The Role of Platelets in Acute Inflammation

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Abstract

Platelets are cell fragments with haemostatic and inflammatory properties which are present in huge numbers in the peripheral blood. Lung inflammation in the Acute Respiratory Distress Syndrome (ARDS) occurs with particular severity in pancytopénie patients who are receiving regular platelet transfusions. Histological and experimental evidence supports a role for platelets in the pathogenesis of this condition. This thesis is primarily concerned with the investigation of the role of platelets in the pathogenesis of vascular endothelial dysfunction in clinical severe inflammatory conditions such as ARDS and the more generalised Systemic Inflammatory Response Syndrome.

An 18 month long prospective study of admissions to the Paediatric Intensive Care Unit (PICU) with ARDS was undertaken to determine the associations, clinical course and value of established severity markers of this and related conditions. Platelets from patients with ARDS and control PICU patients were analysed by flow cytometry to determine circulating levels of platelet activation. This study failed to demonstrate any differences in the two populations. A method was then developed to investigate the binding of platelets to other circulating inflammatory cells (predominantly neutrophils) as a means by which assessment of free platelet activation may underestimate the true extent of platelet activation. This method was subsequently used to investigate the properties of complexes of platelets and neutrophils with respect to adhesive, phagocytic and metabolic functions. Platelet neutrophil complexes were shown to represent activated sub-populations of both cell types. The capacity of platelets to interact with an established model of the vascular endothelium in culture was investigated by flow cytometry. Studies in intensive care patients and in adults with risk factors for cardiovascular disease indicated that levels of circulating platelet-neutrophil complexes may be related to the extent of endothelial activation.
Table of Contents

Abstract .......................... 2
Table of Contents ..................... 3
List of Figures ...................... 8
List of Tables ..................... 11
Abbreviations .................... 12
Acknowledgements .................. 14
Publications ..................... 15

Chapter 1: Platelets and the pathogenesis of vascular endothelial injury in acute severe inflammation

1.1 Introduction .................... 17
1.2 The systemic inflammatory response 18
1.2.1 The vascular endothelium ...... 20
1.2.2 Leukocyte adhesion .......... 20
1.2.3 Thromboresistance .......... 26
1.2.4 Local blood flow .......... 31
1.2.5 Endothelial permeability .... 32
1.2.6 The Integration of Endothelial Functions .... 33
1.2.7 Therapeutic potential .... 34
1.2.8 Conclusion .................. 34
1.3 Platelets ..................... 35
1.3.1 Introduction ................ 35
1.3.2 Platelet structure ........ 35
1.3.3 Platelet function .......... 45
1.3.4 Platelets and coagulation .... 49
1.3.5 Platelets and inflammation ...... 51
1.3.6 Conclusion .................. 53
1.4 Platelet activation in acute respiratory distress syndrome .... 54
1.4.1 Introduction 54
1.4.2 The Importance of Inflammation in ARDS 56
1.4.3 Platelets and ARDS 60
1.4.4 Conclusion 62
1.5 Aims of this thesis 63

**Chapter 2: General methods**

2.1 Introduction 65
2.2 Materials 65
2.2.1 Chemicals and Reagents 65
2.2.2 Monoclonal antibodies 66
2.2.3 HUVEC culture 67
2.2.4 Neutrophil and platelet separation 67
2.3 Endothelial Cell Culture 68
2.3.1 Background 68
2.3.2 HUVEC culture media 68
2.3.3 Isolation of Human Umbilical Vein Endothelial Cells 68
2.3.4 HUVEC primary Culture 70
2.3.5 HUVEC subculture 70
2.3.6 Identification of HUVECs 70
2.4 Neutrophil separation 71
2.5 Platelet separation 71

**Chapter 3: Paediatric Acute Hypoxaemic Respiratory Failure**

3.1 Introduction 74
3.2 Paediatric acute hypoxaemic respiratory failure 74
3.3 Methods 77
3.4 Results 80
3.4.1 Patients 80
3.4.2 Outcome and acute physiological disturbance 82
3.4.3 Outcome related to diagnosis 82
3.4.4 Clinical course 85
3.4.5 Mode of death 85
3.5 Discussion 87
3.5.1 Summary 87
Chapter 6: Function of Platelet Neutrophil Complexes

6.1 Introduction 139
6.2 Methods 140
6.2.1 Whole blood stimulation 140
6.2.2 Phagocytosis study 141
6.2.3 Oxidative burst 141
6.3 Results 143
6.3.1 Adhesion molecule expression 143
6.3.2 Adhesion molecule activation 149
6.3.3 Influence of CD62P blockade 147
6.3.4 Phagocytosis 154
6.3.5 Neutrophil superoxide activity 151
6.4 Discussion 157

Chapter 7: Platelets and the Vascular Endothelium

7.1 Introduction 163
7.2 Methods 165
7.2.1 Platelet induced endothelial adhesion molecule expression 165
7.2.2 Platelet induced neutrophil adhesion 167
7.2.3 Adhesion of platelet neutrophil complexes to HUVECs 167
7.2.4 Platelet neutrophil complexes in type 1 diabetic patients 168
7.3.1 Results 171
7.3.1 Platelet induced endothelial adhesion molecule expression 171
7.3.2 Platelet induced neutrophil adhesion 173
7.3.3 Adhesion of platelet neutrophil complexes to HUVECs 175
7.3.4 Platelet neutrophil complexes in type 1 diabetic patients 177
7.4.1 Discussion 180
7.4.1 Platelet induced endothelial adhesion molecule expression 180
7.4.2 Platelet induced neutrophil adhesion 181
7.4.3 Adhesion of platelet neutrophil complexes to HUVECs 183
7.4.4 Platelet neutrophil complexes in type 1 diabetic patients 184
7.5 Conclusions and Future Work 187
Chapter 8  Platelet-Neutrophil Complexes in Sepsis

| 8.1 | Introduction | 189 |
| 8.2 | Methods | 190 |
| 8.2.1 | Whole blood stimulation | 190 |
| 8.2.2 | Time course of neutrophil and platelet parameters in clinical meningococcal septicaemia | 191 |
| 8.3 | Results | 194 |
| 8.3.1 | Effect of lipopolysaccharide endotoxin on platelet-neutrophil complexes | 194 |
| 8.3.2 | Effect of fMLP on platelet-neutrophil complex formation | 199 |
| 8.3.3 | Mechanisms of platelet-neutrophil complex formation | 200 |
| 8.3.4 | Neutrophil and platelet parameters in meningococcal septicaemia | 203 |
| 8.3.5 | Time course of changes in neutrophil and platelet parameters in meningococcal septicaemia | 206 |
| 8.4 | Discussion | 210 |

Chapter 9  Discussion

| 9.1 | Platelets in Acute Inflammation | 215 |
| 9.2 | Questions addressed in this thesis | 215 |
| 9.3 | Assessing the validity and significance of this project | 219 |
| 9.4 | Conclusions and Future Work | 220 |

References 221

Publications 259
### List of Figures

| Figure 1.1 | The sequence of events involved in leukocyte emigration from the circulation into the surrounding tissues | 21 |
| Figure 1.2 | Principal leukocyte-endothelial interactions incorporating the selectin family of adhesion molecules | 22 |
| Figure 1.3 | Principal leukocyte-endothelial interactions incorporating the integrin and immunoglobulin superfamily of adhesion molecules. | 24 |
| Figure 1.4 | Endothelial cell surface molecules which contribute to coagulation and fibrinolytic pathways | 27 |
| Figure 1.5 | Platelet at rest and following activation. | 36 |
| Figure 1.6 | The interaction of CD62P (P-selectin) and its principal ligand P-Selectin glycoprotein ligand (PSGL-1) | 40 |
| Figure 1.7 | Structure of GpIib/IIIa receptor | 41 |
| Figure 1.8 | The platelet thrombin receptor and the interaction of GpIib/IIIa with ligand and platelet | 43 |
| Figure 1.9 | Schematic drawing of GpIb-V-IX complex | 44 |
| Figure 1.10 | The proposed mechanisms for platelet adhesion to intact vascular endothelium | 46 |
| Figure 1.11 | Platelets and coagulation | 50 |
| Figure 1.12 | Platelets can act to support multi-step leukocyte adhesion at sites of endothelial damage | 52 |
| Figure 1.13 | ARDS in Sepsis | 55 |
| Figure 1.14 | Section of normal human lung | 58 |
| Figure 1.15 | Severe ARDS | 58 |
| Figure 1.16 | Gross appearance of the cut surface of human lung following fibrotic stage of lung injury | 59 |
| Figure 1.17 | Appearance of 'hyaline membranes' | 59 |
| Figure 1.18 | Micro-thrombus obstructing a pulmonary arteriole | 62 |
| Figure 3.1 | Mortality by A) the acute diagnosis precipitating admission and B) by pre-existing diagnosis | 84 |
| Figure 3.2 | Clinical course of AHRF identified by the best daily AaDO2 ratio | 86 |
| Figure 4.1 | The characteristic forward and side scatter pattern of platelets with whole blood flow cytometry | 100 |
Figure 4.2 Patterns of fluorescence staining of platelets with anti-GpIIb/IIIa (PAC-1:FITC) and anti-CD62P:FITC  
Figure 4.3 Circulating platelet GpIIb/IIIa expression (MFI) in single system failure ICU patients and cases of ARDS.  
Figure 4.4 Circulating platelet CD62P expression A) MFI and B) % positive fluorescence in ARDS, at risk and AHRF cases.  
Figure 5.1 Platelet Satellitism  
Figure 5.2 Appearance of erythrocyte / leukocyte population after staining with CD11b:FITC and CD42b:PE.  
Figure 5.3 Flow cytometer forward / side scatter profile for measurement of platelet / neutrophil complexes.  
Figure 5.4 Fluorescence profiles of a typical sample prepared for measurement of platelet / neutrophil complexes.  
Figure 5.5 The appearance of platelet neutrophil complexes on confocal microscopy  
Figure 5.6 The changes in the percentage of neutrophils which are complexed to platelets after stimuli to platelet activation (ADP, thrombin) and neutrophil activation (fMLP).  
Figure 5.7 Intensity of CD42b expression in PNC population.  
Figure 5.8 Kinetics of percentage platelet / neutrophil complex formation and % of platelets expressing CD62P following stimulation with ADP.  
Figure 5.9 The influence of mechanical agitation on the kinetics of A-C percentage platelet-neutrophil complex formation and free platelet CD62P expression following stimulation.  
Figure 5.10 The influence of anticoagulant on %PNC detected at rest and following ADP stimulation.  
Figure 5.11 Influence of co-incubation with CD11a/CD11b/CD18 and CD62P blocking monoclonal antibodies.  
Figure 6.1 Neutrophil adhesion molecule expression  
Figure 6.2 Example of fluorescence profiles for adhesion molecules in free and PNC neutrophils.  
Figure 6.3 Comparison of the changes in CD11b expression with platelet agonists in whole blood and separated neutrophils  
Figure 6.4 Neutrophil Mac-1 activation.
Figure 6.5 Influence of platelet-neutrophil contact on platelet-induced changes in neutrophil adhesion molecules.

Figure 6.6 Comparison of PNC and free neutrophil phagocytosis

Figure 6.7 Oxidative burst activity

Figure 6.8 The influence of G1 blocking antibody on %PNC and neutrophil and PNC DHR fluorescence.

Figure 7.1 Characteristic forward and side scatter appearance of HUVECs

Figure 7.2 Platelet induced endothelial adhesion molecule expression

Figure 7.3 Platelet mediated neutrophil adhesion to HUVECs.

Figure 7.4 PNCs adherence to HUVECs

Figure 7.5 Relationship between flow mediated vasodilatation (FMD) and %PNCs

Figure 7.6 Are platelet neutrophil complexes are primed for endothelial adhesion?

Figure 8.1 A 10 month infant with severe meningococcal septicaemia

Figure 8.2 Lipopolysaccharide endotoxin stimulation of whole blood at 60 min

Figure 8.3 Single donor response PNCs to LPS stimulation at 10ng/ml

Figure 8.4 The influence of co-stimulation of whole blood with LPS and ADP on %PNC

Figure 8.5 The influence of co-stimulation of whole blood with LPS and ADP on free platelet activation

Figure 8.6 Platelet neutrophil complexes after stimulation with ADP and co-stimulation with ADP/fMLP

Figure 8.7 Expression of candidate molecules for neutrophil influence on PNC formation after stimulation with LPS

Figure 8.8 %PNC after stimulation with ADP and co-stimulation ADP/KIM127

Figure 8.9 Time course of changes in neutrophil and platelet parameters in a case of severe meningococcal disease

Figure 8.10 Time course of Neutrophil CD11b, Mab24 CD62L and %PNC in severe meningococcal disease.

Figure 8.11 Time course of platelet activated GpIIb/IIIa expression in severe meningococcal disease
List of Tables

Table 1.1  Definitions of sepsis and related terms  19
Table 1.2  Endothelial derived factors which contribute to altered  28
           haemostasis in SIRS
Table 1.3  Principle platelet adhesion molecules  38
Table 1.4  Platelet granule contents  39
Table 1.5  American European Consensus Conference 1992 Definition of  54
           ARDS
Table 1.6  Histological stages of ARDS  57
Table 1.7  Evidence for Involvement of platelets in the pathogenesis of  61
           ARDS
Table 2.1  Miscellaneous Reagents  65
Table 2.2  Monoclonal antibodies  66
Table 2.3  Chemicals and reagents and materials for HUVEC culture.  67
Table 2.4  Chemicals and reagents for neutrophil and platelet separation.  67
Table 3.1  Inclusion criteria for study  79
Table 3.2  Univariate analysis of respiratory parameters by PICU survival  81
Table 3.3  Multiple logistic regression analysis of physiological parameters  83
           against PICU survival
Table 3.4  Comparison of previously published respiratory severity  88
           parameters
Table 3.5  Cases treated with "new " therapeutic modalities  89
Table 3.6  Details of diagnoses and outcome for the 'immunodeficient' sub-
           group.
Table 7.1  Relation between FMD and PNCs after adjustment for other risk  179
           factors
Figure 8.1  The Glasgow Meningococcal Sepsis Prognostic Score  193
Figure 8.2  Modified Coma Score  193
Figure 8.3  Univariate analysis of neutrophil and platelet parameters on the
           presentation blood count in clinical meningococcal disease  205
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-aDO2</td>
<td>Alveolar-arterial oxygen tension gradient</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AHRF</td>
<td>Acute hypoxaemic respiratory failure</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ATIII</td>
<td>Anti-thrombin III</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulopathy</td>
</tr>
<tr>
<td>ECMO</td>
<td>Extra-corporeal membrane oxygenation</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow activated cell sorter</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluoroscein isothiocyanate</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow-mediated dilatation</td>
</tr>
<tr>
<td>fMLP</td>
<td>Formyl-Methionine-Leucine-Phenyalanine</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GMSPS</td>
<td>Glasgow Meningococcal Sepsis Prognostic Score</td>
</tr>
<tr>
<td>GPRP</td>
<td>Glycine-proline-arginine-proline</td>
</tr>
<tr>
<td>GTN</td>
<td>Glyceryl trinitrate (GTN)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>HFOV</td>
<td>High frequency oscillatory ventilation</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon - gamma</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>iNO</td>
<td>Inhaled nitric oxide</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthetase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide endotoxin</td>
</tr>
<tr>
<td>MAC-1</td>
<td>CD11b/CD18</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean airway pressure</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MODS</td>
<td>Multi-organ dysfunction syndrome</td>
</tr>
<tr>
<td>MOSF</td>
<td>Multiple organ system failure</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OI</td>
<td>Oxygenation index</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Arterial carbon dioxide tension</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Arterial oxygen tension</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end expiratory pressure</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PF3</td>
<td>Platelet factor 3</td>
</tr>
<tr>
<td>PGI2</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PIP</td>
<td>Peak inspiratory pressure</td>
</tr>
<tr>
<td>PNCs</td>
<td>Platelet-neutrophil complexes</td>
</tr>
<tr>
<td>RDGS</td>
<td>arginine-glycine-aspartic acid-serine</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>t-PA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>VI</td>
<td>ventilation index</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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Acknowledgements

This work could not have been initiated without the ideas and encouragement of Robert Tasker and the support of Professor David Hatch and Ian James. I am particularly grateful to Nigel Klein for his endless patience enthusiasm and excitement, his readiness to make helpful suggestions and for teaching me extra-ordinary time-keeping practices.

I would also like to thank Karolena Kotowicz for her thorough and patient teaching of laboratory techniques and Garth Dixon for readily taking more than his share of the on-call workload. I was fortunate in working with Mike Mullen and John Deanfield in the assessment of in-vivo endothelial dysfunction.

Finally, none of this would have got anywhere without Julie Forsythe's constant encouragement to 'stop fussing and get on with it'.
Publications

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


Chapter 1

Platelets and the Pathogenesis of Vascular Endothelial Injury in Acute Severe Inflammation

1.1 Introduction

1.2 The systemic inflammatory response

1.3 Platelets

1.4 Platelet activation in acute respiratory distress syndrome

1.5 Aims of the project
1.1 Introduction

Inflammation is the host response to an invading micro-organism or injury. The non-specific nature of this response has led to the viewpoint that host responses to infection or injury may be responsible for extending tissue injury beyond that which was induced by the initial insult. Unchecked inflammation has been considered to be critical in the pathophysiology of systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS) and multi-organ dysfunction syndrome (MODS) (Bone et al. 1992). Many efforts have been made to limit the intensity of the systemic inflammatory response by therapeutic blockade of mediators known to be involved (Zeni et al. 1997). None have proved effective. The balance between the beneficial and detrimental effects of a host’s inflammatory response remains the subject of debate (Peters et al. 1999; Docke et al. 1997).

