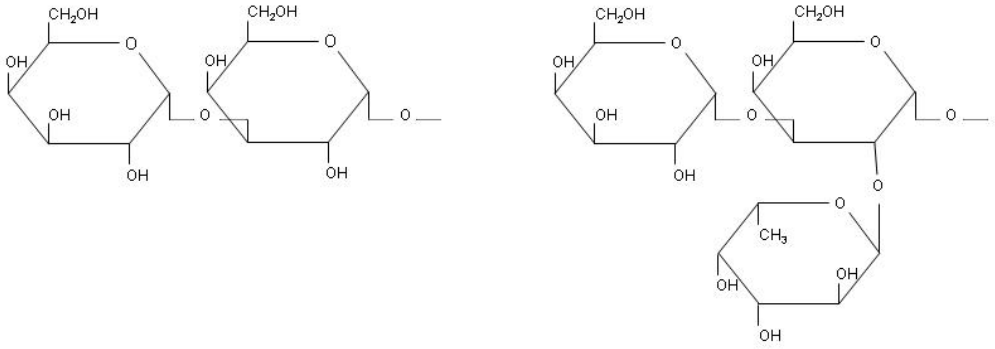

Figure 4.7: Gal and B antigen structure. Schematic representation of the structure of Galα1-3Gal ('Gal'), left and Galα 1-3 (Fuc α 1-2) Gal, right, of the blood group B antigen.

Lastly, there have been several attempts at reducing the consequences of perioperative endotoxaemia founded on the observed association between low EndoCAb IgM and poor outcome (Bennett-Guerrero *et al* 1997; Hamilton-Davies *et al* 1997; Mathew *et al* 2003; Mythen *et al* 1993). These have involved either donor plasma high in endotoxin antibodies, a synthetic endotoxin antagonist or polyclonal commercial gammaglobulin (Bennett-Guerrero *et al* 2007; Friedrich *et al* 2002; Hamilton-Davies *et al* 1996). None of these studies was without risk and have either been stopped prematurely or not significantly altered outcome. If EndoCAb and natural IgM concentrations are related, the

situation is more complex. It is possible that the natural IgMs themselves may alter the immune response regardless of what has triggered it.

Previous studies have noted a considerable spread or range of values in apparently healthy individuals for EndoCAb IgM, gal, phosphorylcholine and A and B blood group IgM antibodies comparable to the population of donors reported here (table 2) (Barclay 1990; Buonomano *et al* 1999; Padilla *et al* 2004; Rieben *et al* 1991; Spalter *et al* 1999). Aside from a few uncommon primary and acquired immune conditions there is little literature discussing the reason for this range. However, an endogenous enzyme from Neutrophils, acyloxyacyl hydrolase, that detoxifies endotoxin can modulate the B cell proliferation and level of polyclonal antibody response to gram negative infection in mice (Erwin and Munford 1990; Hagen *et al* 1991; Lu *et al* 2008; Munford and Hall 1989; Shao *et al* 2007). It is not currently possible to measure this enzyme in humans.

In any study one must consider the possible sources of error. It is possible that the associations described may have resulted from a systemic error, such as sample haemodilution. For example, if blood samples were diluted (e.g. with saline, an anticoagulant or overhydration) then an apparent association between concentrations of antibodies would result. I think this is unlikely for several reasons. Firstly, the median total IgM concentrations are very similar with previously published values: the median level here is 1.2 mg/mL compared to a median of 1.04mg/mL at 40 years of age in a study of over 110,000 samples (Ritchie *et al* 1998). Furthermore, the median EndoCAb IgM value here of 100.6 MU/mL is similar to the reference median of 100 MU/mL in blood donors and in other populations (Barclay 1990; Down *et al* 2004). The blood group A and B antibody concentrations are consistent with their respective blood groups, for example higher A antibody concentrations in volunteers with O and B groups compared to those with A and AB groups (50 arbitrary units vs 3.9 arbitrary units; table 4.2). Lastly, the association between EndoCAb IgM and total IgM is similar to that found in a previous study by Bennett-Guerrero and colleagues (0.57 vs 0.65 respectively) (Bennett-Guerrero *et al* 2001b).

These facts suggest that the assays were working correctly and that there was no haemodilution.

There are several limitations to this study. This study does not answer *why* there is an association between these antibodies. They may recognise, in part, a similar antigen. Their production may be stimulated by a common event or factor, such as previous surgery or illness, present to different degrees in individuals, although this does not answer why all the antibodies might be elevated (Munford 2005). There may be a common control of these antibody concentrations, suggested by the discovery that acyloxyacyl hydrolase alters the level of polyclonal antibody response to gram negative infection in mice (Lu *et al* 2008; Shao *et al* 2007). One significant improvement would be the addition of a characteristic 'immune' antibody- e.g. one present as the deliberate result of vaccination such as tetanus. If concentrations of this 'immune' antibody had no association with EndoCAb IgM this would strengthen the links between EndoCAb IgM and natural IgM.

Conclusion of study 4A

In this study I showed that EndoCAb IgM concentrations are associated with concentrations of other natural IgM antibodies, blood group A, B, gal, and phosphorylcholine, over and above any effect of total IgM. If replicated in a surgical cohort, individuals with low EndoCAb IgM, at the same threshold that defined a poor outcome in chapter 3 would have statistically lower concentrations of the natural antibodies. Whether they themselves would be associated with outcome is not yet known: that question must be answered in another study.

4.6.2 Study 4B: IgM human Hybridomas

In this study I showed that a panel of monoclonal antibodies were able to bind to a panel of natural antigens to varying degrees but only two were able to bind to endotoxin core. These hybridomas had previously been shown to share many characteristics with natural antibodies: they used distinctive Ig V(D)H genes in germline (non mutated) conformation and were able to provide

protection in a model of bacterial infection (Baxendale *et al* 2008). The discovery that they have a broad specificity strengthens the similarity with natural IgM. That some bind to endotoxin core supports the hypothesis that EndoCAb IgM is part of the natural antibody group.

Similarity between these mAbs and natural IgM must be taken with caution. The original clones were selected on the basis of binding to pneumococcal antigens after immunisation and although they have some features in common with natural IgM they may not be representative of the wider group. Most of our knowledge about the origin and specificity of natural IgM comes from the mice, where discrete populations of B cells are more clearly identified (Ehrenstein and Notley 2010).

4.6.3 Study 4C: Mothers and their newborn

I was able to demonstrate the presence of IgM and blood group A, B, EndoCAb and Gal, but not phosphorylcholine IgM in cord blood samples (figure 4.7 and table). The concentrations were low, all less than the 5th adult centile of the 171 blood donors in study 1, even though total IgM concentrations were normal for age, approximately 1/10 of that in the adults and similar to other studies on cord blood (Shah and Yadav 1977; Vick *et al* 1995). The total IgM concentrations were similar to previously published studies suggests this was not due to a technical error.

One of the often quoted features of natural antibodies is that they are present in umbilical cord blood although when measured this has not been a universal finding (Boes 2000; Doenz *et al* 2000; Gyorgy *et al* 2008). Several studies have examined the structure, reactivity, origin and persistence into infancy and adult life of natural IgM found in umbilical cord or neonatal blood (Dighiero *et al* 1985; Guigou *et al* 1991; Kantor *et al* 1997; Mouthon *et al* 1995; Mouthon *et al* 1996; Wuttke *et al* 1997).

The IgM antibodies of the specificity measured in this study have been either not measured before in cord blood or found in some cord samples at low concentrations. There are no published papers of EndoCAb IgM in cord blood,

although it was detected in 59% of neonates at 3 months of age in a study of samples submitted to a virology laboratory for other clinical investigations (Oppenheim *et al* 1994). Wuttke found 11% of 36 cord blood samples (compared to 50% here) had IgM antibodies to A or B blood group antigens, rising to 100% at 8 months of age (Wuttke *et al* 1997). Gal IgM could not be detected in any of 16 cord blood samples whilst phosphorylcholine IgM does not appear to have been detected before 4 months of age in humans (Doenz *et al* 2000; Gray *et al* 1983). The presence of EndoCAb IgM in some cord samples is consistent with the hypothesis of it being a 'natural IgM'.

4.6.4 Study 4D: Longitudinal concentrations

I showed that concentrations of EndoCAb IgM along with A, B, gal and phosphorylcholine IgM in 17 healthy volunteers varied little over a 6 month period. I had previously hypothesized that if EndoCAb IgM changed little over this period (like the 'natural antibody' group) that would support its inclusion as a natural antibody.

Few investigators have examined the longitudinal course of natural IgM in the same individual. Padilla found that phosphorylcholine changed little over 6 weeks with a range of coefficient of variation from 6 to 25% in 40 healthy donors. Over a much larger timeframe, 20 years, there is a decline in B and phosphorylcholine IgM concentrations (Nordenstam *et al* 1989). Aside from the arena of pig to human and ABO incompatible solid organ transplants in which both gal and ABO antibodies are important, there is little literature on longitudinal concentrations of natural IgM in healthy humans.

There is certainly evidence that external events can influence concentrations of natural antibody. In an elegant series of experiments that would probably be considered unethical today, Springer and Horton showed that blood group B antibodies (they was not able to determine which isotype) could be induced by feeding infants under 1 year (many of whom were orphans with diarrhoea) and adults live or killed *E. coli* 086B7 that has structural similarity with the B antigen (Andersson *et al* 1989; Springer and Horton 1969). Infants' phosphorylcholine antibodies rise after pneumococcal carriage and infection, whilst adults do not

change after pneumococcal vaccination (Brown *et al* 1984; Gray *et al* 1983). Padilla showed falls in phosphorylcholine IgM independent of total IgM in patients after isolated limb perfusion, a procedure that causes necrosis and apoptosis to tumour cells (Padilla *et al* 2004). Similarly, Hamilton-Davis showed falls in EndoCAb IgM, independent of total IgM immediately after coronary artery bypass graft surgery. Both these studies suggest that the antibodies can be consumed after exposure to large amounts of the congnate antigen.