Vascular endothelial dysfunction is a constant feature of severe inflammation such as SIRS or ARDS. Dysfunctional endothelium cannot maintain homeostasis in terms of permeability to plasma proteins, changes in local blood flow, cellular adhesion or intravascular coagulation. Each of these functional alterations contributes directly to the clinical picture of severe inflammation.

Platelets arrest very rapidly at sites of endothelial injury. They are the principal component of the primary haemostatic process and provide an essential catalyst for stable clot formation. Evidence for considerable overlap between haemostasis and inflammation is increasing as platelets have been shown to have the capacity to interact with leukocytes (Diacovo et al. 1996b; Rinder HM et al. 1991b) and intact endothelium (Bombeli et al. 1998).

The purpose of the work presented in this thesis was to identify mechanisms by which platelets might induce vascular endothelial activation either directly or through interactions with other inflammatory cells. This was performed alongside a clinical audit of an example of severe inflammation in children, ARDS, which has been
observed to occur in the absence of leukocytes but not platelets (Sivan et al 1990; Ognibene et al 1986).

The introduction will describe our current understanding of the alterations in vascular endothelial function that accompany the onset of systemic inflammation, the structure and function of platelets and the nature of the specific example of severe inflammation; acute respiratory distress syndrome.

1.2 The systemic inflammatory response

Systemic inflammatory response syndrome (SIRS), is defined by changes in body temperature, heart rate, respiratory rate and peripheral white cell counts (Bone, 1996; Bone et al 1992). SIRS includes the systemic response to infection; sepsis, and the similar processes which follow major insults such as trauma, pancreatitis or major burns. SIRS, most commonly in the form of severe sepsis, can progress to multi-organ dysfunction syndrome (MODS) in which all body systems fail, is a major cause of morbidity and the commonest cause of death in intensive care units (table 1.1).

While systemic inflammation is a complex multi-cellular process, it is clear that dysfunction of the vascular endothelium is a central event in the development of the clinical features of sepsis and SIRS. This section of the introduction therefore will focus on the role of the endothelium in the pathogenesis of SIRS.
**Introduction**

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**Table 1.1**

**Definitions of sepsis and related terms.** The consensus criteria for a diagnosis of bacteraemia, SIRS, sepsis, severe sepsis, and multiple organ failure in adults are shown (American College of Chest Physicians and Society of Critical Care Medicine 1992).

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td><strong>Bacteraemia</strong></td>
<td>The presence of viable bacteria in the blood</td>
</tr>
<tr>
<td><strong>Systemic inflammatory response syndrome (SIRS)</strong></td>
<td>The systemic inflammatory response syndrome to a variety of severe clinical insults manifested by ≥2 of: Temp &gt;38 or &lt;36°C, Heart Rate &gt;90 beats per min Resp Rate &gt;29 breaths per min or PaCO₂ &lt;4.3 KPa White blood count &gt;12,000 or &lt;4,000 cells/ml, or &gt;than 10% immature forms</td>
</tr>
<tr>
<td><strong>Sepsis</strong></td>
<td>The systemic response to infection, manifested by ≥2 of Temp &gt;38 or &lt;36°C, Heart Rate &gt;90 beats per min Resp Rate &gt;29 breaths per min or PaCO₂ &lt;4.3 KPa White blood count &gt;12,000 or &lt;4,000 cells/ml, or &gt;than 10% immature forms</td>
</tr>
<tr>
<td><strong>Severe Sepsis</strong></td>
<td>Sepsis associated with organ dysfunction, hypoperfusion and hypotension</td>
</tr>
<tr>
<td><strong>Septic Shock</strong></td>
<td>Sepsis with hypotension despite adequate fluid resuscitation along with perfusion abnormalities</td>
</tr>
<tr>
<td><strong>Multiple organ dysfunction syndrome (MODS)</strong></td>
<td>Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention</td>
</tr>
</tbody>
</table>
1.2.1 The vascular endothelium

In health, the vascular endothelium provides a thromboresistant surface, regulates local blood flow, controls the adhesion and migration of leukocytes and provides a semi-permeable membrane which is essential for sustaining intra-vascular protein and fluid composition.

Each of these functions of the endothelium are altered in SIRS and MODS. Disseminated intravascular coagulopathy (DIC), hypotension, leukopenia and "capillary leak" represent the clinical consequences of severe disruption of each of these functions, and each is consistently associated with a poor outcome (Kornelisse et al 1997; Derkx et al 1996).

This section will describe the current understanding of the causes and nature of these changes in vascular endothelial function and report on recent attempts to manipulate endothelial properties for therapeutic benefit.

1.2.2 Leukocyte Adhesion

The regulation of leukocyte traffic is critical to control of infection and wound healing. Many of the molecular mechanisms that are involved in controlling leukocyte adhesion and migration have now been characterised. Three principal stages (rolling, firm adhesion and transmigration) have been identified involving three families of adhesion molecules: the selectins, the integrins and the immunoglobulin superfamily (figure 1.1).

Leukocyte Rolling

The first stage of "leukocyte rolling" is principally mediated by the selectins; E-selectin (CD62E) and P-selectin (CD62P) present on endothelial cells and L-selectin (CD62L) present on leukocytes (Carlos & Harlan, 1995). These molecules form transient bonds with a variety of carbohydrate or glycoprotein ligands (figure 1.2)
addressins GlyCAM-1 and MAdCAM-1) dictate the sites of leukocyte rolling which is maximal in the high endothelial venules (HEVs) (Carlos & Harlan, 1995).

Rolling leukocytes have the opportunity to sample the endothelial micro-environment. In sepsis this endothelial micro-environment will contain pro-inflammatory cytokines (e.g. interleukin-1, tumour necrosis factor-α, interleukin-6) and chemokines (e.g. interleukin-8) which provide stimuli for the leukocyte to prepare for firmer adhesion (Shanley et al 1995; Arnaout, 1993; Mackay & Imhof, 1993).

**Figure 1.1**

The sequence of events involved in leukocyte emigration from the circulation into the surrounding tissues. Initial ‘rolling’ adhesion is mediated by the selectin adhesion molecule family, prior to firm adhesion and transmigration which requires integrin–immunoglobulin superfamily interactions.
**Figure 1.2**

**Principal leukocyte-endothelial interactions incorporating the selectin family of adhesion molecules** (Adapted from Varki, 1997).

**CD62P** binds to carbohydrates including sialyl Lewis\(^\text{X}\) (CD15s, SLe\(^\text{X}\)) and related molecules with relatively low affinity and to the specific P-selectin glycoprotein ligand -1 (PSGL-1) see platelet structure section 1.3.2.

**CD62E** also binds to tetrasaccharide carbohydrates and to lymphocyte adhesion molecules that may have similar carbohydrate components (CD66, CD62L and \(\beta_2\) integrins and proposed E-selectin ligand -1 ESL-1).

**CD62L** binds to glycosalation dependent cell adhesion molecule-1 (GlyCAM-1) and to Mucosal addressin cell adhesion molecule MAdCAM-1.
Initial selectin-mediated rolling adhesion is followed by firm integrin to members of the immunoglobulin superfamily mediated adhesion and transmigration into the extravascular space. Endothelial activation and chemokine (e.g. IL-8) production is essential for the progression from rolling to firm adhesion.

**Firm adhesion and transmigration**

Firm adhesion and subsequent transendothelial migration of inflammatory cells is largely mediated by the leukocyte β2-integrin molecules (principally LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) and by endothelial expression of the immunoglobulin superfamily ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), VCAM-1 (CD106), PECAM-1 (CD31) and MAdCAM-1 (Carlos & Harlan, 1995; Zimmerman et al. 1995).

The selective recruitment of leukocyte subsets to sites of inflammation is orchestrated by the controlled production and interaction of different inflammatory mediators which are able to direct expression of adhesion molecules and mediator release at the endothelial cell surface. The differences in kinetics of endothelial adhesion molecule expression, such as E-selectin, ICAM-1 and VCAM-1 (figure 1.3), induced by these mediators, may contribute to the selective recruitment of leukocyte subsets to sites of inflammation (Shanley et al. 1995; Zimmerman et al. 1995; Ebisawa et al. 1994; Lukacs et al. 1993). Further fine control of adhesion is achieved by changes in integrin expression and affinity occurring as a result of stimuli to other leukocyte receptors (a similar process occurs with αIIbβ3 figure 1.8) (Smyth et al. 1993). Therefore, the endothelium and circulating leukocytes both exert an influence over the process of firm adhesion. The pro-inflammatory cytokines IL-1 and TNF-α induce expression of all three major adhesion molecules, E-selectin, ICAM-1 and VCAM-1, on endothelium. E-selectin is expressed early and transiently, peaking within 6 hours and leads to preferential attraction of neutrophils. ICAM-1 and VCAM-1 expression occurs later (12-24 hours after stimulation) and is more sustained, resulting in attraction of lymphocytes and monocytes (Bevilacqua, 1993; Thornhill et al. 1991).
Principal leukocyte-endothelial interactions incorporating the integrin and immunoglobulin superfamily of adhesion molecules (Adapted from Varki, 1997). Integrins consisting of combinations of α and β chains adhere to endothelial cell adhesion molecules of the immunoglobulin superfamily. The differing patterns of affinity allow selective recruitment of leukocyte subsets by their characteristic patterns of integrin expression (e.g. VLA-4 α4β1 is expressed by lymphocytes but not neutrophils, and hence VCAM-1 expression will encourage selective recruitment of lymphocytes). MAdCAM-1 is unique as an adhesion molecule as it acts as a ligand for α4β7 and for the selectin CD62L. PECAM-1 appears to have a dual role in leukocyte endothelial interactions as both an agonist receptor and an adhesion receptor (Newman, 1997).
Interleukin-4 (IL-4) is also an important regulator of endothelial cell function and recruitment of leukocyte subsets. Alone, it selectively enhances VCAM-1 (Beekhuizen et al. 1992; Thornhill et al. 1991) leading to adhesion of lymphocytes, monocytes, eosinophils and basophils but not neutrophils (Luscinskas et al. 1995; Luscinskas et al. 1994; Briscoe et al. 1992; Thornhill et al. 1990). In combination with other cytokines, it modulates adhesion molecule expression and leukocyte subsets attracted to the site of inflammation. IL-4 inhibits TNF-α and IL-1 induced E-selectin and ICAM-1 but enhances VCAM-1 favouring the attraction of lymphocytes over neutrophils (Thornhill et al. 1991; Masinovsky et al. 1990; Thornhill & Haskard, 1990). In contrast, Interferon-γ (IFN-γ) augments TNF-α induced E-selectin expression and prolongs its half-life on the cell surface, thus favouring neutrophil attraction (Doukas & Pober, 1990; Leeuwenberg et al. 1990).

Cleavage of molecules such as ICAM-1, VCAM-1 and the selectins may also be part of the mechanism for controlling leukocyte adhesion and transendothelial migration during infection (Carlos & Harlan, 1995; Ishiwata et al. 1994). A number of adhesion molecules appear as cleaved soluble products in the serum and are elevated in patients with various diseases (Katayama et al. 1993). High levels of these soluble products may block leukocyte attachment to mediator activated endothelium and be important in combination with cytokines in directing leukocyte attraction and controlling development, maintenance and downregulation of the inflammatory immune response. Whilst the role of circulating adhesion molecules is still unclear, elevated levels of soluble ICAM-1 indicate endothelial dysfunction in coronary vascular disease (Ridker et al. 1998).

The understanding of control of leukocyte adhesion is constantly changing. There have been recent advances in determining the relationships between structure and function of adhesion molecules (Hogg et al. 1999), the particular relevance of adhesion molecules to specific clinical conditions (Gavrilovskaya et al. 1999; Prestigiacomo et al. 1999; Rosenkranz et al. 1999; Scalia et al. 1999) and the
consequences and signalling mechanisms resulting from adhesion molecule ligation (Schoenwaelder & Burridge, 1999).

In summary, endothelial adhesion molecules are regulated by individual or combinations of cytokines, inflammatory cells and mediators which may be additive, synergistic or antagonistic. These complex regulatory mechanisms lead to the recruitment of the appropriate leukocyte subset at sites of inflammation.

### 1.2.2 Thromboresistance

The healthy vascular endothelium provides a thromboresistant surface which promotes flow across the luminal surface of the blood vessel wall (Heyderman, 1993; Levi et al. 1993;). This function is disrupted in acute sepsis by a reduction in anticoagulant and an increase in pro-coagulant molecule expression, by attenuation of the mechanisms by which the endothelium resists platelet activation and by limitation of fibrinolytic activity (figure 1.4 and table 1.2).

In acute severe sepsis, disseminated intra-vascular coagulopathy (DIC) is associated with a high mortality (Barquet et al. 1997; Kornelisse et al. 1997; Derkx et al 1996). The mechanisms leading to DIC are complex but involve the activation of both fibrinolysis and coagulation (van Deventer et al 1990). The vascular endothelium is directly involved in both of these pathways (figure 1.4) and measures of endothelial activation correlate with the severity of DIC (Okajima et al 1997). In-vivo evidence of the sequence of events in the pathogenesis of DIC has been obtained from experiments in which endotoxin has been administered to healthy volunteers (Pajkrst et al 1997; DeLa Cendra et al 1996; van Deventer et al 1990; Suffredini et al 1989;). Endotoxin induces an initial increase in fibrinolytic activity within 2 hours principally via tissue plasminogen activator, (t-PA). This is rapidly superseded by suppression of fibrinolysis mediated by raised levels of plasminogen activator inhibitor type 1 (PAI-1) (van Deventer et al 1990). Both t-PA and PAI-1 are produced by the vascular endothelium (Sakata et al 1985) (table 1.2). Following simple bolus
Introduction

Figure 1.4

Endothelial cell surface molecules which contribute to coagulation and fibrinolytic pathways The endothelium expresses molecules which have pro-coagulant (e.g. tissue factor) and anti-coagulant (e.g. tissue factor pathway inhibitor, (TFPI) CD39 or thrombomodulin) effects. The net effect of severe endothelial dysfunction is pro-coagulant. Further contributions are made by soluble factors and by the sub endothelial matrix (see text)
**Table 1.2**

*Endothelial derived factors which contribute to altered haemostasis in SIRS.* These changes in the haemostatic balance may lead to intravascular thrombosis. GAGs = glycosaminoglycans, ATIII = Antithrombin III, and iNOS = inducible nitric oxide synthetase.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Action</th>
<th>In Sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulation system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue factor</td>
<td>Activates Factor VII to VIIa. Initiates extrinsic clotting pathway</td>
<td>TF increased on endothelial cells and endothelial bound monocytes</td>
</tr>
<tr>
<td>Factor XII</td>
<td>Activation initiates intrinsic clotting pathway</td>
<td>Activated by exposed sub-endothelium</td>
</tr>
<tr>
<td>Tissue Factor Pathway Inhibitor</td>
<td>Main inhibitor of TF. Endothelial derived. Expressed on surface bound to GAGs</td>
<td>Surface bound TFPI decreased in sepsis. Circulating levels increased</td>
</tr>
<tr>
<td>GAGs and associated molecules (e.g. ATIII)</td>
<td>Maintain endothelial charge and retain anticoagulant molecules at endothelial surface</td>
<td>GAGs and associated molecules lost from endothelial surface</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>Binds and inactivates thrombin. Complex induces fibrinolysis</td>
<td>Decreased endothelial expression</td>
</tr>
<tr>
<td>Fibrinolytic System</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tissue Plasminogen Activator</td>
<td>Promotes fibrinolysis</td>
<td>Initial increase in activity then suppressed</td>
</tr>
<tr>
<td>Plasminogen Activator Inhibitor-1</td>
<td>Inhibits fibrinolysis</td>
<td>Raised</td>
</tr>
<tr>
<td>Protein C &amp; S</td>
<td>Promotes fibrinolysis</td>
<td>Decreased</td>
</tr>
<tr>
<td>Platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD39 (ecto-ADPase)</td>
<td>Endothelial Inhibitor of platelet activation</td>
<td>Decreased expression following activation</td>
</tr>
<tr>
<td>Prostacyclin (PGI2)</td>
<td>Endothelial Inhibitor of platelet activation</td>
<td>Decreased production</td>
</tr>
<tr>
<td>Nitric Oxide</td>
<td>Multiple effects on local blood flow and adhesion molecule expression</td>
<td>Change to iNOS dependent production. Resultant effect on haemostasis</td>
</tr>
</tbody>
</table>
Introduction

The next detectable stage in DIC pathogenesis is the activation of the "extrinsic" or Tissue Factor (TF) coagulation pathway (Levi et al 1993). Tissue factor is a potent pro-coagulant small membrane protein which binds and activates factor VII leading to thrombin generation and clot formation. Tissue factor is expressed by endothelial cells and by monocytes (especially those adherent to activated endothelium) (Collins et al 1995). The primary inhibitor of tissue factor activity: tissue factor pathway inhibitor, (TFPI) is synthesised by healthy endothelium and expressed on the cell surface bound to glycosaminoglycans (GAGs) (Bajaj & Bajaj, 1997). Bound TFPI levels are decreased in sepsis (Bajaj & Bajaj, 1997). Clinical sepsis is a vastly more complicated picture than the response to a single dose of endotoxin, but observational studies have demonstrated similar processes in respect of the fibrinolytic (Brandtzaeg et al 1990) and TF (Shimura et al 1997) pathways in SIRS and MODS. Indeed the importance of TF has been demonstrated in primate models of sepsis in which anti-TF or anti factor VIIa blocking monoclonal antibodies were able to completely inhibit thrombin and fibrin production (Levi et al 1994).