4.7 Conclusion

EndoCAb IgM shares *some* features with natural IgM antibodies. There are positive associations between natural IgM and EndoCAb IgM concentrations, even after accounting for the association with total IgM. Likewise EndoCAb IgM has a similar unfluctuating temporal pattern to the natural IgM group and is present, albeit at low concentrations in some umbilical cord blood samples. Taken together, with caveats, these studies suggest that antibodies directed against endotoxin cores are probably part of both the 'natural' and 'immune' IgM antibody group.

4.8 Further work

There are many possible strands of work that could come from these sets of studies. As demonstrated in chapter 3, low EndoCAb IgM is associated with a poorer postoperative outcome *and* low EndoCAb IgM is associated with low concentrations of the natural IgM panel, it would be interesting to look at the relationship between the natural IgM panel and clinical postoperative outcome. In the same study it would be possible to see whether postoperative concentrations of A, B, gal, phosphorylcholine and EndoCAb IgM remain similar to those seen preoperatively, whether they are depressed or boosted by surgery.

One area of uncertainty is the characteristic cellular origin of natural IgM in humans. One approach could be to sort B cells (e.g. by Fluorescence-activated cell sorting according to surface markers e.g. CD27), culture and examine the antibody concentrations made in the supernatant.

As I mentioned in Chapter 1, a useful tool in elucidating the role of EndoCAb IgM would be the manufacture of an EndoCAb IgM concentrate. This is a big undertaking, but one of the difficulties is that EndoCAb IgM is by necessity a polyvalent/ heterogeneous group of antibodies as it is the result of a 4-antigen ‘cocktail’.

Chapter 5

Endotoxin immunity and the systemic inflammatory response syndrome in critically ill children

5.1 Introduction

Aside from cytokines (chapter 3) and fever response (chapter 6) another way of examining EndoCAb's ability to alter inflammation is by examining the prevalence of the systemic inflammatory response syndrome (SIRS).

Multiple organ failure resulting from systemic inflammation is the main cause of morbidity and mortality on intensive care units, irrespective of the initial illness or insult precipitating admission. The systemic inflammatory response syndrome (SIRS), which may lead to organ dysfunction, can be caused by a variety of clinical scenarios, including trauma, major surgery, infections, cerebral haemorrhage and infarcts, burns, and pancreatitis (Brun-Buisson 2000). However, even when patients are subjected to similar procedures, illnesses or insults, not all of them go on to develop SIRS. Furthermore, whilst the prevalence of SIRS is high not every patient with SIRS goes on to incur measurable organ dysfunction (Brun-Buisson 2000). Common genetic polymorphisms which influence the concentrations of key mediators including mannose-binding lectin (MBL) and TNFα have recently been implicated in determining the incidence and severity of systemic inflammation in critically ill patients (Fidler *et al* 2004; Garred *et al* 2003; Stuber *et al* 1996).

Endotoxin is an important trigger of SIRS. Experimentally, endotoxin can initiate a systemic inflammatory response and is found in large quantities in the colonized human gut. Critically ill patients as well as those undergoing surgery may be exposed to endotoxin from leakage into the systemic circulation via an impaired gastrointestinal barrier, gram-negative infection or as a result of bowel manipulation during surgery.

All adult humans have antibodies directed against the core of endotoxin (EndoCAb) although observed concentrations vary by more than eighty-fold (Barclay 1990). EndoCAb IgG is present at birth, and is probably of maternal origin, acquired trans-placentally. EndoCAb IgM is almost absent in the first month but increases to approximately adult concentrations by a year (Oppenheim *et al* 1994). Higher preoperative concentrations of EndoCAb IgM in adults are associated with a good outcome following surgery (see chapter 1) whilst higher EndoCAb IgG concentrations have been linked to survival in

sepsis (Goldie *et al* 1995; Strutz *et al* 1999). It is not known if this is a causal association with EndoCAb acting to modulate systemic inflammation, or if high EndoCAb titres are simply a marker of a favourable immune state (e.g. via associations with natural antibodies) in patients at risk of systemic inflammation.

I took the opportunity afforded by a previously conducted study (on the relationship between MBL levels and SIRS in critically ill children) to look at the levels of EndoCAb levels in the same population.

Because of the previous literature on EndoCAb, we defined two sub-groups according to the primary reason for Paediatric Intensive Care Unit (PICU) admission: infection and non-infection. The analyses were performed on the combined group and the individual sub-groups.

5.2 Hypothesis

My hypothesis was that children who develop SIRS in their initial period on PICU have lower concentrations of antibodies to endotoxin core.

5.3 Aims

Primary Aims

To examine the relationship between EndoCAb (IgM and IgG) and the development of a Systemic Inflammatory Response Syndrome within 48 hours of admission to a PICU in children with more than one organ failure.

Secondary Aims

To examine the relationship between EndoCAb (IgM and IgG) and

- Serum concentrations of mannose-binding lectin
- Age
- Initial diagnosis on the Paediatric Intensive Care Unit

5.4 Methods

Approval for this observational study was obtained from our institution's ethics committee. Patient data was stored according to the requirements of the Data Protection Act and parental informed consent was obtained. The clinical data, samples and blood for EndoCAb in this chapter were obtained from a previously conducted double blind, observational cohort study in critically ill children. This wider study was conducted by the departments of Anaesthesia, Intensive Therapy and Respiratory Medicine, Immunobiology Unit and Infectious Disease and Microbiology Units, Institute of Child Health, UCL. I performed the EndoCAb assays.

Consecutive admissions to our tertiary multi-disciplinary PICU were recruited, as part of the larger study into the role of mannose-binding lectin, over a 6 month period. On enrolment, cases were assigned to one of two groups, infection or non-infection, according to the principal reason for PICU admission as documented by independent PICU physicians not involved in the study. Within each of these groups, patients were subdivided into those who did or did not develop ‘early’ SIRS i.e. within the first 48 hours of admission. MBL concentrations from the previous study were recorded as a potential confounder of any effect of EndoCAb.

Subject selection

Inclusion criteria were: age between birth and 17 years and the presence of at least one organ system failure for more than 12 hours (or death within the first 12 hours). The following exclusions were applied: presence of multiple congenital abnormalities; known congenital immunodeficiency; known central neurological or neuromuscular disease (all considered to represent major risk factors for PICU admission resulting from infection); persistent pulmonary hypertension of the newborn; weight less than 2.2 kg; informed consent not available; suspected non-accidental injury; repeat PICU admission during the study period; lack of intravenous or intra-arterial access and anticipated short stay (less than 24 hours) on the PICU.

Clinical measurements

Infection was defined as ‘proven’ if a causative organism was isolated from a normally sterile site and ‘presumed’ in those with a history and examination consistent with an infection e.g. fever, cough and coryza combined with chest x-ray changes consistent with pneumonia. Diagnoses of SIRS, sepsis and septic shock were made according to the American College of Chest Physicians/Society of Critical Care Medicine guidelines modified for age (1992). Essentially, SIRS was determined by the presence of 2 or more of the criteria in table 5.1. Respiratory rate was not included as a diagnostic criterion because of the high proportion of cases receiving mechanical ventilation. Cases meeting these criteria for SIRS with ‘proven’ or ‘presumed’ infection were classified as ‘sepsis’ whilst septic shock was diagnosed in cases of sepsis who were hypotensive, defined against age specific values for mean blood pressure after fluid resuscitation requiring treatment with inotropes and/or vasopressor therapy (Fidler *et al* 2004). An electronic patient charting system (Care Vue, Hewlett Packard) was reviewed daily and maximum and minimum ventilator and physiological parameters for each 24-hour period were recorded prospectively onto a Microsoft Access database. Microbiological, biochemical and haematological information was recorded from the PICU and the referring hospital. Paediatric logistic organ dysfunction (PELOD) score, a measurement of the severity of multiple organ dysfunction syndrome was calculated daily (Leteurtre *et al* 2003) (see Appendix 4).

Diagnosis	Criteria	Range for diagnosis
SIRS	Central Temperature	greater than 38.0 ^{o}C or less than 36.0 ^{o}C
	White Cell Count	greater than 12x10^{9} cells/L or less than 4x10^{9} cells/L
	Heart Rate (beats/minute)	newborn to 3 months: 95-145 3-12 months: 110-175 1-3 years: 105-170 3-7 years: 80-140 7-10 years: 70-120 greater than 10 years: 60-100
Sepsis	SIRS with 'proven' or 'presumed' infection	
Septic shock	Patients with sepsis who were *hypotensive after fluid resuscitation *and* treatment with inotropes and/or vasopressors	

Table 5.1: Diagnostic criteria for the Systemic Inflammatory Response Syndrome (SIRS) in children. The presence of SIRS in a patient was determined by the presence of 2 or more of the *age-specific criteria above (1992). Respiratory rate was not included due to the high proportion of cases receiving mechanical ventilation.

Laboratory measurements

Serum samples were taken within 48 hours of admission, spun, separated and the serum stored in aliquots at -80^{o}C until analyzed. The investigators performing the endotoxin-core antibody (EndoCAb) serum concentrations and MBL serum concentrations were blinded to the diagnosis of SIRS. Similarly the clinician acquiring the clinical data was blinded to the laboratory data. IgM and EndoCAb IgG were measured as described in chapter 2. MBL concentrations in serum were previously determined by a symmetrical sandwich ELISA using commercial kits from Antibody Shop, Copenhagen, Denmark according to the manufacturer's instructions by another investigator.

Statistics

As EndoCAb concentrations are not normally distributed, Medians and interquartile ranges are reported. Non parametric analytical statistics (Mann-Whitney U test) were used apart from log transformed data for regression analysis. Normal distribution of the transformed data was confirmed using the Kolmogorov-Smirnov test. All statistical calculations and analyses were conducted using SPSS (SPSS 15, Chicago, IL, USA).

5.5 Results

After informed consent serum was obtained from 139 suitable patients: their characteristics are shown in Table 5.2. 71 children were admitted for non-infectious indications (postoperative management, after head injury or with other non-infectious conditions) whilst 68 were admitted with infection (localised infection, sepsis or septic shock).

In the unselected cases (n=139), IgG concentrations were lower (but the differences were not significant) in those with early SIRS (SIRS median: 99 MU/mL vs Non-SIRS 119; p=0.215). There was no difference in the IgM concentrations between the two groups (87 vs 86 p=0.588). There was a weak correlation between EndoCAb IgG and EndoCAb IgM concentrations (Spearman's Rank Correlation Coefficient, R =0.311, p=<0.001) but not between EndoCAb IgG and MBL level (R =0.071, p=0.406). Of the potential confounding variables (age, sex, initial PELOD score, CRP & MBL) only MBL level was significantly associated with the development of SIRS on univariate analysis (table 5.2).

	All patients				Non-Infection				Infection			
	All	SIRS	Non-SIRS	p value	All	SIRS	Non-SIRS	p value	All	SIRS	Non-SIRS	p value
	n= 139	n=82	n=57		n=71	n=32	n=39		n=68	n=50	n=18	
Age months*	26 (9-121)	25 (9-108)	34 (10-130)	0.389	57 (18-151)	57 (24-139)	64 (12-153)	0.959	14 (3-74)	13 (4-81)	17 (2-29)	0.956
Initial PELOD score*	12 (10-21)	12 (11-21)	12 (2-21)	0.182	12 (10-21)	12 (11-12)	12 (10-21)	0.939	12 (11-21)	16 (11-22)	11 (1-21)	0.106
Diagnosis (%, no)												
Septic Shock	18.7 (26)	31.7 (26)	0		0	0	0		38.2 (26)	52 (26)	0 (0)	
Sepsis	17.3 (24)	29.3 (24)	0		0	0	0		35.3 (24)	48 (24)	0 (0)	
Infection	12.9 (18)	0	31.6 (18)		0	0	0		26.5 (18)	0 (0)	100 (18)	
Postoperative	23.0 (32)	12.2 (10)	38.6 (22)		45 (32)	31.2 (10)	56 (22)		0	0	0	
Head Injury	23.7 (33)	19.5 (16)	29.8 (17)		46.5 (33)	50 (16)	44 (17)		0	0	0	
Other	4.3 (6)	7.3 (6)	0		8.5 (6)	18.8 (6)	0 (0)		0	0	0	
CRP mg/l*	75 (35-137)	84 (41-157)	66 (30-120)	0.149	73 (34-120)	75 (37-147)	62 (34-120)	0.408	76 (41-147)	89 (41-169)	70 (27-83)	0.315
EndoCAb IgM MU/mL*	86 (45-159)	87 (44-152)	86 (51-200)	0.588	98 (57-179)	99 (72-158)	95 (54-208)	0.829	79 (30-138)	60 (30-131)	83 (23-141)	0.906
EndoCAb IgG MU/mL*	101 (46-209)	99 (44-198)	119 (59-229)	0.215	100 (49-198)	72 (43 -153)	131 (80 -210)	0.009	106 (43-229)	111 (49-218)	80 (31-264)	0.518
MBL ng/mL*	1851 (525-3886)	754 (325-2516)	2831 (993-4284)	<0.001	2091 (633-4174)	1185 (408- 3860)	2518 (624-4344)	0.054	947 (293-3284)	682 (163-2379)	3715 (1078-4107)	0.001

Table 5.2 Characteristics of the 139 study patients. Demographic, clinical and other characteristics of the study patients divided into 3 groups; the whole group and the two subgroups according to the primary reason for ICU admission: infection and non-infection. Each of these groups is further divided into those that did or did not develop SIRS in the first 48 hours following admission to PICU. P values were calculated using the Mann-Whitney U test. *Definition of abbreviations*: SIRS=Systemic Inflammatory Response Syndrome; PELOD= Paediatric logistic organ dysfunction; EndoCAb= Endotoxin core antibody; IgM= Immunoglobulin M type; IgG= Immunoglobulin G type; MBL= Mannose-binding lectin; *median, (interquartile range).

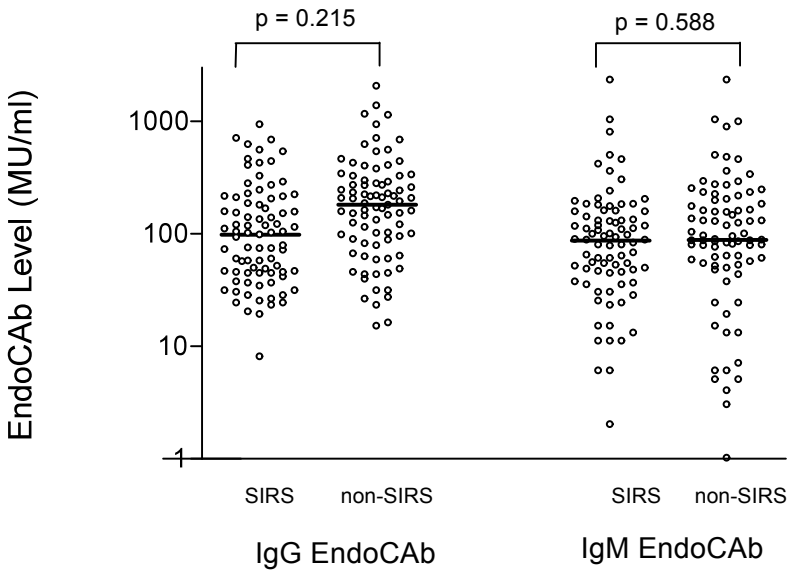

Figure 5.1 EndoCAb and SIRS in the 139 patients. IgG (left) and IgM (right) EndoCAb concentrations of the 139 critically ill children and the subsequent development of SIRS in the first 48 hours following admission to PICU. P values were calculated using the Mann-Whitney U test. No significant differences were seen in either IgG or EndoCAb IgM between patients with and without SIRS. For abbreviations please see table 5.2.

5.5.1 Non- infected Sub-group

The characteristics of the 70 children without infection are shown in table 5.2. EndoCAb IgG concentrations are significantly lower (p=0.009 Mann-Whitney U) in those children developing SIRS. There were no significant differences in EndoCAb IgM concentrations between those with and without early SIRS (p=0.829 Mann Whitney U) (figure 5.2).

Log_{10} EndoCAb IgG remained independently associated with the development of SIRS in the non-infected population in a binary logistic regression analysis after correction for the effects of age, sex, initial PELOD scores, CRP and MBL. The odds ratio of SIRS in this group increases by over 2 ½ times if the EndoCAb IgG is below 57 MU/mL when compared to those patients with an EndoCAb IgG above 57 MU/mL (table 5.3)

	EndoCAb IgG (MU/mL)	
	<57	>57
non-SIRS % (no)	25 (5)	66.6 (34)
SIRS % (no)	75 (15)	33.3 (17)

Table 5.3 SIRS and IgG EndoCAb. The occurrence of SIRS in the first 48 hours following admission to PICU in the non-infected group according to a 'threshold' of IgG EndoCAb: above and below 57 MU/mL. The likelihood of SIRS increases by over 2 ½ times if the EndoCAb IgG is below 57 MU/mL

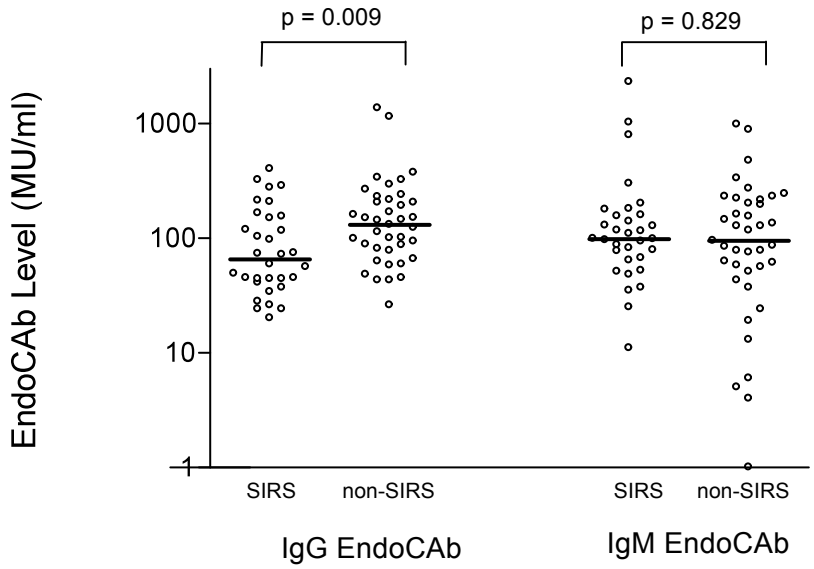

Figure 5.2 EndoCAb and SIRS in the non-infected patients. IgG (left) and IgM (right) EndoCAb concentrations in the 71 non-infected patients according to whether they did or did not develop SIRS in the first 48 hours following admission to PICU. P values were calculated using the Mann-Whitney U test. Significant differences were seen in EndoCAb IgG between patients with and without SIRS (p = 0.009). For abbreviations please see table 5.2.

5.5.2 Infected Sub-group

The characteristics of the 68 children with infection are shown in table 5.1. There were no significant differences in IgG or EndoCAb IgM concentrations between those children developing SIRS and those not (111 vs 80 MU/mL; p=0.518 and 60 vs 83 p=.906 respectively) (figure 5.3). There was no significant difference in the serum IgM & EndoCAb IgG concentrations in patients who had increasing severity of infection (localized infection vs sepsis vs septic shock, p=0.4 Kruskal-Wallis test for both EndoCAb IgG & IgM (data not shown). Of the potential confounding variables (age, sex, ethnicity, initial PELOD score, CRP & MBL) only MBL concentrations were associated with the development of SIRS in this group (p=0.001) as previously reported (Fidler *et al* 2004).