Diffuse widespread clotting factor activation may present a serious clinical problem in the context of reduced fibrinolytic activity. This process may be exacerbated by TNF-α induced downregulation of constitutive endothelial anticoagulant molecules. One such molecule, thrombomodulin (Gando et al 1995; Conway & Rosenberg, 1988; Nawroth et al 1986;) binds thrombin and activates the Protein C and Protein S complex which in turn inhibits factor V and VIII activation and promotes fibrinolysis. Endothelial surface bound GAGs (see below) bind anti-thrombin III (which inactivates thrombin) and heparin co-factor II and TFPI. During SIRS, GAGs are lost from the endothelial cell surface (Klein et al 1992), further reducing the capacity of the endothelium to resist thrombosis (figure 1.4 and table 1.2)

The contact or "intrinsic" coagulation pathway is also activated in acute systemic inflammation via factor XII activation following contact with
activated platelets, the sub-endothelial matrix at sites of endothelial damage or Gram-negative bacterial surfaces (Herwald et al 1998). Blockade of this pathway in a primate model did not alter DIC but attenuated the hypotension induced by experimental septicaemia (presumably by decreasing production of the vasodilator kallikrien which accompanies factor XII activation) (Jansen et al 1996).

The healthy endothelium has three principal mechanisms by which it resists platelet activation. These are 1) endothelial cell surface expression of an ecto-ADPase (CD39) (Marcus et al 1997), 2) constitutive endothelial nitric oxide (NO)(Vallance et al 1997) production and 3) prostacyclin (PGI2) (Heyderman et al 1991) production. Endothelial activation decreases CD39 expression, increases NO production (via a switch from the endogenous NO synthetase to the inducible NO synthetase dependent pathway) and decreases endothelial PGI2 production (Vallance et al 1997). These effects may shift the balance towards to promotion of activated platelet binding to fibrin, sub-endothelial matrix, other platelets and other inflammatory cells. This reinforces the long standing observation that consumption of platelets is a poor prognostic sign in acute sepsis (Derkx et al 1996).

In summary, the endothelium is involved directly in all the processes leading to coagulation abnormalities in acute sepsis. It contributes to the early activation of, and subsequent inhibition of fibrinolysis. In addition the endothelium can directly activate the tissue factor pathway and induce platelet activation. It is increasingly clear that viewing inflammation and haemostasis as distinct entities is a gross oversimplification. The endothelium provides a surface where these processes are indistinguishable, - "inflammatory" changes promote coagulation and "haemostatic" processes accelerate inflammatory cell recruitment.
1.2.3 Local Blood Flow

The normal metabolic activity of the vascular endothelium exerts a vasodilator influence (Hinshaw, 1996). Though many factors influence vascular tone, endothelial-dependent nitric oxide (NO) production via the L-arginine pathway is principally responsible for basal vasodilatation (in addition to the role in the inhibition of platelet activation described above). Endothelial cyclo-oxygenase activity producing prostacyclin, thromboxane A₂ and similar molecules (prostanoids), further contributes to vasoactive and anti-platelet effects. These basal effects can be massively exaggerated in sepsis when pro-inflammatory cytokines induce a widespread increase in nitric oxide synthetase (Moncada & Higgs, 1995) and cyclo-oxygenase activity (Habib et al 1993). These changes contribute to a dysregulated micro-circulation with redistribution of organ blood flow, a generalised reduction in vascular resistance and intravascular pooling (Hinshaw, 1996). The consequences of these effects are clear in SIRS. Excessive vasodilatation is a principal component of the pathogenesis of shock, relative stasis accelerates clotting factor activation and DIC, and loss of fine control of the micro-circulation contributes to organ hypo-perfusion and MODS (Hinshaw, 1996). Attempts have been made to attenuate the profound vasodilatation of sepsis with inhibitors of nitric oxide production. Though these therapies increase systemic vascular resistance they appear to worsen cardiac function providing no overall benefit (Hata & Dellinger, 1995; Zhang et al 1995; Petros et al 1994;). The endothelium does not recover promptly from an inflammatory insult and low levels of vasodilator production persist well into recovery, a process that has been termed "endothelial stunning" (Vallance et al 1997; Bhagat et al 1996). The concept that endothelial dependent vasodilatation and platelet inhibition may be attenuated following sepsis has been proposed as a explanation for the apparent increase in risk for acute cardiovascular events after systemic insults (Vallance et al 1997).
1.2.4 Endothelial Permeability

Normal vascular permeability is maintained by a combination of endothelial cell integrity and basement membrane characteristics which allows the free passage of water and small molecules, while confining larger molecules to the intra-vascular compartment (Lum & Malik, 1994).

The ability of the endothelium to regulate permeability is dictated by the presence of cell surface binding proteins, the inherent leakiness of intracellular junctions, the composition of sub-endothelial matrix proteins, and endothelial cell membrane surface charge (Lum & Malik, 1994; Venkatachalam & Rennke, 1978). The specific contribution of each of these components to vascular permeability varies between vascular beds and with the presence of inflammatory stimuli. However, the presence of negative charged molecules on the luminal surface appears to be of particular importance (Venkatachalam & Rennke, 1978). Experimental neutralisation of endothelial negative charge increases albumin passage across the endothelium as evidenced by increased renal clearance. Indeed the fractional clearance of cationic albumin exceeds that of native albumin by a factor of 300 (Purtell et al 1979). Though there are a number of anions associated with endothelial cells and the sub-endothelial matrix which contribute to the control of vascular permeability, evidence suggests that glycosaminoglycans are particularly important (Kanwar et al 1980). Glycosaminoglycans also have the property of binding and presenting active compounds at optimal locations for functional interactions. Examples include the anticoagulant molecules anti-thrombin III and heparin co-factor II as described above, the reactive oxygen scavenger superoxide dismutase, (Inoue et al 1991) and the cytokine macrophage inflammatory protein-1β (Tanaka et al 1993).

Patients with septic shock invariably have a profound "capillary leak" and may require resuscitation with as much as three times their circulating
volume in 24 hours. The pro-inflammatory cytokines IL-1, TNF-α, and IFN-γ directly alter the distribution and decrease the intensity of the negative charge of the GAGs on the endothelial cell surface (Klein et al 1992). These changes are not compensated for by increased GAG synthesis (Klein et al 1992). This effect is compounded by the loss of the GAG-associated molecules lessening the capacity of the endothelium to resist thrombosis, inflammatory cell adhesion and oxidative stress. In addition inflammatory cells which may become adherent to the septic endothelium such as neutrophils and platelets have the capacity to synthesise cationic proteins which can neutralise surface charge and also can secrete GAG-degrading enzymes.

Therefore in SIRS the control of endothelial permeability is inseparable from the processes of coagulation disturbance and inflammatory cell adhesion.

1.2.5 The Integration of Endothelial Functions

The roles of the endothelium in sepsis are not as easily divided as the structure of this introduction might suggest. Each role impacts on the others. For example while loss or saturation of platelet activation inhibition may contribute to fibrinogen binding and clotting factor consumption, this role also overlaps with inflammatory cell recruitment as platelets are able to bind neutrophils and support the multi-step adhesion and transmigration process via MAC-1 (CD11b/CD18) (Diacovo et al 1996b). Hence established thrombus formation is likely to promote further inflammatory cell adhesion, endothelial damage and clotting factor activation. A further example is how the microvascular changes induced by acute vasodilator excess contribute to intra-vascular pooling with inflammatory cell recruitment encouraged by the lower flow, an effect which would be exaggerated by the viscosity increase following colloid loss via capillary leak (Hinshaw, 1996). The extent of this overlap between the roles of the
endothelium explains the rapidity with which dysfunction of one component is followed by the others in the familiar spiral of multi-organ dysfunction syndrome.

1.2.6 Therapeutic Potential

A range of soluble factors has been investigated in phase II or III clinical trials which have been designed to attenuate the pro-inflammatory cytokine cascade of early SIRS. Antibodies to TNF-α, soluble TNF-α receptors, anti-endotoxin antibodies and Interleukin-1 receptor antagonists have all been disappointing (Zeni et al 1997). Therapies for specific components of the endothelial dysfunction including anti-prostaglandin treatments (Bernard et al 1997b), nitric oxide synthetase inhibitors (Petros et al 1994), recombinant tissue plasminogen activator (Zenz et al 1995) and blocking antibodies against adhesion molecules have yet to demonstrate any efficacy in the clinical situation. Of particular interest however is the recent suggestion that too little pro-inflammatory cytokine too late may be a key component of severe SIRS (Bone, 1996). The future may well see a complex cocktail of therapies to modulate adhesion, promote fibrinolysis, replace clotting factors, and limit nitric oxide production while maintaining adequate immune function with administration of Interferon-γ (Docke et al 1997).

1.2.7 Conclusion

The vascular endothelium may be considered as the tissue responsible for many of the clinical consequences of systemic inflammatory response syndrome. Disruption of its normal function by circulating mediators and inflammatory cell adhesion alters its ability to maintain coagulation homeostasis, control local blood flow, maintain fluid balance and regulate inflammatory cell adhesion.
1.3 Platelets

1.3.1 Introduction

Despite being present in huge numbers in healthy peripheral blood (150 - 500 $\times 10^9$/L) platelets were not described until 1882. The "distinguished investigator of blood formation" Professor Giulo Bizzozero of Turin observed the presence of these new and numerous corpuscles in freshly drawn blood and commented that 'the evidence is very strong that this coagulation...takes place under the direct influence of these particles" (Anon. 1882).

Mammalian platelets appear to have developed from primitive, less specialised cells which have both inflammatory and haemostatic functions. In invertebrates these multi-purpose haemocytes aggregate to limit intravascular fluid loss at sites of injury, phagocytose pathogens and initiate the process of tissue repair following injury. The platelet may be considered to be the descendent of these primitive cells which is now principally responsible for the process of early haemostatic control. However, many characteristics of platelets reflect their origins as less specialised cells capable of a broad range of inflammatory functions.

1.3.2. Platelet structure

Platelets are small (1.5 $\times$ 0.5-1.0 $\mu$m) cells which are derived directly from bone marrow megakaryocytes. A platelet may be considered as a cell fragment as it lacks a nucleus and retains only very limited synthetic capacity from mRNA templates left over from the megakaryocyte precursor. Platelets circulate for 7-10 days and become slowly less dense as the contents of the cytoplasmic granules are expressed or secreted and not replaced (Hanadin, 1998).
Figure 1.5
Platelets at rest and following activation. A) The platelet increases in size, the surface becomes irregular and granule release occurs. In particular alpha granules fuse with the surface within seconds of a stimulus to activation allowing the expression of CD62P. B) Scanning EM of resting and activated platelets.
Platelets possess three types of cytoplasmic granule containing a wide range of bioactive molecules (table 1.4). The contents of all these granules may be released rapidly following a potent stimulus to platelet activation or in a more selective fashion after a sub-maximal stimulus.

Platelet α-granules contain proteins involved primarily in haemostatic functions (e.g. fibrinogen, von Willebrand factor, fibronectin, α2 plasmin inhibitor, plasminogen) whereas the dense or δ-granules store mediators most directly involved in the control of vascular tone (e.g. thromboxane A2, serotonin, calcium and ADP). Lysosomal (λ) granules do not secrete their contents until platelets agonists are present in high concentrations and principally contain proteins involved in thrombolysis (table 1.4).

Platelet structure alters rapidly following a stimulus to activation (figure 1.5). The normal discoid shape swells and becomes increasingly irregular with the formation of long pseudopodia due to rapid changes in the abundant actin filament cytoskeletal network. The granules which are initially randomly scattered through the cytoplasm move towards the centre of the activated platelet where they can fuse with invaginations of the lipid bi-layer membrane to secrete or express their contents.

In addition to the complex system of cytoplasmic granules platelets have the capacity to express a wide range of surface adhesion molecules (table 1.3) which provide ligands for coagulation factors, sub-endothelial matrix proteins and ligands on other inflammatory cells (Hanadin, 1998).
Table 1.3

Principal platelet adhesion molecules.
The main platelet adhesion molecules are shown. Each of the main adhesion molecule families are represented (selectin, integrin and immunoglobulin superfamily) as well as the Gplb-V-IX complex.

<table>
<thead>
<tr>
<th>Adhesion molecule</th>
<th>Location</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD62P (P-Selectin)</td>
<td>α-granules at rest. Rapidly expressed on surface following activation</td>
<td>PSGL-1</td>
</tr>
<tr>
<td>Gpllb/IIIa (αIIbβ3)</td>
<td>Surface and α-granules change conformation on activation</td>
<td>Fibrinogen vWF (mediates plt-plt binding)</td>
</tr>
<tr>
<td>Gplb-V-IX complex (Includes CD42b)</td>
<td>Surface</td>
<td>Sub-endothelial vWF or vWF under shear conditions</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>? Surface</td>
<td>?Fibrinogen/MAC-1</td>
</tr>
</tbody>
</table>
### Table 1.4

**Platelet granule contents.** The many components of the three types of platelet granules are shown.

<table>
<thead>
<tr>
<th>Granule</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>CD62P (p-selectin)</td>
</tr>
<tr>
<td></td>
<td>Transforming Growth Factors $\alpha$ &amp; $\beta$</td>
</tr>
<tr>
<td></td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td></td>
<td>Platelet factor 4</td>
</tr>
<tr>
<td></td>
<td>Factor V</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen</td>
</tr>
<tr>
<td></td>
<td>vWF</td>
</tr>
<tr>
<td></td>
<td>Plasmin activator inhibitor type 1</td>
</tr>
<tr>
<td></td>
<td>Plasminogen</td>
</tr>
<tr>
<td></td>
<td>$\alpha_2$-plasmin inhibitor</td>
</tr>
<tr>
<td></td>
<td>$\beta$-Thromboglobulin</td>
</tr>
<tr>
<td></td>
<td>GpIIb/IIIa</td>
</tr>
<tr>
<td></td>
<td>vitronectin</td>
</tr>
<tr>
<td></td>
<td>Multimerin</td>
</tr>
<tr>
<td>Dense ($\delta$)</td>
<td>ADP</td>
</tr>
<tr>
<td></td>
<td>Serotonin</td>
</tr>
<tr>
<td></td>
<td>Thromboxane $A_2$</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
</tr>
<tr>
<td>Lysosomal ($\lambda$)</td>
<td>Fibrinolytic enzymes</td>
</tr>
</tbody>
</table>

---
In common with vascular endothelial cells, activated platelets express the 140 kDa glycoprotein CD62P (P-selectin, GMP-140, PADGEM). This type 1 transmembrane molecule is stored in α-granules which fuse with the platelet surface within seconds of exposure to agonists (e.g. ADP, thrombin). A similar process occurs with endothelial CD62P which is stored pre-formed in Weibel-Palade cytoplasmic granules and rapidly expressed following inflammatory stimuli. The rapidity with which CD62P is expressed both on platelets and endothelium has focused attention on it as a potential target for anti-adhesion molecule immunotherapy. A soluble form of CD62P has been identified in plasma as a truncated molecule which lacks the transmembrane domain (Ishiwata et al 1994). The function of this soluble form remains unclear. The principle ligand for CD62P identified to date is CD162 (P-selectin glycoprotein ligand-1, PSGL-1) which is a 240kDa homodimeric (disulphide linked) molecule present on most myeloid cells and some lymphocytes (figure 1.6).

\[\text{Figure 1.6}\]

The interaction of CD62P (P-selectin) and its principal ligand P-Selectin glycoprotein ligand (PSGL-1). PSGL-1 is a glycosalated transmembrane protein that is the only high affinity ligand for CD62P identified to date. Figure reproduced from McEver & Cummings, 1997.
Platelets also express a range of adhesion molecules whose principal ligands are extra-cellular matrix proteins (table1.3). The most important of which are GpIIb/IIIa (αIIbβ3) and CD42 (Gp1b-V-IX complex). Unlike CD62P these molecules also have important roles in platelet haemostatic functions and congenital absence causes the bleeding disorders Glanzmann thrombasthenia (GpIIb/IIIa) (Kato, 1997; Coller \textit{et al} 1994). and Bernard-Soulier syndrome (CD42) (Clemetson \& Clemetson, 1994; Bernard, 1983).