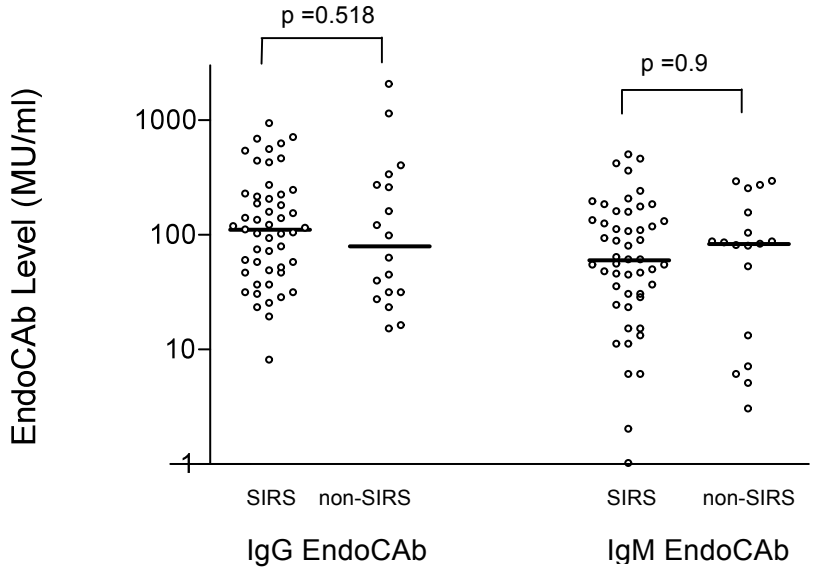

Figure 5.3 EndoCAb and SIRS in the infected patients. IgG (left) and IgM (right) EndoCAb concentrations in the 68 infected patients according to whether they did or did not develop SIRS in the first 48 hours following admission to PICU. P values were calculated using the Mann-Whitney U test. No significant differences were seen in either IgG or EndoCAb IgM between patients with SIRS and without SIRS. For abbreviations please see table 5.2.

5.6 Discussion

This is the first study to investigate the concentrations of antibodies against endotoxin core in a mixed population of critically ill children. I have shown that cases admitted following trauma or surgery (or for other non-infectious reasons) who go on to develop an early systemic inflammatory response have lower EndoCAb IgG concentrations than those who do not develop SIRS. In addition it confirms that children have similar concentrations to those seen in large adult studies (Bennett-Guerrero *et al* 1997; Bennett-Guerrero *et al* 2001b; Goldie *et al* 1995; Mathew *et al* 2003; Strutz *et al* 1999).

Several studies have found that high *preoperative* concentrations of these antibodies are associated with better outcomes, consistent with the theory that

they are capable of neutralising endotoxin (see chapter 1, introduction) (Bennett-Guerrero *et al* 1997; Bennett-Guerrero *et al* 2001b; Hamilton-Davies *et al* 1997; Mathew *et al* 2003; Mythen *et al* 1993).

In contrast to studies in surgical patients the relationship between initial EndoCAb level and outcome is not so clear in sepsis (Goldie *et al* 1995; Maury *et al* 2003; Strutz *et al* 1999). Initial EndoCAb IgM concentrations in 146 septic adults were higher in survivors though this may have been explained by other factors as it was not predictive of mortality in a multivariate analysis (Goldie *et al* 1995). Their study found a subset of patients with very low initial EndoCAb IgG level (less than 10% of the median level in healthy adults) had greater mortality. In 205 ICU adult patients with sepsis, Nys found a strong relationship between the absence of IgM anti-core antibodies and subsequent septic shock whilst rising IgG anti-core antibodies were associated with a positive outcome (Nys *et al* 1993). Depressed concentrations of antibody to endotoxin core have also been associated with outcome in children with acute lymphoblastic leukaemia and adults with pancreatitis or abdominal sepsis (Bose *et al* 2002; Jackson *et al* 1990; Wakefield *et al* 1998; Windsor *et al* 1993). Clearly there are many influences on the development of SIRS in critically ill individuals. There include the infecting dose of pathogen (e.g. in *Neisseria meningitidis*), the nature, length and severity of the insult (e.g. surgery, cardio-pulmonary bypass) and specific deficiencies in aspects of coagulation and innate immunity (Brandtzaeg *et al* 1989; Fidler *et al* 2004; Fourrier *et al* 1992; Khabar *et al* 1997; Powars *et al* 1993).

This study has several potential limitations. Firstly, the study was originally designed to look at the effect of MBL on the incidence and severity of SIRS and as such these results should be interpreted with caution. Data was not collected on whether patients received plasma products, which contain antibodies with the same concentrations and range as the donor adult population. Addition of hyperimmune plasma can raise EndoCAb concentrations, so any patient who receives plasma from a donor high (or low) in EndoCAb may have their level

altered (Hamilton-Davies *et al* 1996; Rashid *et al* 2004). Patients with sepsis may be more likely to receive plasma products due to the coagulopathy and this may alter their level and in turn remove the effect of their apparent EndoCAb on outcome. Likewise patients receiving crystalloids or colloids (human albumin solution should not contain antibodies) may have their level of EndoCAb lowered.

A second limitation is that the effect of EndoCAb IgG on SIRS may not be a specific effect, but a reflection of total IgG or associated with other factors. In chapter 4 (table 4.3 and 4.4) I showed that concentrations of EndoCAb IgM were associated with both total IgM and a panel of natural antibodies. I was not able to measure the natural antibody panel in this study due to the small size of the samples. Previous studies in adults however, have found that total IgG and IgM and even 'reference antigens' such as anti-tetanus antibodies have been unrelated to EndoCAb IgG or IgM concentrations, although this does not preclude an effect in children (Bennett-Guerrero *et al* 1997; Bennett-Guerrero *et al* 2001b; Hamilton-Davies *et al* 1997). Furthermore in a previous study EndoCAb concentrations were independent of cardiovascular variables such as heat rate, blood pressure, cardiac output and stroke volume (Hamilton-Davies *et al* 1997). Thirdly, EndoCAb concentrations have been reported to vary with age in children: IgM and EndoCAb IgG climb from 3 months towards adult values by one year (Barclay 1995). In this study there are no significant differences in age between the group that developed SIRS and those that did not. Furthermore, in the multivariate analysis of the non-infected group, age did not alter the effect of EndoCAb IgG on the development of SIRS. Lastly, our numbers are too small to detect an effect on mortality whilst the study population. However, despite all these potential limitations there was still a detectable effect of EndoCAb IgG on the development of SIRS in the non-infected group.

The hypothesis that EndoCAb has a direct protective role by mopping up endotoxin is appealing but not yet proven. If this were to be a specific effect of

EndoCAb IgG this opens up the question of immunotherapy aimed at reducing systemic inflammation even after the patients have entered the ICU.

5.7 Conclusion

This study shows that in children with at least one organ failure admitted to the intensive care after head injury, surgery or for other non-infectious reasons, depressed EndoCAb IgG concentrations are associated with the early development of the systemic inflammatory response syndrome. Whilst it does not show causation, why EndoCAb IgG not IgM is associated or whether this is necessarily a specific effect, the information provides a potential mechanism by which elevated EndoCAb concentrations are associated with an improved outcome.

5.8 Further work

Following on from chapter 4, where I showed a significant relationship between EndoCAb IgM and the natural antibody panel, it seems obvious to ask in the light of this chapters result “does a similar relationship exist between EndoCAb IgG and IgG natural antibodies?”. This was not originally performed as the majority of previous literature suggested that EndoCAb IgM, not IgG, was associated with perioperative outcome (Chapter 1, table 1.2). A similar study could be done where those receiving plasma products could be excluded – at least in the postoperative group, where that limitation might not exclude such numbers as to make the study too impractical to run.

What this study does not answer is the wider question as to whether more EndoCAb *causes* lower endotoxin concentrations which in turn results in less inflammation and a better outcome. That requires a different model, where the timing and concentrations of (putative) endotoxin binding substances are known.

Chapter 6

Low dose endotoxin and variability in the inflammatory response in healthy volunteers

6.1 Introduction

As described in chapter 1, endotoxin is implicated as a cause of inflammation and organ dysfunction after surgery. Clinically, patients differ in their response to apparently broadly similar external insults such as infections or surgery, leading to differences in outcome.

One of the problems in testing potential factors that modify or alter the response to endotoxin (whether drugs, genetic or blood born factors) is the *current model* of endotoxin volunteer studies. Currently volunteers receive a 4ng/kg endotoxin bolus, causing a significant inflammatory response and symptoms in *almost all* the volunteers. Aside from its unpleasantness, this 4ng/kg bolus does not result in a big *range or variability* of responses. This traditional human model of endotoxin challenge (4 ng/kg) also may not mirror the *inter-individual variability, spread or dispersion* in systemic inflammation that is characteristic of natural exposure and so might not be the best model for studying variability in the inflammatory response to endotoxin (Bunnell *et al* 2000; De Winter *et al* 1995; Elin and Csako 1989; Kumar *et al* 2004; Pajkrt *et al* 1997; Pernerstorfer *et al* 1999; Rodrick *et al* 1992; Rossignol and Lynn 2002; Soop *et al* 2003; Suffredini *et al* 1989; Suffredini *et al* 1995; Wyshock *et al* 1995; Zijlstra *et al* 2004).