![Figure 1.7](image)

**Figure 1.7**

**Structure of GpIIb/IIIa receptor.** (Reproduced from Topol \textit{et al} 1999).

GpIIb/IIIa is a heterodimer of the α subunit (CD41 containing disulphide linked heavy and light chains which are the product of intracellular proteolysis of a single gene product (Poncz \textit{et al} 1987)) and a β subunit (CD61 which is common to other β3 integrins). Both subunits the cell membrane. Activation is accompanied by conformational changes and clustering of copies of the receptor on the platelet surface.
GpIb/Ilia is expressed only on platelets (and megakaryocytes) but is present on the surface at a very high density (~80,000 copies in total <200 Angstroms apart) as well as a further store in the \(\alpha\)-granules. Both the \(\alpha\) and \(\beta\) chains are type I transmembrane proteins. Stimuli to platelet activation (ADP, serotonin, thromboxane A2, thrombin etc.) initiate changes in GpIb/Ilia to a high affinity receptor for fibrinogen or vWF. The mechanisms of signal transduction leading to activation of GpIb/Ilia are complex and incompletely understood (Shattil & Ginsberg, 1997b; Fox et al 1996b; Leong et al 1995b; Clark et al 1994b) (figure 1.8). Activated GpIb/Ilia is the only adhesion molecule able to mediate platelet-to-platelet binding which represents a key stage in haemostasis (Shattil et al 1997).

Therapeutic blockade of GpIb/Ilia function with monoclonal antibodies or oligo-peptide binding site antagonists has been under intensive investigation (Topol et al 1999). Several such agents reduce the incidence of reocclusion after coronary angioplasty (Coller, 1997).

The principal vWF receptor is provided by the CD42 (GpIb-V-IX) complex. This is also abundant on the unactivated platelet membrane (~25,000 copies per platelet). The complex contains four polypeptides GpIX, (22kDa) GpIb\(_{\alpha}\) (150kDa) (which contains the binding site for vWF and thrombin), GpIb\(_{\beta}\) (27kDa) and weakly non-covalently associated GpV (82 kDa) in the ratio 2:2:2:1 respectively (Lopez et al 1998; Lopez & Dong, 1997). Expression of this complex changes little following platelet activation (Clemetson, 1997) (figure 1.9).
Figure 1.8

The platelet thrombin receptor and the interaction of Gpllb/IIIa with ligand and platelet.

The thrombin receptor is a seven transmembrane domain receptor which is cleaved by thrombin to provide its own tethered ligand. Biochemical signals (including protein tyrosine kinases) trigger inside-out activation of Gpllb/IIIa probably by interaction with a regulatory protein attached to the integrin cytoplasmic tails. These changes result in the exposure of the fibrinogen binding site. Subsequent fibrinogen binding causes a complex process of outside-in signalling enabling platelet shape change via interaction with the cytoskeleton. (Shattil 1997)
Figure 1.9
Schematic drawing of Gplb-V-IX complex (adapted from Lopez et al 1998). This complex is responsible for the primary event in haemostasis, the adhesion of a flowing platelet to subendothelial von-Willebrand factor under high shear stress conditions. Intracellular signalling events following vWF binding to this complex have recently been described (Lopez et al 1998). Abnormal function of this receptor leads to a severe bleeding disorder (Bernard, 1983).
1.3.3. Platelet function

Platelets are well recognised to be highly specialised haemostatic cells which bind rapidly to exposed sub-endothelial matrix and soluble coagulation factors in order to provide early control of haemorrhage. The role of the platelet in haemostasis has been well described and may be considered in the stages: adhesion, activation, aggregation and secretion. Although many of these processes take place simultaneously or in very rapid succession, I will consider them in turn.

Platelet adhesion

Platelet adhesion is typically described to the sub-endothelial matrix following endothelial injury. The primary event in platelet adhesion is GpIb complex mediated adhesion to sub-endothelial vWF (Roth, 1992). This interaction appears to require high shear stress which is provided by the tethering of vWF to other sub-endothelial components (such as collagen) and hence spontaneous interaction between circulating vWF and GpIb complex is avoided. CD42 ligation appears to initiate platelet activation via non-receptor tyrosine kinases and may contribute to the shape change that accompanies activation via interactions with membrane associated cytoskeleton. Recent data suggest that CD42-vWF binding alone may not be adequate for firm platelet adhesion and GpIIb/IIIa binding to fibrinogen may be required before adhesion is firmly established. This process is similar to leukocyte rolling and subsequent firm adhesion (Savage et al 1996).

Recent work has identified the capacity for activated platelets to adhere to intact vascular endothelium (Bombeli et al 1998; Li et al 1996) and not just to surface bound or exposed extra-cellular matrix proteins. This adhesive mechanism appears to be complex, requiring activated platelet GpIIb/IIIa binding to fibrinogen (or other adhesive proteins) which are in turn bound to endothelial adhesion molecules such as ICAM-1 or αvβ3 (figure 1.10).
There is now evidence that non-activated platelets can bind to endothelial cells undergoing apoptosis although the mechanisms have yet to be defined (Bombeli et al 1999).

**Figure 1.10**

The proposed mechanisms for platelet adhesion to intact vascular endothelium (Bombeli et al 1998). Adhesive proteins such as fibrinogen or VWF act as a bridge between endothelial adhesion molecules and the activated form of GpIIb/IIIa on the surface of activated platelets.

![Diagram of platelet adhesion](image)

Endothelial cell

The adhesion of activated platelets to other circulating cell types has been recently described (Rinder HM et al 1991a; Rinder HM et al 1991b; Larsen et al 1989b). The mechanism for these interactions is principally platelet CD62P dependent, (Peters et al 1997; Larsen et al 1989) and hence platelet activation is an essential step in the formation of these heterotypic cell aggregates. Activated GpIIb/IIIa may also contribute to the formation of
heterotypic cell aggregates via a fibrinogen bridging mechanism as observed between activated platelets and the intact endothelium (Bombeli et al 1998). (figure 1.10).

Platelet activation

Following platelet activation a number of changes occur. Secretion of bioactive molecules (table 1.4), shape change (figure 1.5) and the alteration of the adhesion molecule profile (Shattil et al 1985) transform the platelet into a dynamic cell which interacts profoundly with its immediate environment. Many stimuli can initiate platelet activation but two are particularly important.

Thrombin, the serine protease generated from prothrombin by factors Xa and V in the coagulation cascade, has a potent direct agonist effect on platelets via a complex polypeptide receptor with 7 membrane spanning domains and possibly via Gp1b (Brass & Molino, 1997; Jamieson, 1997). Thrombin receptor activation on platelets activates protein kinase C and inhibits adenyl cyclase activity, causing increased intra-cellular Ca^{2+} levels. The production of a range of phospholipases (C,A2 and D) is also induced (Jamieson, 1997). These changes result in maximal platelet activation with shape change, activation and secretion.

ADP is secreted from activated platelet dense granules and has effects on local blood flow via groups of receptors (P2Y, P2X) present on endothelial and smooth muscle cells amongst others (Gachet et al 1997). The effects on platelets themselves may be mediated by two sub-types of the above receptors, one responsible for an increase in intra-cellular Ca^{2+} levels (P2X1) and the other for inhibition of adenyl cyclase activity (P2Y1). ADP has been referred to as a weak agonist of platelet activation in comparison to thrombin. Other platelet agonists include adrenaline in 90% of people (Scrutton et al 1981), thromboxane A2, serotonin (via 5-HT2) and anti-
diuretic hormone as well as components of the extra-cellular matrix, vWF and collagen (Hanadin, 1998).

**Platelet Aggregation**

The formation of platelet to platelet bonds is the critical step in primary haemostasis. Fibrinogen or vWF binding to activated GpIIb/IIIa cross-links adjacent platelets to form a firm platelet plug. As described in section 1.3.2. GpIIb/IIIa requires activation into its high affinity conformation by a stimulus to platelet activation (e.g. exposure to thrombin). This occurs by an “inside-out” signalling process which is complex and tightly regulated so as to avoid spontaneous platelet thrombus production (Shattil et al 1997). Following GpIIb/IIIa ligation “outside-in” signalling acts via tyrosine kinase activity (Clark et al 1994; Huang et al 1993) and results in cytoskeletal reorganisation. These processes are essential for maximal platelet aggregation, platelet spreading on the extra-cellular matrix and for clot retraction (Schoenwaelder et al 1997). Patients who cannot perform this outside-in signalling present with variant thrombopenia (Chen et al 1992).

**Platelet secretion**

Contents of platelet cytoplasmic granules are listed in table 1.4. In addition platelets are reported to have the capacity to generate toxic reactive oxygen metabolites, a range of unique antimicrobial peptides and the lipid pro-inflammatory mediator platelet activating factor (PAF) (Yeaman, 1997).
1.3.4. Platelets and Coagulation.

In addition to their role in primary haemostasis with the production of a haemostatic plug, platelets are also involved in the complex process of coagulation. This cascade of activation of soluble serine proteases ('clotting factors') in response to tissue factor or contact with the sub-endothelium results in fibrin production from fibrinogen. Platelets catalyse the cascade at many points (figure 1.11) by soluble factors (e.g. PF3) but most importantly provide a surface for the assembly of coagulation factor complexes. Factors IXa and VIIIa form an intrinsic tenase complex on the platelet surface with calcium which can activate Factor X (Hirsh & Weitz, 1999). Thrombin production from prothrombin which requires factors Xa, and Va is accelerated several thousand times on the platelet surface in the prothrombinase complex (Hirsh & Weitz, 1999; Billy et al 1997). Platelet secretion of factor Va (Camire et al 1998) adds to the intensely prothrombotic locale of the activated platelet. Platelets also act to promote clot formation by retarding local fibrinolysis via the production of plasminogen activator inhibitor type 1 (Fay et al 1994; Booth et al 1988). The platelet surface prothrombinase complex is also relatively protected from the actions of the circulating coagulation inhibitors antithrombin III and protein C (Weitz et al 1990). Indeed platelet surface bound coagulation factors are also protected from therapeutic anticoagulants such as unfractionated heparin (Hirsh & Weitz, 1999).

In summary activated platelets accelerate clot formation and retard clot lysis by a range of mechanisms which are predominantly active in their immediate surroundings.
Platelets and coagulation. Platelets interact with coagulation factors to accelerate fibrin production by the formation of coagulation factor complexes on the platelet surface which have increased activity. Platelets also retard fibrinolysis by PAI-1 production.
1.3.5 Platelets and Inflammation

The extent of the involvement of platelets in the process of acute inflammation remains the subject of debate. In acute systemic inflammation such as *Neisseria meningitidis* sepsis and multi-organ failure, a low circulating platelet count is a consistent marker of disease severity and poor outcome (Derkx *et al* 1996).

Platelets have adhesion molecules which to allow them to accumulate very rapidly at sites of endothelial injury. It has recently become clear that platelets do not simply form an inert plug following their initial adhesion and aggregation to limit intra-vascular fluid loss. Rather, activated platelets are capable of interacting with leukocytes to support leukocyte rolling, firm adhesion and transmigration in a manner similar to the interactions between leukocytes and the vascular endothelium (Weber & Springer, 1997; Diacovo *et al* 1996) (figure 1.12).

The contents of platelet granules also support the role of activated platelets in the inflammatory response. Local changes in blood flow occur as part of inflammation (Hinshaw, 1996) and platelet dense granules are well equipped to contribute to this process via secretion of ADP, serotonin, and thromboxane A2. Platelets can generate toxic oxygen metabolites which can cause local tissue injury and contribute to microbial killing. They also produce small amounts of proteases and even a range of unique microbicidal proteins (platelet microbicidal proteins PMP 1-5) (Yeaman, 1997).

Recent data have suggested that attenuation of severe inflammation can be achieved by therapies aimed at the haemostatic imbalance of DIC. Administration of protein C,* (Murakami *et al* 1997; Murakami *et al* 1996; Rintala *et al* 1996) rt-PA (Zenz *et al* 1995) and PGI2 have all been reported to confer benefit, though the evidence is anecdotal to date (Duncan, 1997).
reduced mortality by 20-40% in small trials (Eisele et al 1998) but results of large studies are awaited.

As described in section 1.2.2, the Contact or "intrinsic" coagulation pathway which is activated via factor XII following contact with platelets, or the sub-endothelial matrix (Herwald et al 1998) is also implicated in the pathophysiology of acute inflammation and shock (Jansen et al 1996).

Figure 1.12
Platelets can act to support multi-step leukocyte adhesion at sites of endothelial damage. Key: The endothelial cell is blue, platelets are yellow, neutrophils white and sub-endothelial matrix grey. Rolling occurs via the CD62P-PSGL-1 interaction and firm adhesion may occur via platelet bound fibrinogen-Mac-1 interactions (adapted from Diacovo et al 1996b).
Recent evidence has demonstrated the extent to which activated platelets may activate endothelium via surface expression of the pro-inflammatory TNF-like molecule CD154 (Henn et al. 1998).

Therefore the processes of haemostasis and inflammation are linked (Marcus et al. 1995). Platelets possess many of the mechanisms that would be required to contribute to inflammation as well as haemostasis, but to date, there is no direct evidence of the extent to which these mechanisms act in-vivo. Further there is not yet any evidence to suggest that the contribution of platelets to acute inflammation may be harmful (Heffner, 1997).

1.3.6 Conclusion

Platelets are cell fragments with both haemostatic and inflammatory properties. The contribution of platelets to haemostasis has been defined in some detail, whereas the mechanisms by which they influence inflammation directly through the action of numerous granule contents and indirectly through adhesion to other inflammatory cells are still being defined.
1.4 Platelet activation in acute respiratory distress syndrome

1.4.1 Introduction

In the previous sections endothelial, leukocyte and platelet activation have been described. The particular importance of activation of these inflammatory cells in examples of acute inflammation such as systemic inflammatory response syndrome has been highlighted. One example of acute inflammation that has early and potentially fatal clinical consequences for the host is the acute respiratory distress syndrome (ARDS). ARDS is defined as the rapid onset of a diffuse lung disease, which produces severe disruption of gas exchange in the absence of left heart failure (Bernard et al 1994) (table 1.5). Clinically ARDS is manifest as the rapid development of reduced lung compliance, loss of lung volume, ventilation/perfusion mismatching and hypoxaemia. There is widespread alveolar oedema on chest x-ray (figure 1.13) and mechanical ventilatory support is usually required.

Table 1.5
American European Consensus Conference Definition of ARDS.
This simple clinical and radiological definition of ARDS has been widely adopted in preference to more complex definitions requiring respiratory function data. (Bernard et al 1994).

<table>
<thead>
<tr>
<th>Acute respiratory distress syndrome (ARDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid onset (over less than 48 hours)</td>
</tr>
<tr>
<td>diffuse lung disease</td>
</tr>
<tr>
<td>(characteristic appearance on chest x-ray) (see figure 1.13)</td>
</tr>
<tr>
<td>severe disruption of gas exchange</td>
</tr>
<tr>
<td>(arterial oxygen tension / fraction of inspired oxygen &lt;200 mmHg)</td>
</tr>
<tr>
<td>No left atrial hypertension</td>
</tr>
</tbody>
</table>
These processes may be localised to the lung following direct lung injury (e.g. aspiration or *Pneumocystis carinii* pneumonia) or represent the pulmonary component of MODS following a systemic stimulus to inflammation such multiple trauma, burns, or systemic sepsis. ARDS carries a significant mortality of around 50% (Bernard *et al* 1994) although this appears may have been improving slowly in recent years (Milberg *et al* 1995).

Figure 1.13.
**ARDS in Sepsis.** Chest x-ray of severe acute respiratory distress syndrome with diffuse interstitial airspace shadowing. Example shown is of a young adult with MODS from meningococcal septicaemia.
1.4.2 The Importance of Inflammation in ARDS

The pathophysiology of ARDS is highly complex. Increased circulating (Shanley et al 1995; Roumen et al 1993) or bronchoalveolar fluid (Timmons et al 1995) levels of pro-inflammatory mediators (TNF-α and IL-1) are a consistent finding. Many cell types including macrophages and eosinophils have been implicated, and complement activation has been reported (Nichols et al 1992). However neutrophils appear to play a major role (Windsor et al 1993; Repine & Beehler, 1991) in the majority of cases. High bronchoalveolar fluid levels of the neutrophil chemotactic peptide interleukin-8 are associated with an increased risk of developing ARDS (Donnelly et al 1993). High circulating levels of neutrophil elastase (Donnelly et al 1995) and soluble CD62L (Donnelly et al 1994) correlate with the severity of subsequent ARDS. Animal models of lung injury induced by cobra venom have been shown to be neutrophil-dependent (Mulligan et al 1992). Neutrophil reconstitution often appears to worsen ARDS in neutropenic patients (Rinaldo & Borovetz, 1985). The balance between oxidant and anti-oxidant activity may be of particular importance in determining the development of ARDS (Pacht et al 1991; Brigham, 1986).

Histological examination of the lungs from patients who died with ARDS has revealed a consistent pattern of changes which strongly support the inflammatory aetiology. These changes evolve following the insult (table 1.6). Neutrophils feature in the early exudative stage when in contrast to healthy lungs (figure 1.13) the acutely injured lung is heavy and there are prominent intra-alveolar and intra-luminal aggregates of neutrophils as well as fibrin and platelets (figure 1.14 and 1.15). The injured lung then attempts to heal - a process which may result in vigorous collagen production and fibrosis. This can lead to distortion of the lung structure with the formation of multiple cystic areas (figure 1.16) and persistent abnormalities of gas exchange across the thickened alveolar walls.
Table 1.6: Histological stages of ARDS. The characteristic appearances of ARDS are listed below. Note that these appearances are independent of the causative mechanism adapted from (Meduri, 1996) & (Nichols et al 1992).

<table>
<thead>
<tr>
<th>Timing (from initial injury)</th>
<th>Exudative</th>
<th>Proliferative</th>
<th>Fibrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early (&lt;1 week)</td>
<td>Rigid, heavy</td>
<td>Firm, consolidated</td>
<td>Spongy</td>
</tr>
<tr>
<td>Haemorrhagic</td>
<td></td>
<td>Pale grey</td>
<td>Cystic</td>
</tr>
<tr>
<td>Intermediate</td>
<td></td>
<td>Pale</td>
<td></td>
</tr>
<tr>
<td>Late (&gt;3 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Macroscopic:                 | Endothelial injury (mild)      | Endothelial injury           | Endothelial injury        |
|                             | Congestion                     | Intimal fibroproliferation   | Distortion                |
|                             | Intraluminal aggregation of    | Medial hypertrophy           | Compression               |
|                             | platelets, fibrin & neutrophils| Thrombi                      | Proliferation             |
|                             |                                 |                              |                           |

| Microscopic:                 | Type 1 pneumocyte necrosis,    | Type 2 pneumocyte            | Fibrosis                  |
|                             | Hyaline membranes              | proliferation,               |                           |
|                             | Partial collapse               | Myofibroblast invasion       |                           |
|                             |                                 | Collagen deposition          |                           |
|                             |                                 |                              |                           |

| Basement membrane           | Denuded                        | Gaps with myofibroblast      | Disruption                |
|                             |                                 | invasion                     |                           |
|                             |                                 |                              |                           |

| Alveolar wall               | Oedema                         | Myofibroblast proliferation  | Thick collagen            |
|                             |                                 |                              |                           |

| Interstitium                | Volume+ Oedema                 | Volume++ myofibroblast       | Volume+++ Fibrosis        |
|                             |                                 | proliferation               |                           |
Figure 1.14
Section of normal human lung. The alveolar walls are thin, with numerous air spaces.

Figure 1.15
Severe ARDS. Lung section from patient suffering from fatal *Pneumocystis carinii* pneumonia. The air spaces are almost completely obliterated with dense cellular inflammatory infiltrates.
Figure 1.16
Gross appearance of the cut surface of human lung following fibrotic stage of lung injury. There are numerous cystic areas and an increase in interstitial tissue.

Figure 1.17
Appearance of 'hyaline membranes' A thick layer of fibrin, fibronectin and cellular debris which adhere over denuded epithelium. This appearance is characteristic of surfactant deficiency and epithelial damage.
1.4.3 Platelets and ARDS

There is a high incidence of severe inflammatory lung disease in children suffering from congenital or acquired immunodeficiency. This condition is particularly prevalent in the post bone marrow transplantation period when severe lung disease is well recognised to carry a dismal prognosis (Nichols et al 1994; Psiachou et al 1994; Denardo et al 1989) with a mortality rate approaching 100%. Severe post-bone marrow transplant lung disease occurring during pancytopenia suggests that mechanisms other than neutrophil mediated injury are likely to be contributing (Sivan et al 1990; Denardo et al 1989; Ognibene et al 1986; Braude et al 1985). Although circulating monocyte, neutrophil and lymphocyte counts may be absent or at least greatly suppressed for one or more weeks following bone marrow transplant, platelet counts are supported by regular transfusions to reduce the risk of haemorrhage. Post-mortem examination of the lungs of 26 severely neutropenic children who died from ARDS revealed a complete absence of neutrophils in 85% (22/26) of cases and no leukocytes in 46% (12/26) of cases (Sivan et al 1990). Therefore neutrophil independent mechanisms for the production of severe lung injury must exist. The histology of non-neutropenic ARDS is also consistent with a role for platelets. In the early exudative phase (table 1.6) platelets form part of the micro-vascular aggregates and pulmonary arteriolar thrombi (figure 1.18) are frequently seen. Such lesions contribute to the ventilation / perfusion mis-matching that is responsible for the hypoxaemia of ARDS. Indeed therapies aimed at promoting fibrinolysis with the aim of improving pulmonary perfusion have shown some promise (Murakami et al 1997).

There is also indirect evidence of involvement of platelets in the pathogenesis of ARDS (Heffner, 1997). Autologous radiolabelled platelet transfusions have been shown to be consumed much more rapidly by the lungs in patients with ARDS than in healthy controls (Schneider et al 1980). In a rabbit model of traumatic ARDS, increased platelet aggregation in the
exchange were observed (Jansson et al 1985). In a cobra venom induced rat lung injury model, blocking antibodies against or soluble ligands to CD62P attenuated the neutrophil dependent injury (Mulligan et al 1993; Mulligan et al 1992) although the relative contributions of endothelial or platelet CD62P were not assessed. In one study of adult intensive care patients, more CD62P expression on circulating platelets was detected amongst those with ARDS than controls (George et al 1986). We have discussed above (chapter 1.3.5) that platelets have a clear capacity to contribute to inflammation via the wide range of vasoactive molecules, cytokines, free radicals and proteases that they produce (Yeaman, 1997). In addition the capacity of platelets to induce endothelial cell activation through soluble and surface factors (Henn et al 1998; Kaplanski et al 1997) has been described. The interactions of platelets with leukocytes (Diacovo et al 1996b) are currently being investigated but recent work suggests that there may be a stimulatory 'cross-talk' between these cell types (Ostrovsky et al 1998; Chignard & Renesto, 1994).

**Table 1.7 Evidence for the involvement of platelets in the pathogenesis of ARDS.** The five main lines of evidence supporting a role for platelets in ARDS are shown.

<table>
<thead>
<tr>
<th>Evidence for Involvement of platelets in the pathogenesis of ARDS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ARDS occurs in severe neutropenia (Sivan et al 1990)</strong></td>
</tr>
<tr>
<td><strong>Histology (Meduri, 1996)</strong></td>
</tr>
<tr>
<td>• CD62P function responsible for severity of neutrophil dependent injury (Mulligan et al 1992)</td>
</tr>
<tr>
<td><strong>Platelets consumed by ARDS lungs (Schneider et al 1980)</strong></td>
</tr>
<tr>
<td><strong>Increased circulating platelet activation in ARDS (George et al 1986)</strong></td>
</tr>
</tbody>
</table>
Figure 1.18

*Micro-thrombus obstructing a pulmonary arteriole.* This is a case of septic MODS with disseminated intra-vascular coagulopathy. This is a common finding in ARDS.

1.4.4 Conclusion

Acute respiratory distress syndrome is an acute inflammatory condition which is seen following severe systemic or direct lung insults. Many components of the host response to these insults are involved in pathophysiology but neutrophils appear to play a major role. The contribution of haemostatic and thrombotic mechanisms to the condition remain unclear although there are a several lines of evidence that strongly support a major role for platelets in the acute inflammatory processes ARDS (table 1.7). The main aim of this thesis is to explore the possibility of platelet induced endothelial dysfunction in inflammatory conditions.
1.5 Aims of this thesis

The main hypothesis to be explored in this thesis is that platelets contribute to the inflammatory processes that induce vascular endothelial dysfunction in conditions such as systemic inflammatory response syndrome and acute respiratory distress syndrome.

To investigate this hypothesis the following principal aims were identified:

1. To detail the clinical course and assess the importance of host factors in children with acute hypoxaemic respiratory failure and acute respiratory distress syndrome and to identify factors associated with a poor outcome.

2. To assess circulating platelet activation in cases of acute respiratory distress syndrome.

3. To explore the effects of activated platelets on vascular endothelial cell function.

4. To explore the effects of activated platelets on neutrophil function.

5. To explore the interactions of platelets and neutrophils in severe inflammation.
Chapter 2

General Methods

2.1 Introduction

2.2 Reagents

2.3 HUVEC culture

2.4 Neutrophil separation

2.5 Platelet separation
2.1 Introduction

The experimental work presented in this thesis required the development of new methods and modification of existing techniques. These methods have been described in the appropriate results chapters. This chapter will describe the methods used in several sections of the thesis which did not require refinement.

2.2 Materials

2.2.1 Chemicals and Reagents

Table 2.1

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine diphosphate</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
<tr>
<td>fMLP</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
<tr>
<td>Gly-pro-arg-pro (GPRP)</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
<tr>
<td>Arg-Gly-Asp-Ser (RDGS)</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
<tr>
<td>Lipopolysaccharide (E. coli)</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
</tbody>
</table>
### 2.2.2 Monoclonal antibodies

All antibodies described are mouse anti-human.

#### Table 2.2 Monoclonal antibodies

<table>
<thead>
<tr>
<th>Antibody Specificity, Fluorochrome, isotype (clone)</th>
<th>Source</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control FITC/PE IgG1/IgG2α</td>
<td>BD</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>CD62P:FITC, IgG1, (AK6)</td>
<td>Serotec</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>Activated GpIib/IIIa: FITC, IgM (PAC-1)</td>
<td>Dr S Shattil &amp; later BD</td>
<td>Univ. of Penn. USA Oxford UK</td>
</tr>
<tr>
<td>Control IgG1:FITC</td>
<td>Serotec</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>CD66b :FITC, IgM (G10F5)</td>
<td>Pharmingen,</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>CD42b:PE, IgG2a (AN51)</td>
<td>DAKO Ltd,</td>
<td>High Wycombe, UK</td>
</tr>
<tr>
<td>CD11b:FITC, IgG1 (ICRF44)</td>
<td>Serotec</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>CD11a, IgG1 (H52) ,</td>
<td>Dr. N. Hogg,</td>
<td>ICRF, London, UK</td>
</tr>
<tr>
<td>CD15: FITC, IgG1</td>
<td>Serotec</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>Active site of β-2 integrin (Mab24)</td>
<td>Dr. N. Hogg,</td>
<td>ICRF, London, UK</td>
</tr>
<tr>
<td>CD18 IgG1(6.5E)</td>
<td>Dr M. Robinson</td>
<td>Celltech Therapeutics Ltd, Slough, UK</td>
</tr>
<tr>
<td>CD11b IgG1, (KIM 247),</td>
<td>Dr M. Robinson</td>
<td>Celltech</td>
</tr>
<tr>
<td>CD62E, IgG1 (1.2B6)</td>
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<td>Oxford, UK</td>
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<tr>
<td>VCAM, IgG1 (1.11B1)</td>
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<td>ICAM-1, IgG1</td>
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<tr>
<td>CD62L:PE IgG1 (Dreg56)</td>
<td>Pharmingen,</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Activator of β-2 integrins, IgG1, (KIM127)</td>
<td>Dr M. Robinson</td>
<td>Celltech</td>
</tr>
<tr>
<td>CD61:PerCP, IgG1</td>
<td>BD</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>PSGL-1 IgG1 ( PL-1)</td>
<td>Coulter</td>
<td>Luton, UK</td>
</tr>
<tr>
<td>PSGL-1 IgG1( PL-2)</td>
<td>Coulter</td>
<td>Luton, UK</td>
</tr>
<tr>
<td>CD62P, IgG1, (G1)</td>
<td>Dr R. McEver</td>
<td>Univ. of Oklahoma</td>
</tr>
</tbody>
</table>

#### 2.2.3 HUVEC culture
Table 2.3

Chemical and reagents and materials for HUVEC culture

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<thead>
<tr>
<th>Reagent</th>
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<td>Collagenase type II</td>
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<td>RPMI1640</td>
<td>Gibco</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>GlobePharm</td>
<td>Esher, UK</td>
</tr>
<tr>
<td>Penicillin / Streptomycin</td>
<td>Gibco</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Trypsin-EDTA</td>
<td>Gibco</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>MCDB131</td>
<td>Gibco</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Gibco</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Roussel</td>
<td>Dublin, Eire</td>
</tr>
<tr>
<td>Plastic filling tubes</td>
<td>Avon Medicals</td>
<td>Bristol, UK</td>
</tr>
<tr>
<td>Falcon Primaria culture flasks and plates</td>
<td>Becton Dickinson</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>endothelial cell attachment factor</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
<tr>
<td>endothelial cell growth factor</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
</tbody>
</table>

2.2.4 Neutrophil and platelet separation

Table 2.4

Chemicals and reagents for Neutrophil and platelet separation

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<thead>
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</tr>
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<td>Mono-poly resolving medium</td>
<td>ICN Biomedical</td>
<td>Thame, UK</td>
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<tr>
<td>Hanks Buffered salt solution</td>
<td>Gibco</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Epoprostanol sodium (Flolan)</td>
<td>Wellcome</td>
<td>Greenford, UK</td>
</tr>
<tr>
<td>Heparin</td>
<td>CP Pharmaceuticals</td>
<td>Wrexham, UK</td>
</tr>
<tr>
<td>trisodium citrate</td>
<td>Sigma</td>
<td>London, UK</td>
</tr>
</tbody>
</table>
2.3 Endothelial Cell Culture

2.3.1 Background

Endothelial cells have been cultured from various areas of vascular anatomy from a number of species and may require specialised techniques for each cell type. Human umbilical vein endothelial cells were first cultured successfully in 1963 (Fryer et al. 1966) and refinements of this approach have been widely used (Klein et al. 1993a; Klein et al. 1992). The method of isolation and culture of HUVEC used in this study is presented below.

2.3.2 HUVEC Culture media

Media were purchased as described in table 2.3. RPM1640 containing 1% L-glutamine, 80μg/ml gentamicin, and 100μg/ml penicillin / streptomycin was used for collecting the umbilical cords. Subsequent washing steps were performed with RPM1640 with the same supplements and 5% heat inactivated fetal calf serum. Primary and secondary culture was performed with MCBD131 with 1% L-glutamine, 100μg/ml penicillin / streptomycin, and 20% heat inactivated fetal calf serum. On some occasions endothelial cell growth factor was added to the culture medium to enhance the yield of HUVECs (Minter et al. 1992).

Fetal calf serum was inactivated at 56°C for 30 minutes prior to use. HUVEC require more serum (20% by volume) than many cell culture systems (Gajdusek & Schwartz, 1982; Gallagher et al. 1980).

2.3.3 Isolation of Human Umbilical Vein Endothelial Cells

Cords were cultured at a maximum of 72 hours from collection. All cell culture and manipulations were performed with an aseptic technique in a class II cabinet. All instruments were sterilised with 70% IMS and
glassware sterilised by autoclaving (120°C for 15 minutes). Culture media and enzymes were warmed to 37°C before use. HUVECs were isolated as follows:

A) The cord was inspected for external damage, and blood contained in the vessels expressed. The cord was cleaned and any damaged areas discarded.

B) Both end of the cord were cut to reveal a clean surface. One end was clamped with an artery forceps and the other examined carefully to identify the umbilical vein. The vein was then dilated with blunt forceps and cannulated with a plastic filling tube. The tube was then secured to the vein with cotton thread.

C) The cord was then slowly filled with RPMI1640 with medium to dilate the vein and any sites of leakage were carefully clamped with artery forceps.

D) The vein was then flushed through with the wash medium to remove any remaining blood and the clamp was replaced.

E) 0.1% Collagenase II was then injected into the vein until it was well dilated (usual volume 10-40 ml) and the filling tube clamped with artery forceps.

F) The cord was incubated at 37°C for approximately 10 minutes.

G) Following incubation, the collagenase solution was carefully removed and the vein gently flushed with a further similar volume of RPMI to remove any remaining non-adherent cells. The recovered solution was collected into 50ml conical centrifuge tubes.

H) The cells were sedimented at 200g for 7 min at room temperature. A small white pellet was recovered with a variable small amount of red blood cell contamination. The supernatant was discarded and the cells resuspended in MCDB131 culture medium (5-10 ml) to establish HUVECs in primary culture.
2.3.4 HUVEC primary culture

The cell suspension derived from centrifugation of the collagenase digest was seeded into 25 cm² surface modified polystyrene flasks and incubated for primary culture (37°C, 5% CO₂). HUVECs adhered to the plastic flasks in 2-6 hours in small clusters which gradually coalesced to form a monolayer in 24 - 72 hours (Gimbrone, Jr. et al 1974). Cultures that did not reach confluence in 72 hours often arrested and deteriorated.

2.3.5 HUVEC subculture

When HUVEC primary cultures were observed to be approaching confluence, (>90%) they were washed twice with sterile, warmed PBS to remove non-adherent cells and the FCS containing culture medium. 0.25% Trypsin-1mM sodium EDTA was then added and the HUVECs removed from the flask with gentle mechanical action. Secondary culture was performed on 24 well flat bottom plates (approximately 24 wells per 25 cm² flask) that had been prepared with endothelial cell attachment factor (a commercially available preparation containing gelatin and adhesive proteins including fibronectin in a buffered salt solution).

In view of the changes in HUVEC morphology and function that have been observed over time and passage number (Jaffe et al 1973; Fryer et al 1966) the cells used in this study were always from the first subculture passage and within 72 hours of reaching confluence.

2.3.6 Identification of HUVECs

Endothelial cells were identified by their characteristic cobblestone appearance on phase contrast microscopy (Jaffe et al 1973; Fryer et al 1966). Previous work on this model in our laboratory employed CD31 and CD105 monoclonal antibodies to confirm this phenotypic identification of
endothelial cells. Significant contamination with fibroblasts or epithelial cells were not seen in HUVEC cultures.

2.4 Neutrophil Separation

Neutrophils were separated from heparinised (10 iu/ml) venous blood. One ml of blood was carefully layered onto 3 ml of warmed (37°C) monoply resolving medium (Ferrante & Thong, 1978) in a polypropylene FACS tube. This was then centrifuged at 400g for 30-45 minutes with the brake off. The polymorphonuclear cells formed a visible fraction just below the plasma medium interface which could be drawn off using a sterile pastette. The cells were washed with warmed (37°C) HBSS without Ca$^{2+}$/Mg$^{2+}$ or phenol red and viable cells were counted on a haemocytometer after staining with trypan blue (De Luca, 1965). Preparations in which there were significant (>5%) mononuclear cell or erythrocyte contamination were discarded as were samples in which a high proportion (>10%) of the observed neutrophils were not viable. If the neutrophils were not used immediately they were resuspended in HBSS with 5% FCS, kept at 37°C and used within two hours.