A lower dose may cause a significant response in some but not in others - allowing us to examine the difference in responders and non-responders (e.g. in EndoCAb levels). This aim of this alternative approach to investigate the human response to endotoxin involves a model using a lower dose of endotoxin to cause the largest *range* of change (variability or dispersion) in inflammatory markers. This could allow us to explore variations in threshold of effects. In such an ideal model some volunteers would exhibit *no* inflammatory symptoms or have no raised inflammatory markers whilst others would. This model might better allow us to

- Investigate the differences between individuals in endogenous factors (in plasma and genotype) that alter the response to endotoxin. This may take the form of asking the question “what are the factors that make some individuals display an exaggerated inflammatory response to endotoxin challenge whilst others appear to show little or no response”?
- Model the postoperative clinical situation in which some patients get an overt SIRS whilst others appear not to
- Avoid the very unpleasant 4 ng/kg dose with attendant symptoms and rare serious effects (van Eijk *et al* 2004)

A low dose of endotoxin may cause optimum inter-individual variability in markers of systemic inflammation by not ‘overwhelming’ the natural endotoxin binding capacity of the serum (Bennett-Guerrero *et al* 2001a). Previous studies that have used relatively low doses of endotoxin down to 0.8-1.0 ng/kg have shown a dose-response effect (i.e. less endotoxin, less change in inflammatory markers and symptoms), but have not focussed on the variability in response between individuals (Elin *et al* 1981; Korth *et al* 1996; Krabbe *et al* 2001a; Krabbe *et al* 2001b).

A second way to increase *inter-individual variability* in systemic inflammation *may* be to use a slow infusion (as opposed to a bolus) of endotoxin. An infusion may better mimic the clinical scenario whereby endotoxin is *thought* to be released systemically in gram negative sepsis or by a compromised gastrointestinal tract over a period of time. It may be that by delivering a lower rate of endotoxin to the volunteer we may allow more time for endotoxin binding in plasma (e.g. BPI / HDL-Cholesterol and antibodies), resulting in a lower ‘free’ endotoxin level and so not cause as much inflammation. This approach has not been investigated before.

As the minimal pyrogenic dose is thought to lie below 0.5 ng/kg we decided to use 0.25 ng/kg and 0.75 ng/kg as either a bolus or an infusion (Elin *et al* 1981). Our aim was to describe the variability between individuals in inflammatory

markers in the first 10 hours following these regimens. Unfortunately one of the research team responsible for collecting data was subsequently found to have been manufacturing some data. The whole of this suspect data (white cell count, C reactive protein and platelet counts) have not therefore been included (see 'ethics and safety' later in 6.4).

6.2 Hypothesis

My primary hypothesis was that the low dose endotoxin dosing regimens would result in a significant change in inflammatory markers compared to baseline.

My secondary hypothesis was that low dose endotoxin dosing regimens can elicit variability in systemic inflammation i.e. changes (rises or falls) in inflammatory markers in some subjects but not in others.

My third hypothesis was that infusions of endotoxin would produce greater variability in inflammatory marker changes (rises or falls) than boluses at the same total dose.

6.3 Aim

My aim was to measure the changes in inflammatory markers following low dose endotoxin dosing regimens.

6.4 Methods

This was a prospective volunteer study performed in collaboration with Dr Elliott Bennett-Guerrero and his team at the department of Cardiovascular Anaesthesia, Columbia Presbyterian Hospital New York, USA.

Endotoxin

Escherichia coli O:113 endotoxin (EC-6) was a kind gift from the National Institutes of Health, USA (Suffredini *et al* 1999). EC-6 is supplied as a lyophilized powder, and was reconstituted to the appropriate dilution according

to the manufacturer's instructions by the investigational pharmacy at Columbia Presbyterian Hospital to a concentration of 200 ng/mL (2000 EU/mL) in pyrogen free water (US Pharmacopeia, Abbott Laboratories, Abbott Park, IL, USA). It was then further diluted in pyrogen free water (according to the volunteers weight and dosing regimen) to use in a syringe driver as an infusion over 30 minutes or as a bolus over 1 minute (Table 2) by the investigational pharmacy. EC-6 is the standard endotoxin used in volunteer studies by the National Institutes of Health, USA and was prepared under good manufacturing practices specifically for experimental administration to humans.

Volunteers

After ethical approval, healthy male and female ambulant adults between the ages of 18 and 55 years were enrolled who, after written informed consent, underwent a screening examination (physical examination, electrocardiograph), blood tests (full blood count, electrolytes, liver function, cholesterol) and a urinary pregnancy test. Volunteers were excluded if they had any of the conditions listed in table 6.1 and were paid for their time.

Criteria
Significant abnormal results on history, physical examination or ECG
Abnormal baseline laboratory tests
A positive pregnancy test in the previous week
Chronic disease or hospitalisation within the past year
Prescription drug use on a regular basis
Any acute illness including 'flu or 'flu like symptoms
Known or suspected controlled substance use
Participation in another research protocol within the previous 2 months
Receipt of a blood transfusion in the previous month
Receipt of vaccination or known endotoxin within the past three months

Table 6.1: Exclusion criteria. Volunteers were excluded from participation in the study if they fulfilled any of the criteria above. ECG, electrocardiogram.

Study course

Volunteers were starved from midnight on the study day. On the morning of the study, two intravenous catheters were inserted into each volunteer, (one for blood sampling, the other in the opposite arm for the dosing regimen) after which baseline observations and bloods were taken. Using a computer-generated schedule, subjects were randomised to receive one of 6 dosing regimens described in table 6.2. To ensure blinding (as the timing of the two treatments, bolus or infusion could not be hidden) a 'double-dummy' procedure was used. Each volunteer received a bolus (10 mL over 1 min) and an infusion (50 mL over 30 min) of either water or endotoxin according to which study group they were assigned to (see table 6.2). Because the effects of 4 ng and 0 ng/kg endotoxin are very well described, most of the volunteers were randomised to the 0.25 and 0.75 ng/kg dosing regimens. Over the next 10 h, subjects' vital signs were monitored by an investigator and symptoms (headache, 'I feel like shivering', myalgia and nausea) were recorded hourly by the volunteer on a self-reporting visual analogue scale from 0 to 10. For example, volunteers were asked "on a scale on 0 to 10, (0 being not present at all, 10 being the worst you can imagine), how nauseated do you feel?" To prevent dehydration, volunteers were given 30 mL/kg intravenous saline over the 10 hour study period, equal to 2100 mL for a 70 kg individual.

Outcomes

The primary marker of inflammation was temperature (^{o}F). Original secondary outcomes included white cell count (WCC), platelet count, Interleukin-6, 10, TNFα and C-reactive protein (CRP), but WCC, CRP and platelets have been removed (see 'ethics and safety later in 6.4). Heart rate and blood pressure were also measured for safety reasons and to allow comparison with previous other endotoxin dosing volunteer studies.

Measurements

Blood samples were taken into sterile, additive-free tubes (Becton Dickinson, Franklin Lakes, NJ, USA), every half hour for the first 2 hours, hourly until the 6^{th} hour, then at 8^{th} and 10^{th} hour (See Figure 6.1). They were allowed to clot at 4°C, spun at 2000 rpm for 10 minutes and the serum aliquoted and frozen at $^{-}$80 °C for later analysis. Heart rate and blood pressure (Dynascope DS 5100E, Fukuda Denshi, Tokyo, Japan), oral temperature (IVAX 2080A, IVAC, San Diego) white cell count, platelet count, C-reactive protein (CRP), tumour necrosis factor (TNF α), interleukin 6 (IL-6) and interleukin 10 (IL-10) were measured but WCC, CRP and platelets are not included here. Clinical and laboratory measurements were performed blinded to the subjects' endotoxin dose group. Baseline screening tests, white cell counts, platelet counts and CRP concentrations were performed at the New York Presbyterian Medical Centre (New York, NY, USA) according to methods from the College of American Pathologists. Cytokine assays were performed by myself using Biosource ELISA Cytoset kits (Biosource International CA, USA) according to the manufacturer's instructions by myself, a single investigator, in duplicate, and in as large a batch as possible to reduce assay variability (see Methods, Chapter 2). A standard curve provided by the manufacturer and a quality control serum were included on each ELISA plate.

dosing regimen	number of volunteers	endotoxin	
		bolus[1]	infusion[2]
Positive control	3	4 ng/kg	water
0.75 ng/kg bolus	5	0.75 ng/kg	water
0.75 ng/kg infusion	7	water	0.75 ng/kg
0.25 ng/kg bolus	7	0.25 ng/kg	water
0.25 ng/kg infusion	6	water	0.25 ng/kg
Placebo	2	water	water

Table 6.2: **Infusions and bolus in each of the dosing regimens.** A 'double-dummy' design was used as the timing of the two treatments (bolus or infusion) could not be concealed. [1]Bolus (in 20 mL over 1 minute) and [2]Infusion (in 50 mL over 30 minutes).

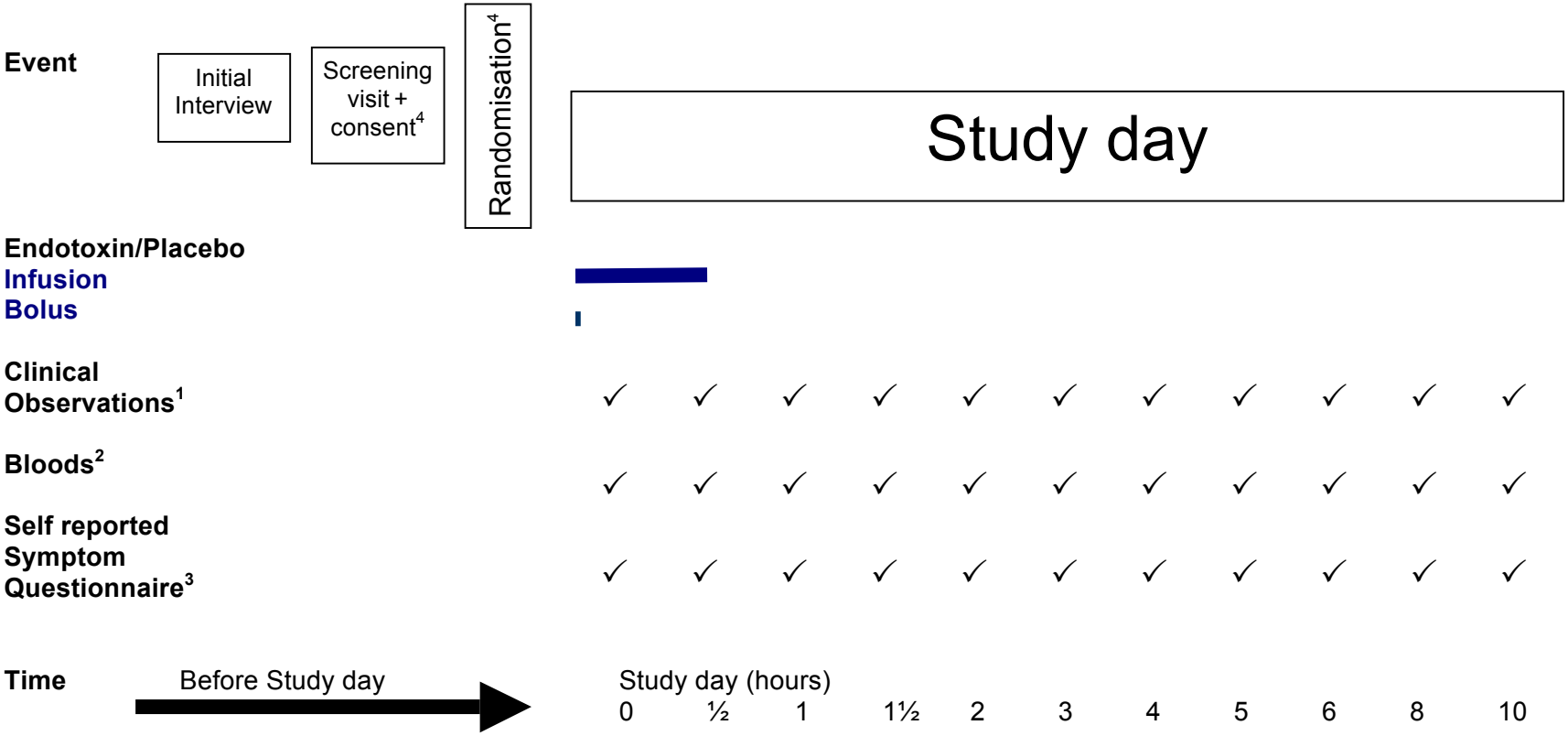

Figure 6.1: Summary timeframe of volunteer protocol. [1]Heart rate, Blood pressure, temperature were taken every 30 minutes. [2]Tumour necrosis factor alpha, interleukin 6 and interleukin 10. [3]Nausea, vomiting, myalgia, headache, shivering were self reported hourly. [4]Screening visit, consent and randomisation were performed before the study day.

Statistics

As the data were not normally distributed non-parametric tests were applied. Changes from baseline were examined by the Wilcoxon Signed Ranks test. Within-group variability of the inflammatory markers was assessed by visual (semiquantitative) examination of the graphed results and ranges (excluding top and bottom values). Statistics were calculated using SPSS (Statistical Package for the Social Sciences 15, Chicago, IL, USA).

Ethics and Safety

The clinical part of the study was performed in a research institute (Irving Clinical Centre, Columbia Presbyterian Hospital, New York, USA) designed for human volunteer research according to the Institutions regulations**.** Ethical permission was obtained from both the institution where the volunteers received the regimens (Irving Clinical Centre) and the institution where the cytokines were measured and data analysed (Departments of Immunobiology, Infectious Disease and Microbiology, Institute of Child Health, University College London, London, UK). Some years after the study had been completed the Columbia Presbyterian Hospital started to have concerns about one of the local researchers in the team. Their investigation (which took several years) ended in January 2013 and concluded *that they were unable to vouch for some of the data***.** The committee were unable to account for 29% of the white cell counts, platelet counts and C reactive protein measurements. These had been collected as surrogate measurements of inflammation and to allow comparison with previous endotoxin volunteer studies. Because of this the institution has asked that I, as corresponding author, withdraw the paper (Stephens *et al* 2005). At that stage (January 2013) this thesis was being revised after the viva at the request of the examiners. At the time of final writing (April 2013) I am waiting for a response from the journals editors.

6.5 Results

Thirty one volunteers were screened: one was excluded prior to randomisation because of an abnormal electrocardiogram. The other thirty were consented and randomised to one of six dosing regimens (table 6.2). Their characteristics are presented in table 6.3.

6.5.1 Outcome changes from baseline

Maximum increases from baseline in temperature, TNFα, IL-6 and IL-10 are shown in figure 6.2. Each bar represents a volunteer, with each volunteer appearing in the same order in all the graphs, allowing for a comparison of the results.

Characteristic		Result
Age (median, interquartile range) in years		25 (21–32)
Gender: number of males, females		10, 20
Racial group (number)	White Caucasian African-American Hispanic Other Asian	14 8 4 3 1
Screening HDL-cholesterol (median, interquartile range) in mg/dl		53 (45–59)
Initial temperature (median, interquartile range) in °F		97.7 (97.1–98.2)

Table 6.3: Summary of selected characteristics of the 30 volunteers. *Abbreviations*: HDL-cholesterol= High-density lipoprotein cholesterol.

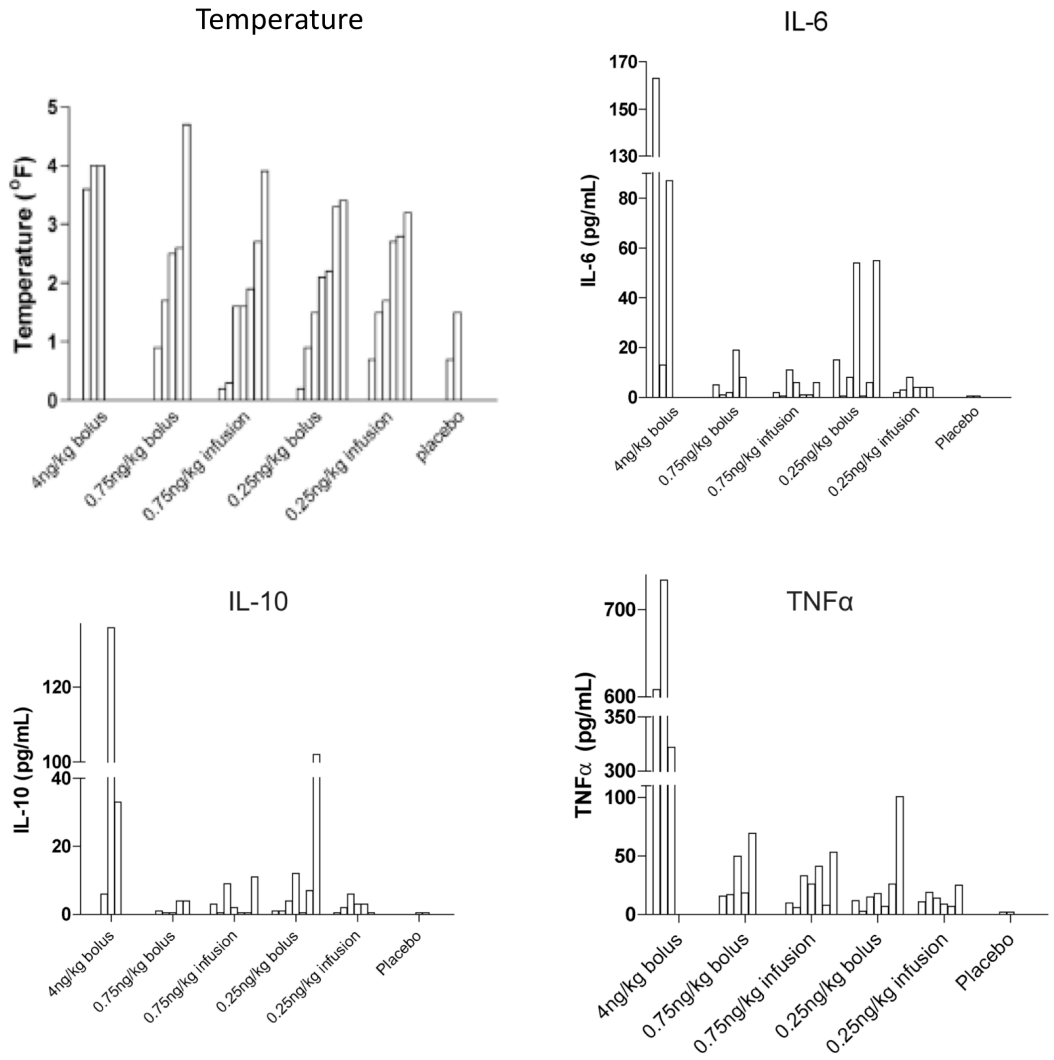

Figure 6.2: Temperature, Tumour Necrosis Factor alpha, Interleukin 6 and Interleukin 10. Maximum increase from baseline in temperature, Tumour Necrosis Factor alpha (TNFα), Interleukin 6 (IL-6) and Interleukin 10 (IL-10) grouped according to the 6 dosing regimens. Each bar represents a volunteer, with each volunteer appearing in the same order in all the graphs, allowing for a comparison of the results The groups are presented in order of descending dose concentration, and within each dosing regimen, the results are in order of ascending temperature rise. Volunteers show a range of responses within dosing groups.

Those randomised to the 4 ng/kg endotoxin dosing regimen (positive control) experienced significant symptoms and had rises in inflammatory markers, whilst those subjects that received normal saline did not (figures 6.2 and tables 6.4, 6.5).