2.5 Platelet Separation

Platelets were separated from citrated (0.38% final conc.) venous blood immediately after collection (Mannucci, 1972). Blood was taken from a free flowing venepuncture after the first 3-5 ml had been discarded. Epoprostanol (2μg/ml) was added to limit artefactual platelet activation during preparation (Read et al 1985; Vargas et al 1982). The blood was centrifuged at 300g for 20 minutes to obtain platelet rich plasma. The top two-thirds of the platelet rich plasma was collected, a further 0.3μg/ml epoprostanol was added and the mixture was centrifuged at 900g for 10 minutes. A small pale platelet pellet then could be identified, this was resuspended in HBSS without Ca$^{2+}$/Mg$^{2+}$ or phenol red and centrifuged
again at 900g for 10 minutes. The final pellet was resuspended in HBSS or culture medium depending on the nature of the experiment and the platelet count ascertained with an automated Coulter counter (Immunotech, UK).
Chapter 3:

Paediatric Acute Hypoxaemic Respiratory Failure

3.1 Introduction

3.2 Paediatric Acute Hypoxaemic Respiratory Failure

3.3 Methods

3.4 Results

3.5 Discussion

3.6 Conclusion
3.1 Introduction

The definition, clinical features and pathophysiology of acute respiratory distress syndrome are outlined in Chapter 1.4. This inflammatory condition which follows severe local or systemic insults appears to have a lower mortality rate in recent years (Ferring & Vincent, 1997). While this may be attributable to changes in intensive care practices, the consequence is that the severity of the initial lung disease has now been observed to have little relationship to survival in adult cases of this condition (Vincent, 1994; Jimenez et al 1994). Therefore, attention has become focused on host factors, such as the underlying diagnosis, rather than the severity of the acute lung disease as being the major determinate of mortality. Such observations have not, to date, been made in paediatric practice. The majority of published series of paediatric respiratory failure and ARDS are small and now several years out-of-date in what is a rapidly evolving field (Tamburro et al 1991; Timmons et al 1991; Rivera et al 1990). This chapter describes a large prospective (n=120) audit of acute hypoxaemic respiratory failure (AHRF) and ARDS. Cases of ARDS constitute a major subset (n=52) of this patient population. This study was carried out on the intensive care units at Great Ormond Street Hospital for Children NHS Trust in order to determine the incidence, clinical associations and factors which relate to outcome from paediatric AHRF.

3.2 Paediatric Acute Hypoxaemic Respiratory Failure

Acute hypoxaemic respiratory failure remains a common reason for emergency paediatric intensive care. Even with currently available ventilatory support it retains a significant morbidity and mortality with the ARDS subgroup having a mortality of 40-75% (Tamburro et al 1991; Rivera et al 1990; Lyrene & Truog, 1981). Promising new interventions
have been described with the purpose of rescuing survivors from severe pathology (Timmons et al 1995).

The modern management of patients with acute respiratory failure is directed towards preventing ventilator induced lung injury or, in the specific case or ARDS, of limiting the progression from acutely inflamed (exudative phase) to scarred lungs (fibrotic phase). Recent years have seen the recognition that the final resultant injury to the lung is the sum of the initial insult plus ventilator induced lung damage from high pressures, large tidal volumes and high inspired oxygen concentrations (Bernard et al 1994). Hence a number of techniques have been developed to limit these factors to attenuate the iatrogenic component of lung injury including prone positioning, (Stocker et al 1997; Amato et al 1995) permissive hypercarbia (Hudson, 1998; Nordenhem & Wiman, 1997; Amato et al 1995;) and high frequency oscillatory ventilation (Arnold et al 1994).

Improving ventilation/perfusion matching by the administration of inhaled nitric oxide as a selective pulmonary vasodilator (Dellinger et al 1998; Troncy et al 1998), or surfactant (Anzueto et al 1996) or perfluorocarbon administration (Hirschl et al 1998) to increase lung volume recruitment have not been shown to improve outcome. Optimising respiratory support may be responsible for the gradual improvement in outcome for cases of ARDS that has been observed in recent years (Milberg et al 1995). However these techniques are aimed at preventing secondary lung injury and cannot be considered therapies for the condition itself (Vincent, 1994).

Therapies aimed at limiting the primary lung injury have yet to prove themselves effective. Anti-endotoxin antibody (Bigatello et al 1994), cyclooxygenase inhibitors (Steinberg et al 1990), pro-inflammatory cytokine blockade (IL-1, PAF), pentoxifylline (Bigatello et al 1994), anti-oxidants (Bernard et al 1997a; Redl et al 1988a), anti-proteases (Tuxen & Cade, 1986), amongst others have been attempted. So far none has been
shown to improve clinical outcomes (Bernard et al. 1994). However recent animal work has suggested that therapies which promote fibrinolysis (e.g. Protein C administration) may have a beneficial effect (Murakami et al. 1997). Glucocorticoid therapy has been widely used in an attempt to limit the progression from exudative to fibrotic stages. Evidence for a beneficial effect is not compelling except in the specific case of ARDS related to *Pneumocystis carinii* pneumonia (Meduri, 1996). A recent study demonstrated that in adults with established lung injury for seven days, methyl prednisolone therapy significantly improved morbidity and mortality (Meduri et al. 1998).

In order to perform effective clinical trials of such therapies objective assessments of disease severity are needed. Therefore, based on the hypothesis that the magnitude of acute severe physiological derangement equates with subsequent mortality, attempts have been made to identify which acute physiological parameters may effectively predict outcome (Timmons et al. 1995; Davis et al. 1993; Timmons et al. 1991; Rivera et al. 1990; Lyrene & Truog, 1981). However the usefulness of predictors so generated may be limited by the variance in clinical practice between institutions and the case mix of the populations studied.

This study was undertaken in order to understand better the factors which determine the severity of paediatric respiratory failure. This chapter describes the largest, prospective, single institution study of AHRF and ARDS in children to date. Published predictors of mortality in this population are assessed. The clinical course, associated and underlying disease processes are described and where the acute physiological parameters fail to accurately predict outcome in these patients we suggest that outcome from respiratory failure in modern paediatric intensive care is determined by the nature of the host and the severity of associated pathology and no longer by the severity of respiratory failure.
3.3 Methods

Approval for this observational study was obtained from our institution's ethics committee and patient data was stored according to the requirements of the Data Protection Act.

Between August 1 1995 and March 31 1997, all children older than one month of age admitted to the PICU at Great Ormond Street Hospital for Children, London, were eligible for inclusion in this prospective observational study. Inclusion criteria were modified from the American-European Consensus Conference diagnostic criteria for ARDS (Bernard et al 1994) described in chapter 1 (table 1.5). Patients were included in this study if they had an acute onset of respiratory failure over less than 48 hours with evidence of a severe oxygenation defect for at least six consecutive hours on the day of admission. Patients who had been ventilated for more than 48 hours for this episode in another Intensive Care Unit were excluded from the study as were cases with clinical or echocardiographic evidence of raised left atrial end diastolic pressure. Chest x-ray appearances were noted and cases with the characteristic four quadrant interstitial showing of ARDS were distinguished from cases without this pattern which were termed AHRF (table 3.1).

Details of the patients' acute diagnoses as well as any underlying conditions were recorded. An electronic patient charting system (Carevue, Hewlett Packard) was reviewed daily and ventilator and physiological parameters recorded and stored on a separate database. Every blood gas analysis performed throughout the patients' admissions was reviewed and the oxygenation index (O.I. = mean airway pressure x FiO2/PaO2), alveolar-arterial oxygen tension gradient (A-aDO2) PaO2/FiO2 ratio and ventilation index (V.I.= resp rate x arterial carbon dioxide tension x peak inspiratory pressure / 1000) were calculated for each measurement. Analyses were carried out using the best and worst
values obtained over the period under assessment. For comparison with previously reported studies every blood gas was reviewed and the relevant study criteria applied for patient selection.

The ventilatory strategy employed was one of permissive hypercarbia ($\text{PaCO}_2 \leq 8\ \text{kPa}$, provided $\text{pH} \geq 7.25$), with limitation of peak inspiratory pressure ($\leq 35\ \text{cmH}_2\text{O}$), while employing high mean airway pressures to ensure maximum lung volume recruitment via the use of peak end expiratory pressure and inverse inspiratory:expiratory ratios. High frequency oscillatory ventilation was employed if oxygenation was inadequate with a mean airway pressure of $20\ \text{cmH}_2\text{O}$ or greater. The use of inhaled nitric oxide therapy throughout the last 12 months of the study was controlled by an institution approved randomisation protocol. Extra-corporeal membrane oxygenation was employed when no stability could be achieved with the above techniques. Death or survival to discharge from the paediatric intensive care unit were the endpoints of the study. In children who died, the mode of death was recorded (failed resuscitation, limitation or withdrawal of support or brain death).

Data were stored on a Microsoft Access 2.0 database and analysed with Microsoft Excel 7.0 and statistical software (Statistic package for social sciences 6.13). Comparisons between survivor and non-survivor data were performed with an independent sample t-test after transformation to normality if required. Comparison with published predictors employed the one sample test for a proportion with correction for sample size. Multiple logistic regression analysis was performed with the statistical software package.
### Inclusion Criteria

| Acute onset of respiratory failure over less than 48 hours; |
| Evidence of a severe defect in oxygenation (arterial oxygen tension to fraction of inspired oxygen ratio (PaO2/FiO2) of less than 200mmHg) for at least six consecutive hours on the day of PICU admission; |
| No evidence of left atrial hypertension; |
| Four quadrant interstitial shadowing on chest X-ray. (Children without the characteristic chest X-ray appearances of ARDS were described as cases of AHRF) |
| Less than 48 hours ventilation for this episode in another institution prior to admission |
| Greater than one month post-natal age. |

### Table 3.1

**Inclusion criteria for study.** These criteria are modified from the American-European Consensus Criteria for ARDS (Bernard 1994, Table 1.5). Absence of left atrial hypertension was predominantly a clinical diagnosis, supported by right atrial pressure measurements in the majority of cases and echocardiography in the majority of cases of multi-organ dysfunction syndrome. Pulmonary artery catheters were used infrequently in this series (n=4).
3.4 Results

3.4.1 Patients

Out of 850 admissions to the PICU, 118 patients were admitted with AHRF over the 20 months of the study. The median age was 9 months (range 1-167 months), and weight 4.3 kg (1-53 kg). The median length of PICU stay was 8 days (0-80 days). The overall PICU or 28 day mortality amongst the AHRF cases was 22% (26/118). One child died within 6 hours of admission, having been inappropriately intubated and resuscitated: since treatment was limited from the time of admission his respiratory indices were excluded from analysis. Fifty-two children who fulfilled the full criteria for ARDS in addition to AHRF had a significantly higher mortality in comparison to the non-ARDS AHRF patients (36.5% (19/52), vs. 10.6% (7/66), p<0.0001).

Table 3.2 (overleaf)

**Univariate analysis of respiratory parameters by PICU survival.** The best daily respiratory parameters are analysed for the first three days of admission. Unlike in previous studies, early measures of the severity of disruption of gas exchange are similar in eventual survivors and non-survivors. Oxygenation index (OI).= mean airway pressure x FiO2/ PaO2, ventilation index (VI) = resp rate x arterial carbon dioxide tension x peak inspiratory pressure / 1000. No correction for multiple comparisons has been applied but none of the markers of severity of AHRF described to date (OI, VI, A-aDO2 or PaO2/FiO2) were significantly different in the two groups. Any trend towards significance would be decreased by adjustment for multiple comparisons.
Table 3.2
Univariate analysis of respiratory parameters by PICU survival.

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivors (91)</td>
<td>Non-survivors (26)</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Survivors (90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Survivors (84)</td>
</tr>
<tr>
<td>A-aDO2 (mmHg)</td>
<td>234 (159-335)</td>
<td>263 (197-324)</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>146 (94-243)</td>
<td>207 (137-324)</td>
<td>0.253</td>
</tr>
<tr>
<td>PaO2/FiO2</td>
<td>170 (112-240)</td>
<td>145 (99-272)</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>217 (154-297)</td>
<td>178 (86-281)</td>
<td>0.25</td>
</tr>
<tr>
<td>OI</td>
<td>6.4 (6-18)</td>
<td>9.2 (5-15)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>18 (10-27)</td>
<td>11.5 (5.6-14.3)</td>
<td>0.09</td>
</tr>
<tr>
<td>VI</td>
<td>23 (16-31)</td>
<td>24 (19-49)</td>
<td>0.84</td>
</tr>
<tr>
<td>PIP (cmH2O)</td>
<td>30 (26-34)</td>
<td>34 (30-38)</td>
<td>0.01</td>
</tr>
<tr>
<td>PEEP (cmH2O)</td>
<td>6 (5-8)</td>
<td>8 (5-10)</td>
<td>0.20</td>
</tr>
<tr>
<td>pH</td>
<td>7.28 (7.19-7.41)</td>
<td>7.17 (7.0-7.31)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>7.38 (7.30-7.48)</td>
<td>7.32 (7.26-7.45)</td>
<td>0.31</td>
</tr>
<tr>
<td>MODS (&gt;2systems)</td>
<td>41% (38/92)</td>
<td>73% (19/26)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>MODS (&gt;2systems)</td>
<td>24% (22/92)</td>
<td>69% (18/26)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
3.4.2 Outcome and acute physiological disturbance

The range of physiological parameters most widely suggested to be associated with outcome are shown as a univariate analysis (table 3.2) for the day of admission (day 0), and the first two complete subsequent days on the PICU. The eventual survivors do not differ significantly from non-survivors in terms of A-aD02, PaO2/FiO2, OI, or VI (the best values in the time period are used). There are significant differences between survivors and non-survivors in the maximum mean airway pressure employed on day 0 & day 1, the highest peak inspiratory pressures employed on day 0 and the worst arterial pH on day 0.

Multiple logistic regression analysis of physiological parameters against PICU survival reveals that best or worst values of OI, VI, A-aDO2, PaO2/FiO2, or the worst values for MAP, PIP, PEEP, PaCO2 or pH recorded on admission days 0 to 2 are unable to predict outcome in AHRF regardless of correction for age, weight or number of organ systems failing (Table 3.3).

3.4.3 Outcome related to diagnosis

The acute diagnosis associated with admission and the mortality in each category is shown in Figure 3.1A, with the underlying or associated diagnosis in Figure 3.1B. The presence of a second (or more) organ system failing was the only pattern of acute diagnosis significantly associated with death (18/40, 45% mortality, odds ratio 4.4, 95%ci = 1.5-13.5). Note the favourable outcome for previously healthy children with 3 deaths from 35 cases (9.6 %) compared to the cases with pre-existing diagnoses (23/83, 27.7%, p<0.0001). As with previous reports (Davis et al 1993; Tamburro et al 1991; Rivera et al 1990; Lyrene & Truog, 1981) the outcome for immunodeficient children who develop AHRF was
Table 3.3

Multiple logistic regression analysis of physiological parameters against PICU survival. Note that only the number of organ systems failing (MODS) (Wilkinson et al. 1986) on days 1 and 2 is related to outcome.

<table>
<thead>
<tr>
<th>variable</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.01 (-0.04 - 0.02)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Weight</td>
<td>-0.09 (-0.27 -0.09)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAP</td>
<td>0.04 (-0.14 - 0.22)</td>
<td>-0.01 (-0.21-0.18)</td>
<td>0.03 (-0.65 - 0.72)</td>
</tr>
<tr>
<td>MODS</td>
<td>0.91 (-0.18 - 2.01)</td>
<td>1.22 (0.32 - 2.11)</td>
<td>2.05 (0.33 - 3.75)</td>
</tr>
<tr>
<td>AaDO2 best</td>
<td>-0.01(-0.021 - 0.007)</td>
<td>0.00 (-0.001 - 0.015)</td>
<td>0.14 (-0.012 - 0.027)</td>
</tr>
<tr>
<td>OI best</td>
<td>0.42 (-0.18 - 0.26)</td>
<td>0.11 (-0.15 - 0.36)</td>
<td>0.38 (-0.09 - 0.85)</td>
</tr>
<tr>
<td>PaO2/FiO2</td>
<td>0.00 (-0.005 - 0.01)</td>
<td>0.01 (-0.003 - 0.012)</td>
<td>0.00 (-0.02 - 0.02)</td>
</tr>
<tr>
<td>PIP</td>
<td>0.16 (-0.03 - 0.35)</td>
<td>0.042 (-0.09 - 0.17)</td>
<td>-0.38 (-0.85 - 0.10)</td>
</tr>
<tr>
<td>PEEP</td>
<td>0.021 (-0.31 - 0.35)</td>
<td>-0.25 (-0.64 - 0.14)</td>
<td>-0.64 (-1.59 - 0.31)</td>
</tr>
<tr>
<td>PaCO2</td>
<td>-0.32 (-0.65 - 0.02)</td>
<td>0.03 (-0.32 - 0.38)</td>
<td>-0.14 (-0.73 - 0.45)</td>
</tr>
</tbody>
</table>
Figure 3.1

Mortality by A) the acute diagnosis precipitating admission and B) by pre-existing diagnosis. The percentage of non-survivors in each group is shown to the left of zero and survivors to the right of zero. LRTD=lower respiratory tract disease, MODS= multiple organ dysfunction syndrome (≥ 2 systems failing). Comparisons are made between the diagnostic groups and the rest of the population. Note the significantly worse outcome for immunodeficient children.

A)

- Head injury (2) ns
- Non-infective LTRD (21) p<0.001
- Infective LRTD (44) p<0.001
- Int. Pneumonitis (11) ns
- All (118) MODS (40) p<0.001

B)

- Neuromuscular (10) p<0.001
- Normal (35) p<0.001
- Ex-Prem (22) NS
- Miscellaneous (22) NS
- All (118) Immunodef (29) p<0.0001
3.5.4 Clinical course

The clinical course of patients with AHRF was categorised according to the severity of the disruption of gas exchange (best daily A-aDO2 is displayed but the classification is identical if PaO2/FiO2 or OI is used in our patients) into five distinct groups, (figure 3.2). Survivors follow a course of mild disease only (Group A: n = 45), early improvement over ≤ 3 days (Group B: n = 23), late improvement after more than 3 days (Group C: n = 24). Non-survivors die with persistent severe hypoxaemic respiratory failure (Group D: n = 17) or during or following resolution of AHRF (Group E: n = 9).

3.5.5 Mode of death

The children who died in group E most frequently did so from severe cerebral injury (5/9 from brain death including the three previously normal children who died), the other four cases had support withdrawn or limited because of the severity of associated diseases. Of the children in group D, none were previously healthy children (10 immunodeficient, 3 ex-premature infants & 4 others with inherited metabolic disorders or major chromosomal abnormalities). Further, only 6/17 (35%) of group D died whilst receiving full supportive treatment including CPR, the remainder had support withdrawn (4/17 24%) or a limitation of intensive care therapy (7/17 41%) because of the severity of the associated conditions or coincident organ failure. Therefore only six cases of with AHRF, none of whom were previously normal children, reached a point of unsupportable respiratory failure.
**Figure 3.2**

Clinical course of AHRF identified by the best daily AaDO2 ratio. All patients fitted in one of 5 patterns of clinical course, 3 (A-C) amongst survivors and 2 (D&E) amongst non-survivors. The largest group (A) never develop severe gas exchange disruption, Group B recover promptly from severe physiological derangement (<3 days), whereas the severe disruption of gas exchange is more persistent in group C (who recover in greater than 3 days). The majority of deaths occur in children with persistently severely abnormal gas exchange (group D) but a significant number of deaths (9) occur amongst children in whom the initial abnormalities of gas exchange have partially or completely resolved (group E).
3.5 Discussion

3.5.1 Summary

The principal observation in this audit is that associated or underlying diagnoses - case mix - have a significant bearing on population outcomes. Not surprisingly, children with ARDS had a poorer outcome, and when using a defined ventilator strategy, which here emphasised permissive hypercarbia and lung volume recruitment, severity of ventilatory parameters (i.e. high mean airway pressure) rather than indices of gas exchange, reflected better the likelihood of poor outcome. Most importantly, where the acute physiological parameters fail to differ between good and poor outcome patients, this study suggests that in modern paediatric practice the severity of pre-existing disease or associated pathology, rather than the severity of respiratory failure alone is associated with outcome.

3.5.2 Comparative assessment of respiratory indices

Comparisons with previously published predictors of outcome from studies of AHRF and ARDS in childhood are shown in table 3.4. None of the proposed physiological correlates of outcome were applicable to this series. Reviewing every blood gas and applying the published criteria demonstrated that in each case the predictor overestimated the patients' risk of mortality, excepting the very severe criteria from the Melbourne study in 1991 which used a peak inspiratory pressure >40 cmH₂O and A-aDO₂ >580 mmHg. These were rarely achieved in this population (7 cases) and hence the confidence intervals remain so wide (18-90%) that no useful conclusion could be drawn.
Table 3.4
Comparison of previously published respiratory severity parameters.

The performance of these predictors in estimating outcome is compared with the present study (one sample test for proportion with correction for sample size). Each published predictor significantly over-estimated mortality in the present study excepting the Melbourne criteria (Rivera et al 1990) of PIP >40cmH2O and AaDO2>580mmHg which failed to achieve significance with only 7 cases fulfilling these criteria.

<table>
<thead>
<tr>
<th>Study</th>
<th>Proposed Predictors</th>
<th>Positive predictive value for mortality</th>
<th>Present study PPV (95% ci)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melbourne 1991 AHRF(Rivera et al 1990)</td>
<td>VI &gt; 40 &amp; OI &gt;40</td>
<td>77%</td>
<td>6/15 = 40% (16-68%)</td>
</tr>
<tr>
<td></td>
<td>PIP&gt;40 &amp; AaDO2 &gt;580</td>
<td>81%</td>
<td>4/7 = 57% (18-90%)</td>
</tr>
<tr>
<td>Memphis 1991 A-aDO2 &gt;449 for 24 hours (Tamburro et al 1991)</td>
<td>A-aDO2 &gt;449 for 24 hours</td>
<td>100%</td>
<td>9/22 = 41% (21-64%)</td>
</tr>
<tr>
<td>Philadelphia 1993 ARDS(Davis et al 1993)</td>
<td>A-aDO2 &gt; 420</td>
<td>87%</td>
<td>17/44 = 40% (24-55%)</td>
</tr>
<tr>
<td>Salt Lake City 1991 ARDS(Timmons et al 1991)</td>
<td>A-aDO2 &gt;470</td>
<td>81%</td>
<td>17/42 = 42% (26-57%)</td>
</tr>
<tr>
<td></td>
<td>MAP &gt;23</td>
<td>90%</td>
<td>8/22 = 32% (17-59%)</td>
</tr>
</tbody>
</table>
The largest study of paediatric AHRF, a multi-centre retrospective study including 470 cases from 1991 (Timmons et al 1995) identified an association between acute respiratory physiological disturbance and outcome. However children who became brain dead or had treatment withdrawn - in the setting of severe neurological insult - were excluded from the subsequent analysis. Such an approach, (included as the study was principally designed to identify ECMO candidates) would have excluded from our analysis all the normal children who died. Brain injury is a possible complication of severe hypoxaemia or the disease processes that initiate hypoxaemia, our view was that these patients must be included in our attempt to identify factors associated with outcome.

**Table 3.5**

**Cases treated with "new " therapeutic modalities.** Note that cases following the clinical patterns C and E (see text) in which outcome is contrary to the severity of gas exchange parameters, frequently received these therapies. HFOV = high frequency oscillatory ventilation.

<table>
<thead>
<tr>
<th>Therapy (total no. of cases)</th>
<th>Cases (118)</th>
<th>Deaths (26)</th>
<th>Pattern C cases (24)</th>
<th>Pattern E Cases (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhaled nitric oxide (or inclusion in iNO trial)</td>
<td>38</td>
<td>12</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>HFOV</td>
<td>25</td>
<td>9</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Surfactant</td>
<td>15</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>ECMO</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3.6
Details of diagnoses and outcome for the 'immunodeficient' subgroup. 29 patients were identified as immunodeficient from a variety of congenital, acquired or iatrogenic causes. The mortality amongst this group as a whole was significantly higher than the rest of the population (figure 3.1). One explanation for this might be a degree of chronic lung disease in these cases prior to the acute episode recorded in this study. Chronic lung disease was recorded in a proportion of cases (5/29) and deaths (3/13)

<table>
<thead>
<tr>
<th>Nature of Immunodeficiency</th>
<th>Number (survivors)</th>
<th>Documented prior lung disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Immunodeficiency Virus</td>
<td>6 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Acute Leukaemia</td>
<td>5 (4)</td>
<td>0</td>
</tr>
<tr>
<td>Severe Combined Immunodeficiency or Wiskott-Aldrich Syndrome</td>
<td>3 (0)</td>
<td>1</td>
</tr>
<tr>
<td>Combined Immunodeficiency</td>
<td>4 (1)</td>
<td>2</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>1 (1)</td>
<td>1</td>
</tr>
<tr>
<td>others</td>
<td>3 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Immunosuppressive therapy</td>
<td>7 (5)</td>
<td>1</td>
</tr>
<tr>
<td>Totals</td>
<td>29 (16)</td>
<td>5</td>
</tr>
</tbody>
</table>
3.5.3 Patterns of disease and outcome.

The patients in this series exhibited one of five patterns in their clinical course. Deaths amongst children admitted in AHRF can occur with active and progressive lung disease (pattern D) or in spite of resolving lung disease (pattern E). In children who survive, recovery may be slow or fairly rapid. On inspecting the data it is apparent that there are similarities in the initial respiratory indices in children who survive despite severe prolonged gas exchange disruption (pattern C) and those who die despite improving or improved gas exchange disruption (pattern E). The relative proportion of the patients with these patterns in a population being studied will clearly determine the utility of gas exchange parameters in predicting survival; our experience when including patients with underlying immunodeficiency or other associated diseases is that these proportions confound their use. Of further note is the mode of death in children with persistently severely abnormal gas exchange (pattern D): cases rarely reached a level of respiratory failure which was unsupportable by current techniques. Instead in the majority of cases (11/17) treatment was discontinued or limited as a result of other aspects of the clinical situation. It is worth re-emphasising that no previously normal child died of unsupportable respiratory failure.

Observations from adult intensive care studies of lung injury have indicated that outcome is not necessarily related to the level of arterial oxygenation (Jimenez et al 1994; Vincent, 1994). In contrast, many previous paediatric studies in defined populations have supported a contrary notion. In this study, the mortality from respiratory failure appears to be related to associated disease rather than the severity of initial gas exchange disruption. The implications of such a conclusion are wide. Is there much to be gained by refining respiratory support further (e.g. by the development of perfluorocarbon assisted gas exchange) when mortality is frequently determined by non-pulmonary factors? Indeed it has been
suggested that mechanical ventilation should now be considered less a form of treatment than a form of organ support during disease resolution (Ferring & Vincent, 1997). Secondly, can severity scoring systems that employ acute pulmonary physiological parameters and do not incorporate underlying aetiology be used to good effect in paediatric AHRF? Our findings suggest that perhaps the reported value of such pulmonary physiological parameters, with their institution specificity, are more a reflection of physician behaviour, i.e. patient selection and local ventilatory strategy than patient pulmonary pathophysiology. More recently developed non-linear, multiple logistic regression models, that predict the risk of death for children less aged than 16 years (e.g. the ‘Pediatric Risk of Mortality III’ (Pollack et al. 1996) and the ‘Paediatric Index of Mortality’ (Shann et al. 1997) may overcome this failing since they incorporate diagnostic/disease categories as well as neurological and respiratory parameters. Thirdly, how can clinical trials be designed to assess the impact of new respiratory therapies? We suspect that a comparison between heterogeneous treated and control groups using only early respiratory parameters to confirm similarity of disease severity is invalid given the distinct patterns of AHRF we have observed. Furthermore, we would be surprised if any randomised controlled trial so designed showed an effect attributable to treatment. It is more likely that, in keeping with other reports, case mix should not be ignored e.g., AHRF in an ex-premature infant with respiratory syncytial virus bronchiolitis is not the same as AHRF in an infant with aspiration pneumonia - even if the respiratory indices suggest that they are similar. This suggests that it is time to reconsider disease-specific stratification criteria in any future evaluations of treatment, even though this will inevitably mean that studies will take much longer to recruit sufficient patients. Finally, since death in a previously normal child is now an infrequent end-point in AHRF our data supports the view that other markers of ventilator-related outcome should be sought (Petros et al. 1995).
3.6 Conclusions

The data presented in this chapter confirm the observations that prompted this project, namely that acute respiratory failure and particularly ARDS in immunodeficient patients carries a very poor prognosis. This remains the case despite an apparent highly significant improvement in the outcome of severe paediatric respiratory failure including ARDS as a whole in comparison to historical controls (Davis et al 1993, Tamburro et al 1991, Timmons et al 1991, Rivera et al 1990).

It is clear that the associated or underlying disease processes are now essential determinates of outcome independent of the severity of the physiological disturbance. This suggests that the nature of the host rather than the nature of the acute insult may be the critical factor that determines outcome. In part, this observation may reflect the behaviour of clinicians who limit escalation of respiratory support in a child who is perceived to have an underlying illness with a poor prognosis. Interpreting the contribution of an abnormal host inflammatory response is less straightforward. There are many reasons why an immunodeficient child may be at greater risk of a poor outcome than an normal child including nutritional status, colonisation with resistant micro-organisms and the presence of chronic organ dysfunction (especially respiratory) prior to the acute insult assessed in the this study. Although the majority of deaths recorded here in immunodeficient children were cases in which prior lung disease had not been documented, subclinical disease in these complex cases cannot be excluded. Controlling for such factors in this heterogeneous population (table 3.6) is outside the scope of this study.

The other possibility is that the nature of the inflammatory response may be more severe in those hosts who develop the most severe respiratory failure. Although this may not appear consistent with the observation of worse outcome in immunodeficiency, it can be appreciated that a child
with an immunodeficiency or chronic lung disease of prematurity may not be able to clear an insult as rapidly as a healthy child. There may be persistence of the pathogen and hence a more prolonged and abnormal inflammatory response.

There are data from investigations of specific immunodeficiencies that support this interpretation that an abnormal immune response in an immunodeficient patient may indeed lead to a more severe result following a clinical insult. One such example is CD154 deficiency or Hyper-IgM syndrome (Korthauer et al, 1993). CD154 (CD40L) is a member of the tumour necrosis factor (TNF) gene family, and is preferentially expressed on activated CD4 T cells, mast cells and eosinophils. The ligand for CD154 is CD40 which is expressed on the surface of B cells, monocytes, dendritic cells and endothelial cells. Hyper-IgM patients have mutations in the CD154 gene resulting in defective expression of CD154 on activated T cells (Noelle, 1996). These patients suffer from repeated and severe bacterial infections, *Pneumocystis carinii* pneumonia, *Cryptosporidium* enteropathy and sclerosing cholangitis. The mechanisms responsible for the susceptibility and sequelae of CD154 deficiency to *Cryptosporidium parvum* infection are now becoming clear. Much of the information has come from experiments conducted with knockout mice, which have shown that several pathways are important in this infection. In severe combined immunodeficiency mice with no T or B cells, *C. parvum* infection was sometimes spontaneously cleared and only mild inflammation was detected in the liver. In contrast, CD154 and CD40 deficient mice could not clear the infection and developed florid hepatitis and sclerosing cholangitis. IFN gamma deficient mice had severe or fatal enteritis and had histological evidence of bile duct sclerosis. Double knockout mice deficient in both CD40 and IFN gamma had bile duct sclerosis but no enteritis (Stephens et al, 1999).
Chronic granulomatous disease also provides support for this interpretation that the nature of the host inflammatory response may determine the clinical phenotype. This is an inherited deficiency of an enzyme which is essential in the production of reactive oxygen metabolites. The killing of ingested micro-organisms is much reduced and these patients have frequent skin and gastro-intestinal infections. A recent study investigated the influence of known common polymorphisms of genes for a range of important immunological factors (including pro-inflammatory cytokines and immunoglobulin receptors) in these patients (Foster et al, 1998). Different polymorphisms were associated with different patterns of infective complications with, for example, widely varying rates of gastro-intestinal complications.

Both these examples indicate that apparently minor differences in the nature of a host response to an insult may have a major effect on the pattern and severity of the resulting clinical problem. A similar pattern could be envisaged in ARDS in which patients with abnormal immunity generate a more clinically severe response to a relatively minor insult.

This speculation that the nature of the host, and particularly factors that influence the nature of the inflammatory response remains central to the outcome from AHRF/ARDS is consistent with the hypothesis that platelets may have a role in the pathogenesis of acute respiratory distress syndrome.
Chapter 4

Measurement of Platelet Activation in ARDS

4.1 Introduction

4.2 Methods

4.3 Discussion

4.4 Conclusions and Future work
4.1 Introduction

In chapter 3 the clinical features and associations of poor outcome of acute respiratory distress syndrome in a paediatric population were described. The observation that host factors such as prior immunodeficiency are clearly related to outcome when the severity of gas exchange disruption is not, is consistent with the view of ARDS as a severe inflammatory response. In chapter 1 the evidence for the inflammatory basis for this condition was reviewed as was the clinical, histological and experimental evidence for the involvement of platelets in the pathogenesis of ARDS (table 1.7). Much of this evidence has been generated from animal models or from adult patient populations. This chapter describes the testing of the hypothesis that circulating platelets are more activated in children with ARDS than in control cases.

4.2 Methods

4.2.1 Methods for the assessment of in-vivo platelet activation.

The development of a sensitive and specific test to detect platelet activation in-vivo has been described as one of the "holy grails" of haemostasis-thrombosis research (Abrams & Shattil, 1991). For many years the levels of platelet granule release products including β-thromboglobulin (Fitzgerald et al 1988; Reilly et al 1986) and platelet factor -4 (Collins et al 1994) or metabolites of thromboxane A2 (Catella et al 1986; Fitzgerald et al 1986) have been used. These techniques have been described as having limited clinical use because of the great care that is required during sample collection and processing to limit artefactual platelet activation and granule release (Abrams et al 1990). Other techniques employed include the estimation of platelet density (van Oost et al 1983) which exploits the decrease in platelet density following granule release as a marker of activation, and a method which compares
platelet counts in formaldehyde fixative or EDTA as an indication of the presence of circulating platelet aggregates in vivo (Wu & Hoak, 1974). The aggregometry method described by Born in 1962 (Born, 1962) and refined since (Simi et al 1987) has been widely used in the investigation of platelet function but is very time consuming and hence is of limited value in the analysis of patient samples.