All 4 low dose regimens produced statistically significant changes within the 10 hour period in most inflammatory markers (temperature and tumour necrosis factor alpha) and physiological measurements (heart rate, systolic and diastolic blood pressure) but not in interleukin-6 and interleukin-10 concentrations (table 6.5).

dosing regimen	Headache (%)	'Shivering' (%)	Myalgia (%)	Nausea (%)	Any Symptom (%)
Positive Control	100	100	100	100	100
0.75 ng/kg bolus	33	50	50	20	80
0.75 ng/kg infusion	29	29	14	0	43
0.25 ng/kg bolus	29	29	29	14	43
0.25 ng/kg infusion	67	33	33	17	67
Placebo	0	0	0	0	0

Table 6.4: **Volunteers' symptoms.** The percentage of volunteers in each dosing regimen with a symptom scored at 1 (or more) out of 10 for at least 2 consecutive time-points on a visual analogue scale recorded every hour.

dosing regimen (ng/kg)	Temperature (°F)	TNF α (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	Heart Rate (bpm)	Systolic BP (mmHg)	Diastolic BP (mmHg)
0.75 bolus	2.5 (0.9-4.6)*	18 (16-70)*	5 (0-19)	1 (0-4)	20 (11-35)*	18 (10-30)*	4 (0-20)
0.75 infusion	1.6 (0.2-3.9)*	26 (6-53)*	2 (0-11)	2 (0-11)	16 (0-20)*	10 (0-24)*	10 (0-12)*
0.25 bolus	2.1 (0.2-3.4)*	15 (3-101)*	8 (0-54)*	4 (0-102)*	18 (6-32)*	10 (4-24)*	10 (0-18)*
0.25 infusion	2.2 (0.7-3.2)*	14 (7-25)*	4 (2-8)*	2 (0-6)	24 (6-39)*	16 (8-28)*	18 (0-20)*

Table 6.5: Changes from baseline in the measured variables. Median change in (range lowest to highest) temperature, Tumour Necrosis Factor alpha, Interleukin 6, Interleukin 10, heart rate, systolic and diastolic blood pressure in the 4 low dose regimens. ^The units are cells $x10^9$ per litre. Cytokine values are quoted to the nearest whole unit.

*= $p < 0.05$ for the change from baseline in a Wilcoxon Signed Ranks test. Systolic and diastolic blood pressure fell, whilst the other outcomes rose.

Abbreviations: bpm, beats per minute; mmHg, millimetres of mercury; mg, milligramme; mL, millilitre; pg, picogramme; BP, blood pressure; ng, nanogramme; kg, kilogramme. TNFα, tumour necrosis factor alpha; IL-6, interleukin 6 and IL-10, interleukin 10.

6.5.2 Variability inflammatory outcomes

Subjects administered infusions or boluses of endotoxin at both 0.25 and 0.75 ng/kg doses exhibited changes in most inflammatory markers (figure 6.2 and table 6.5). In all four of these dosing regimens there was considerable within-group range (variability) in response to endotoxin, *i.e*. some volunteers showed changes in temperature as small as some of those given saline placebo, whilst responses in others were as large as some of those in the 4 ng/kg group (figure 6.2).

Although there was a range of responses, there was no evidence that any one of the 4 regimens caused a greater variability in inflammatory markers than the others (ranges in table 6.5), apart from in IL-6.

There is no evidence on either inspecting the graphed data (figure 6.2) or in terms of the range (table 6.6) that infusions (at either 0.25 or 0.75 ng/kg) of endotoxin produced greater variability in inflammatory markers than boluses at the same total dose.

6.6 Discussion

This is the first human volunteer study to identify several low-dose endotoxin regimens that elicit variability (spread or dispersion) in the inflammatory response between individuals. Other investigators have used relatively low doses of endotoxin (down to 0.8-1.0 ng/kg) before (Elin *et al* 1981; Korth *et al* 1996; Krabbe *et al* 2001a; Krabbe *et al* 2001b). These have shown a dose-response effect (i.e. less endotoxin, less change in inflammatory markers), but have not examined variability, whilst others have either used endotoxin other than the standard US *E coli* 0113 lot EC-5 or EC -6, used different extraction methods, chemically altered endotoxins, not specified the endotoxin, used male volunteers only or ‘old’ endotoxin, making direct comparisons difficult (Elin and Csako 1989; Fong *et al* 1990; Harris *et al* 2002; Korth *et al* 1996; Pollmacher *et al* 1996; Vedder *et al* 1999). However, the results are comparable to previously

reported values (Hochstein *et al* 1994; Kuhns *et al* 1995; Suffredini *et al* 1999). The results for the 1 ng/kg group in Suffredini's 1999 reference study using similar endotoxin and methods are shown for comparison (with table 6.5) in table 6.6.

Variability in groups as small as this is difficult to quantify precisely. If there was no variability within groups, the dispersion would be zero (i.e. if there were a uniform identical change in inflammatory markers with an endotoxin dosing regimen). Given the small number of observations, there was insufficent power to analyse the data using repeated measures analysis of variance (ANOVA). Therefore I decided it would be more appropriate not to statistically over analyse the data. I used the following to examine variability: visual and graphic (semiquantitative) examination of the raw values (figure 6.2) and ranges (table 6.5).

Semiquantitative examination of the raw values (6.2) reveals that in the primary outcome measure (temperature) all 4 of the low dose regimens had volunteers who had maximum temperature rises within 20% of the *median* positive (4 ng/kg) control *and* had volunteers that had rises as low as *median* placebo (0 ng/kg). In some of the secondary outcome measures (eg Interleukin 10) the majority of low dose regimes had changes as large as the smallest change in the 4 ng/kg group, but not as large as the *median* change in the positive control. So in the secondary outcomes there is a range of results, but none of the dosing regimens caused within-group variability as large as with fever: none had changes in inflammatory markers comparable with both the positive *and* negative (placebo) controls. In the case of IL-6 and the 0.25 ng/kg bolus the same one individual had the largest rise in IL-6, IL-10 and TNFα of all the low dose regimens and was the only person in that regimen to experience nausea.

Temperature (°F)	TNFα*	IL-6*	IL-10*
1.9	118	251	not measured

Table 6.6: **Mean maximum changes with 1 ng/kg compared to placebo**. Presented for comparison are the results from volunteers receiving 1 ng/kg (n=4) in the paper from Suffredini and colleagues (Suffredini *et al* 1999) using *Escherichia coli* O:113 lot EC-6. Tumour necrosis factor alpha and interleukin had peaked by 2 hrs from the study start. IL-10 was not recorded in their paper (Suffredini *et al* 1999). The units are *picogrammes per millilitre (IL-6, IL-10 and TNFα). Abbreviations: see table 6.5

There are several factors that strengthen the observations in this study. Firstly, the design included both positive (4 ng/kg) and negative placebo (0 ng/kg) controls. Those receiving the 4 ng/kg (positive control) regimen had symptoms and rises in many inflammatory markers comparable to those reported in previous studies (Elin *et al* 1981; Suffredini *et al* 1999) suggesting that there was no binding of endotoxin to the dosing equipment, the volunteers received the designed doses and the assays were working correctly, were appropriately sensitive and blood was correctly taken from the right person at the right time. Secondly the fact that volunteers receiving the 0 ng/kg (placebo) dosing regimen experienced no symptoms and had no rise in serum markers of inflammation suggests there was no significant contamination in either the dosing equipment or the assays. The intermediate changes seen in the 0.25 and 0.75 ng/kg dosing regimens support this conclusion. Lastly the study design was further strengthened by the fact that both volunteers and investigators were blinded to study group until after the measurements and assays had been completed. These facts also support the idea that blood was correctly taken from the right person at the right time and the research fraud in some measurements (white cell counts, platelet counts and C reactive protein) did not spill over to fever or cytokine measurements.

The removal of the suspect data has reduced the number of inflammatory markers available reducing the study's impact. However this chapter still has

some relevance in determining the fever threshold and that variability is possible with low endotoxin doses as the primary outcome (fever) and cytokines still allow comparison with other volunteer studies.

Although several different dose regimens cause variability, there are limitations to the study. I have not been able to demonstrate that any one regimen results in greater variability than the others. This is partly because there is no generally accepted measure of 'variability' in this context and due to the small numbers necessary for practical reasons. Also, the smaller numbers of positive controls (4 ng/kg), whilst ethically justifiable (as it is unpleasant, has been associated with serious effects and has been studied before) precludes reliable statistical comparison with the other regimens (van Eijk *et al* 2004). A further limitation is that I could not detect any differences between infusion and bolus regimens. Prior to the study, I had speculated that there may be differences in the systemic inflammatory response between infusions and bolus dosing regimens perhaps because an infusion over 30 minutes may allow more time for potential endotoxin neutralizing factors to act, but the results do not support this hypothesis.

One potential drawback of a low dose model is that it may not produce *enough* inflammation. A model designed to induce no or little inflammation in some subjects whilst producing some inflammatory marker activation in others may not be suitable for all purposes. For example, even though the 4 ng/kg endotoxin model is generally considered a good mimic of some of the early responses in septic shock it does not cause even low level changes (at the present rate of detection anyhow) in all the pathophysiological effects of sepsis eg microvascular permeability (Anel and Kumar 2005; van Eijk *et al* 2005). Whilst a low dose model *may* induce enough inflammation to examine some of the predictors of the inflammatory response, it probably will not cause enough inflammation to examine them all.

In addition to causing variability in inflammatory responses there may be other potential benefits of this model. The 4 ng/kg model is unpleasant, causes nasty side effects and occasionally results in serious sequelae. Like many rare side

effects, it is likely that not all of these are reported. I know of one unpublished UK- based case where 1 volunteer had to be admitted overnight with renal dysfunction (raised markers or renal function with reduced urine output) after endotoxin dosing (Stephens 2006). Of course, these side effects may partly be due to other factors such as starvation or fluid restriction, resulting in some to recommend to providing intravenous or oral fluid during the study, as we did (van Eijk *et al* 2004). The recent cases involving the role of healthy military 'volunteers' and the unanticipated severe side effects of 'TGN1412', an anti-CD28 monoclonal antibody given to healthy volunteers have led to increased awareness of the necessity to reduce any potential harm to human volunteers (Lee *et al* 2004; Sharp 2006; Suntharalingam *et al* 2006). A lower dose endotoxin model may play a part in this.

Lastly, in an era where increasing research fraud is being uncovered in my own speciality (Hospital Presents Results of Final Report: Committee Completes Investigation in the Case of Dr Boldt, 2012) the sad discovery of research misadventure in someone known to me has only caused me to try to be as clear and honest as I can be, especially where it really matters- with patients.

6.7 Conclusions

In this study (which had to be altered due to research ethics concerns) I have shown that the effects of low dose EC-6 are broadly consistent with previous published studies. At doses of 0.25 and 0.75 ng/kg, whether by bolus or infusion over 30 minutes, markers of inflammation rise and volunteers experience symptoms. Furthermore the fever threshold of this endotoxin is less than 0.25 ng/kg. Low dose endotoxin regimens do cause variability in the inflammatory response as judged by fever, but less variability in other outcomes such as interleukin 6, 10 and tumour necrosis factor alpha. A low dose regime may be a useful and more palatable tool to investigate the differences between individuals in endogenous factors (plasma, receptor, pathway and genotype) that alter the response to endotoxin.

6.8 Further work

One obvious application of a low dose endotoxin regimen such as 0.25 ng/kg is in teasing apart the various factors that alter an individuals' response to endotoxin. Such a study, if appropriately powered may be able to answer whether specific antibodies (eg anti-0113) or more generic ones (e.g. EndoCAb or natural IgMs) are more important in the response to endotoxin, if at all. Of course, the choice of endotoxin may well be relevant in that study. Furthermore the purpose of that study could also be to elucidate the relative importance of other endotoxin binding substances such as HDL-Cholesterol, Bacterial Permeability-Increasing protein, Lipopolysaccharide Binding Protein and other factors in the endotoxin signalling cascade in preventing endotoxin induced inflammation. Endotoxin dosing of volunteers has been more commonly used as a model of the effects of endotoxin during sepsis and after inflammatory insults on the host. Such a low dose regimen using 0.25 ng/kg endotoxin may not result in enough inflammation to cause, for example, cardiovascular or renal effects. An appropriately powered study using volunteers may be outside the constraints of realistic cost.

Chapter 7

Summary and final conclusions

7.1 Aims of the thesis

My original aim was to re-examine the original reported association between postoperative outcome and EndoCAb IgM in an observational study and then attempt to look at this association to see if the hypothesised mechanism - an exaggerated inflammatory response in those patients with low EndoCAb IgM - was present. In the end this I was only partially able to achieve these aims.

7.2 Summary of main findings

In the methods chapter 2 I showed I could repeatedly and accurately perform the assays needed for this thesis.

In chapter 3, I showed that low EndoCAb IgM was associated with a poor outcome after first time coronary artery bypass graft surgery, independent of the other predictors (Euroscore and Diabetes Mellitus). Other conclusions should be taken with more caution as this chapter was performed by analysing data from another study and using stored blood samples.

In chapter 4 I had intended to examine EndoCAb IgM's effects with IgM fractions taken from different individuals in an in-vitro study using FACS markers and cytokines (in an endotoxin stimulation model) as the outcome. However I was not able to include those methods or experiments as although I was able to manufacture IgM fractions from people with low and high EndoCAb IgM, I was not able to make it in a *sterile* fashion. In conjunction with one of my supervisors (Dr Helen Baxendale) I decided to examine another hypothesis: that EndoCAb IgM shared similarities with the 'natural IgM' pool. In this I had partial success. I was able to show there were significant associations between natural IgM levels (A, B, gal and phosphorylcholine) and EndoCAb IgM. I also found that some umbilical cord blood had measurable levels of EndoCAb and the natural IgM panel- a stated feature of natural IgM. Some (2 out of 7) of the natural IgM hybridomas bound to EndoCAb IgM.

I looked in chapter 5 at another measure of inflammation- SIRS. Again although limited by the fact that I used data and stored blood samples from a previous study, I showed that low EndoCAb IgG was associated with more SIRS after non-septic admissions to the paediatric critical care unit.

Lastly in Chapter 6, (despite ethical concerns) I showed that it was possible to use a novel low dose endotoxin dosing regimen (near the fever threshold) and get variability in response between volunteers – at least as judged by fever as a measure of inflammation.

7.3 Further work

In many ways this thesis raises more questions than it answered. Following on from chapters 3 and 4 it would be interesting to see if levels of the Natural IgM panel were associated with clinical outcome, SIRS or cytokine levels after surgery. To do this more robustly, sequential measurements (rather than a 6 and 24 cytokine level) should be made. Perhaps levels of an 'immune' (ie not a natural) IgM such as tetanus could be included too.

In chapter 4 I originally wanted to manufacture an IgM serum with different EndoCAb IgM levels in but was unable to do so in a sterile environment. Having shown that IgM EndoCAb levels are associated with levels of the Natural IgM panel this may not be such a useful too as a high IgM EndoCAb serum would be expected to also have high natural IgM levels.

Ideally, following on from chapter 6 I would perform an appropriately powered endotoxin infusion study looking at levels of all the potential variables that modify endotoxin-induced inflammation. This would require a large number (>100) of volunteers: a more practical alternative would be an in vitro endotoxin study.

7.4 Conclusions

I have shown that low EndoCAb IgM is associated with poorer outcome after first time CABG surgery. In healthy blood donors, there were significant associations between natural IgM (A, B, gal and phosphorylcholine) and EndoCAb IgM levels, pointing to new mechanisms. Some umbilical cord blood

had measurable levels of EndoCAb and the natural IgM panel. Critically ill (non-septic) children with lower IgG EndoCAb had more SIRS than those with higher levels. Lastly, low dose endotoxin dose regimens can cause varying inflammatory effects. Taken overall EndoCAb is clinically relevant, although I have not been able to show it causes the outcome differences seen between those with low and high levels.

Appendix 1: NYHA Classification

NYHA Class	Functional activity	Symptoms
I	No limitation of activity	Not from ordinary activities
II	Slight, mild limitation of activity	Comfortable with rest or with mild exertion
III	Marked limitation of activity	Comfortable only at rest
IV	Patient should be at complete rest, confined to bed or chair	Any physical activity brings on discomfort; symptoms occur at rest

New York Heart Association (NYHA) classification for Congestive cardiac failure. A functional and therapeutic classification for prescription of physical activity for cardiac patients (Miller-Davis *et al* 2006).

Appendix 2: EuroSCORE

Factor	Criteria	Score
Patient-related factors		
Age	(per 5 years or part thereof over 60 years)	1
Sex	female	1
Chronic pulmonary disease	long term use of bronchodilators or steroids for lung disease	1
Extracardiac arteriopathy	*claudication, carotid occlusion or >50% stenosis, previous or planned intervention on the abdominal aorta, limb arteries or carotids	2
Neurological dysfunction	severely affecting ambulation or day-to-day functioning	2
Previous cardiac surgery	requiring opening of the pericardium	3
Serum creatinine	>200μmol/L preoperatively	2
Active endocarditis	patient receiving antibiotics for endocarditis at the time of surgery	3
Critical preoperative state	*ventricular tachycardia or fibrillation or aborted sudden death, preoperative cardiac massage, ventilation, inotropes, IABP or ARF	3
Cardiac-related factors		
Unstable angina	angina requiring intravenous nitrates until arrival in the anaesthetic room	2
LV dysfunction	moderate or LVEF 30-50%	1
	poor or LVEF <30%	3
Recent myocardial infarct	less than 90 days ago	2
Pulmonary hypertension	Systolic Pulmonary Artery pressure>60 mmHg	2
Operation- related factors		
Emergency	carried out on referral before the beginning of the next working day	2
Other than isolated CABG	major cardiac procedure other than or in addition to CABG	2
Surgery on thoracic aorta	for disorder of ascending, arch or descending aorta	3
Postinfarct septal rupture		4

The components of the European System for Cardiac Operative Risk Evaluation 'EuroSCORE'. *Abbreviations:* IABP=Intra-aortic balloon pump; ARF=Acute renal failure; LVEF= Left ventricular ejection fraction. CABG= Coronary artery bypass graft. *= any one or more of the criteria. (Nashef *et al* 1999)

Appendix 3: Parsonnet Score

Factor	Criteria
Patient-related factors	
Age	
Sex	
Morbid Obesity	> 1.5 times ideal body weight
Diabetes	
Hypertension	> 140/90 mmHg
Renal Dialysis	
Catastrophic states	For example acute structural defect, cardiogenic shock or acute renal failure
Cardiac-related factors	
Ejection Fraction	Good or > 50%, moderate or 30-50%, poor or <30%
Recent myocardial infarct	less than 90 days ago
Preoperative intra Aortic Balloon Pump	
Operation- related factors	
Reoperation	
Emergency	
CABG	At the same time as valve s
Aortic valve surgery	+/- gradient > 120 mmHg
Mitral Valve surgery	+/- gradient > 60 mmHg
Left Ventricular Aneurysm	

The components of the Parsonnet Score. *Abbreviations:* CABG= Coronary artery bypass graft.

(Parsonnet *et al* 1989)

Appendix 4: Paediatric Logistic Organ Dysfunction score: PELOD

Factor	Criteria
Cardiovascular factors	
Heart rate	Age specific thresholds
Systolic blood pressure	Age specific thresholds
Respiratory factors	
$Pa0_2/Fi0_2$	<9.31 kPa
$PaC0_2$	>11.7 kPa
Mechanical Ventilation	Presence of invasive mechanical ventilation
Hepatic factors	
Aspartate transaminase	>950iu/L
International Normalised Ratio	>1.4
Neurological factors	
Glasgow Coma Score	3, 4-6, 7-11, 12-15
Pupillary reactions	Both reactive, both fixed
Haematological factors	
Total White cell count	<1.5, 1.5- 4.4, >4.5 $x10^9$/L
Platelet count	<35 $x10^9$/L
Renal factors	
Creatinine	Age specific ranges

The components of the Paediatric Logistic Organ Dysfunction (PELOD) score (Leteurtre *et al* 2003).

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