4.2.2 Whole blood platelet flow cytometry.

The direct detection of activated platelets by flow cytometry following the addition of specific monoclonal antibodies against surface bound markers of activation was first described in 1987 (Shattil et al 1987). In this way the α-granule component CD62P, the activated form of GpIIb/IIIa or changes in fibrinogen binding capacity (Warkentin et al 1990) can be detected on platelets within seconds of a stimulus to activation. Flow cytometry also allows the rapid analysis of large number of individual platelets rather the whole population. The minimal sample preparation required by this technique has made it suitable for the investigation of platelet activation in-vivo.

Platelet flow cytometry has been undertaken in a number of clinical conditions where significant platelet activation is suspected (Janes et al 1995; Tschoepe et al 1995; Collins et al 1994). In one study of adult intensive care patients, greater CD62P and activated GpIIb/IIIa expression on circulating platelets was detected amongst cases of ARDS than controls (George et al 1986).

4.2.3 Sample Preparation

Whole blood flow cytometry for assessment of platelet activation was undertaken according to established methods (Warkentin et al 1990; Shattil et al 1987). Monoclonal antibodies against CD62P or the activated
form of GpIIb/IIIa, directly conjugated to FITC were purchased (details in Section 2.2). The following solutions were prepared and sterilised by passage through a 0.2μ filter: sodium citrate 3.8%, platelet buffer (10 mmol/l HEPES, 145 mmol/l NaCl, 5 mmol/l, 1 mmol/l MgSO₄, pH 7.4), and platelet fixative (0.2% formaldehyde in PBS) (Warkentin et al 1990). Blood samples were collected without the use of a tourniquet (after discarding the initial 2 ml of blood taken in order to limit artefactual activation) and were immediately added to sodium citrate (final conc.0.38%) anticoagulant. Five μl of blood was added to 50 μl of warmed platelet buffer in a polystyrene FACS tube containing the monoclonal antibodies against CD62P, GpIIb/IIIa or isotype matched control antibodies. Following gentle resuspension, the sample was incubated at room temperature for 20 minutes before the addition of 200μl of platelet fix solution.

4.2.4 Flow cytometry.

Samples prepared as above were analysed within one hour of fixation. Flow cytometric analysis was performed on Becton Dickinson FACSAn and later on FACScalibur. Laser excitation was at 488nm and data were collected on FITC fluorescence at 515nm. Forward and side scatter measurements were made with gain settings in logarithmic mode and the platelet population could easily be identified by the characteristic appearances on forward and side scatter profiles (figure 4.1). Platelet sized events were selected on the forward / side scatter profile and the accuracy of the gating confirmed by staining with antibody against CD42b (>98%). Titration of antibody dose revealed that optimal staining was achieved at saturating concentrations with 5μl of AK6 CD62P antibody, 10μl of PAC-1, GpIIb/IIIa antibody from Dr Shattil but 20μl of PAC-1 antibody from Becton Dickinson (protein concentrations not given) for each 5μl of blood.
Figure 4.1

The characteristic forward and side scatter pattern of platelets with whole blood flow cytometry. The platelets (population defined by displayed region) are clearly visible as a population distinct from the larger, more granular erythrocyte and leukocyte populations. In filtered solutions, platelets can easily be distinguished from contaminating smaller particles.
Figure 4.2

Patterns of fluorescence staining of platelets with anti-GpIib/IIIa (PAC-1:FITC) and anti-CD62P:FITC. The appearance of isotype matched control antibody non-specific staining is shown along with an unstimulated sample. This is compared to the staining seen after 1 minute of exposure to ADP (final conc. 10μM).
GpIIb/IIIa staining is expressed as median fluorescence intensity (MFI) and CD62P staining as MFI and percentage of antibody-positive platelets, defined as those with a fluorescence intensity exceeding that of 98% of the platelets stained with control antibody. The capacity of the system to detect platelet activation was assessed by in-vitro stimulation with the platelet agonists ADP (final conc. 10μM) or thrombin (0.4iu/ml) in the presence of 1mM GPRP peptide to prevent fibrinogen cross-linking (Laudano & Doolittle, 1980) (figure 4.2).

No reproducibility data are described in the original descriptions of assessment of platelet activation by flow cytometry (Shattil et al, 1987, Warkentin et al, 1990). Initial investigations on samples taken from the author demonstrated that there was good reproducibility with <3% variability in the MFI (<1%, %PF) for re-analysis of a single sample and <6% variability in MFI (<1%, %PF) replicated samples.

The performance of the flow cytometer was monitored throughout the period of this thesis with weekly calibration checks. These were undertaken with commercially available latex beads of known size, granularity and amounts of attached fluorochrome (FITC, PE and PerCP) (CaliBrite3 Beads, Becton Dickinson, UK). These were analysed with the automated calibration programme, FACSComp (BD, UK). The performance of the forward, side scatter and fluorescence detectors was therefore monitored against the manufacturer's criteria throughout the studies performed in this thesis. No significant drift of detector performance was apparent during the time these studies were performed. The laser was replaced in the second year of this thesis but all parameters assessed continued to fall within specified limits.
4.2.5 Patients

All children greater than one month post-natal age admitted to the Paediatric Intensive Care Unit between October 1995 and June 1996 were considered for inclusion in this study. ARDS was defined as before according to the American European Consensus Conference criteria (table 1.5). In all cases an in-dwelling arterial catheter was an essential requirement in order to standardise the technique of sample collection.

The study consisted of two stages. In the first stage A), control patients were provided by cases admitted to the intensive care unit with single system failure (isolated upper airway obstruction, head injury) and no evidence of systemic inflammatory response syndrome (table 1.1). These were then compared to patients in the early 'exudative' phase (see section 1.4.2) of ARDS (assessed by a history of less than 3 days of established respiratory failure). The administration of platelet transfusions was not considered a contra-indication to inclusion in this study. This study protocol was approved by the Research Ethics committee. Blood samples were collected from the arterial cannula at the time of a clinically indicated blood gas estimation and the first two ml of blood discarded. The blood was added to the mixture of buffer and monoclonal antibodies within 15 min of collection. In the subsequent phase of the study B), controls were provided by cases of respiratory failure who did not meet the full criteria for ARDS (i.e. localised lung disease, designated acute hypoxaemic respiratory failure, 'AHRF'). This was done to control for hypoxaemia and therapeutic interventions such as would be seen in ARDS cases (e.g. ventilation, fluid restriction). Further controls were those cases with conditions recognised to be at risk for the subsequent development of ARDS who did not meet criteria (e.g. cases of sepsis or aspiration with well preserved respiratory function). Cases were, as in study A), subjects in the acute phase of ARDS. Studies A and B were performed consecutively with no overlap of patients. GpIIb/IIIa was not studied in B
because of a shortage of the PAC-1 antibody which was not commercially available at this stage.

To determine if the measured levels of platelet activation seen amongst children in intensive care were altered compared to normal, the values of healthy adult laboratory staff who denied taking any medication for at least two weeks prior to sampling were included in the analysis. Although not collected simultaneously with the patient samples, these healthy controls were studied on the same FACScalibur analyser with the same settings and antibody batches.

Platelet transfusions are widely used on intensive care in cases with disseminated intravascular coagulopathy or bone marrow failure who are at high risk of severe lung disease. There is evidence for the presence of a 'storage lesion' in platelet concentrates with increased activation over time (Funheer et al 1990). This is a potentially serious confounder of any results obtained from the PICU patients. It was not possible to control for platelet transfusion dose, timing, indication etc.. Therefore, stored platelet concentrates were examined for CD62P expression on the day on which they were considered to be too old for clinical use by the transfusion laboratory in an attempt to assess the size of this potential problem.
4.3 Results

4.3.1 Study A. Comparison of platelet activation in established ARDS with control PICU cases.

The expression of the activated form of GpIIb/IIIa as determined by the binding of monoclonal antibody PAC-1, was not significantly different between 11 single system failure cases (mean 122 95% ci 101-142) and 6 cases of ARDS (96, 81-110, p=0.09, unpaired t-test) (figure 4.3). Indeed there was a non-significant trend towards lower activated GpIIb/IIIa expression amongst ARDS patients than controls.

4.3.2 Study B). Comparison of platelet activation in AHFR, 'at risk' and ARDS cases.

In the second part of the study where 'At Risk' and 'AHFR' groups provided controls, no difference was seen between the PICU groups ARDS (n=8), at risk (n=8) or AHFR (n=12) groups (ANOVA, P>0.05) and the healthy adult control population for CD62P MFI or percentage positive fluorescence (figure 4.4). The two cases 'at risk' who subsequently developed ARDS were not distinguishable from the rest of the 'at risk' population. When samples were stimulated with ADP in-vivo, all demonstrated a marked increase in CD62P MFI and percentage positive fluorescence (data not shown).

Platelet transfusion packs (n=8) on the day of removal from availability for clinical use were significantly more activated than the platelets in whole blood from healthy adult controls (transfusion pack median CD62P MFI = 4.3 vs. 1.15 in healthy controls, p<0.001 Mann-Whitney test).
**Figure 4.3**

Circulating platelet GpIib/IIIa expression (MFI) in single system failure ICU patients and cases of ARDS. Values of individual measurements (open squares) and the means and 95%ci (black square and error bars) of the groups are shown. There are no significant differences in GpIib/IIIa expression between these two groups.
Figure 4.4
Circulating platelet CD62P expression A) MFI and B) % positive fluorescence in Adult controls, ARDS, at risk and AHRF cases. Individual measurements and means and 95% ci are shown. There are no significant differences in the levels of CD62P expression between healthy adults or each of the PICU populations. Very low levels of platelet activation are seen in all cases.
4.4 Discussion

In these two studies we found no evidence that platelets were able to contribute to inflammatory processes directly. Levels of platelet activation as assessed by flow cytometry were no different in patients with ARDS from other paediatric intensive care populations or from healthy adult laboratory controls. This result was unexpected in that there is considerable evidence suggesting a role for platelets in the pathogenesis of this condition as discussed previously (chapter 1.4.3). Indeed one adult study suggested that platelets from cases of ARDS had higher levels of surface CD62P and GpIIb/IIIa (George et al 1986). However, this was performed with a different technique involving monoclonal antibodies linked to radiolabelled iodine. There are several possible explanations for the observations made in this chapter. Firstly platelets might not contribute to the development of ARDS. Alternatively, the flow cytometry technique may not be sensitive enough to detect changes in platelet activation. Platelet flow cytometry has been used widely; it appears to be a sensitive technique which detects brisk responses to platelet agonists while examining a large number of cells very rapidly. However the technique may be not be sensitive in another respect in that activated platelets express CD62P and GpIIb/IIIa and hence in-vivo have an increased capacity to bind to sites of endothelial damage, other platelets or even other cell types. Therefore, free platelets may represent a population of cells which have been already selected as less activated. Aggregated platelets found in the pulmonary micro-vasculature in cases of ARDS may indeed be activated but would not be detectable with this technique. The observation that platelets from ARDS cases could be activated in-vitro with ADP supports this possibility. Local platelet activation occurring in the lungs which is not detectable in the circulating pool is an appealing explanation which is consistent with the trend towards lower levels of activated GpIIb/IIIa expression seen in cases of ARDS. It is possible to imagine that the platelets expressing the higher
levels of activated GpIib/IIa are more likely to be recruited by activated pulmonary vascular endothelium, which would leave the circulating platelet pool depleted of this more activated population. This is consistent with the observations in a rabbit model of trauma induced lung injury (Jansson et al 1985) where the injury lung consumed a large quantity of labelled transfused platelets. Similar observations were made in a small human study of acute respiratory failure patients in comparison to healthy adult controls (Schneider et al 1980). This explanation does not address the difference between these results and those seen in the adult study mentioned above (George et al 1986). One potential confounding factor may be the frequent use of therapeutic heterologous platelet transfusion in the paediatric population. Thrombocytopenia accompanying sepsis or due to an underlying haematological diagnosis is a frequent occurrence in paediatric intensive care and may have contributed to an underestimation of platelet activation. This confounding factor is likely to have been more frequent in the cases of ARDS than in the less severely ill control groups. Data on recent platelet transfusion were not recorded on these subjects which contributes to the difficulty in interpreting these limited studies. It is not clear from the analysis of platelet transfusion packs what the effect of platelet transfusion on the detectable levels of platelet activation are likely to be.
4.5 Conclusions and future work

This chapter has described two studies designed to investigate the role of free platelets in inflammation. Circulating platelets from critically ill paediatric patients with the severe inflammatory condition ARDS were not significantly more activated than controls. There are many possible explanations for this finding including that the study may have not had sufficient power to detect a small change in the levels of activation detectable using flow cytometry. The inevitable heterogeneity of the patients will limit the conclusions that can be drawn from these data.

If these data do reflect the true state of activation of circulating platelets in this complex patient group, then it suggests that any mechanism by which platelets might exert an influence in the pathogenesis of ARDS is likely to be indirect, requiring the presence of other inflammatory cells. A possible influence of platelets in the pathogenesis of endothelial injury cannot be excluded unless interactions with other cells are considered. Interactions with neutrophils and directly with cultured endothelium in a laboratory model were therefore investigated.
Chapter 5:

Platelet-Neutrophil Interactions in Whole Blood.

5.1 Introduction

5.2 Methods

5.3 Results

5.4 Discussion
5.1 Introduction

In chapter 4 the use of free platelet flow cytometry to assess the state of *in-vivo* platelet activation was discussed. The extent of changes in platelet activation seen using this method have been small in many clinical studies (Collins *et al* 1994; Rinder HM *et al* 1994; Abrams & Shattil, 1991; Rinder CS *et al* 1991). One explanation for this could be the selection of activated platelets away from the circulating pool by virtue of their more activated adhesion molecule profile (table 1.3) by adhesion to the sub-endothelial matrix, adhesive proteins (Bombeli *et al* 1998), intact endothelium (Bombeli *et al* 1999) or other cell types.

When platelets were first described in 1882, they were noted to be "*for the most part aggregated around the colourless corpuscles*" (Anon. 1882). This observation was revisited in 1963 when platelets were recorded as binding to leukocytes with a characteristic appearance of platelet rosetting around polymorphonuclear leukocytes on a blood film (Field & MacLeod, 1963) (figure 5.1).

![Platelet Satellitism](image)

**Figure 5.1**
**Platelet Satellitism.** (Shahab & Evans, 1998). This appearance of platelet rosetting around neutrophils is an *in-vitro* artefact that only occurs in EDTA anti-coagulated blood in the presence of IgG anti-platelet and anti-neutrophil antibodies.
The appearance was later recognised to represent an artefact that only occurs in EDTA anti-coagulated blood in the presence of anti-platelet and anti-neutrophil antibodies (Kashiwagi et al 1997; Bizzaro, 1991). The principal importance of this phenomenon is that it may be misinterpreted by an automated cell counter and result in the initiation of inappropriate investigation or therapies for thrombocytopenia (Bizzaro, 1995).

More recently the capacity of platelets to interact with other cells including neutrophils, monocytes, lymphocytes and endothelial cells has been described (Spangenberg, 1994; Rinder HM et al 1991). These studies employed separated cell techniques to demonstrate heterotypic binding between platelets and leukocytes. Although one study demonstrated binding between platelets and leukocytes in whole blood, the method described extensive sample preparation employing multiple washing steps (Rinder HM et al 1991b). Although many detailed observations were made about the response to agonists and kinetics of these interactions it remains unclear whether this pattern of interaction occurs in-vivo. The demonstration of increased platelet-neutrophil complexes in experimental models of systemic inflammation (Rinder CS et al 1992) suggests that analysis of such complexes may provide an additional means of assessing platelet activation both in-vitro and in-vivo. Hence the relevance of these interactions has yet to be fully determined, but communication between platelets and these inflammatory cells is likely to provide an important link between thrombosis and inflammation. This chapter describes the development of a simple and reproducible method for the measurement of platelet-neutrophil complexes (PNC) in whole blood by flow cytometry. This method is used to assess the contribution of platelet and neutrophil activation to the formation of these complexes and, when applied to in-vitro stimulation, suggests that free platelet flow cytometry alone may indeed underestimate the true nature of platelet activation as suggested above. This technique also allows simultaneous measurement of platelet and neutrophil antigens, providing a means of
examining platelet / neutrophil activation in the early stages of severe inflammation.

5.2. Materials and Methods

5.2.1 Materials

Monoclonal antibodies against CD62P, CD11b, CD42b, CD66b and isotype matched controls were purchased as described in Section 2.2. CD11/CD18 antibodies were kindly provided by Dr. N. Hogg, ICRF, London, UK and Dr M. Robinson, Celltech Therapeutics Ltd, Slough, UK. FACSlyse solution was purchased from Becton Dickinson.

5.2.2 Development of Method

The main consideration in the development of this method was that it should require minimal sample preparation to limit any artefactual platelet activation. This would also have the advantage that it could then be widely applied to clinical practice in order to refine the detection of platelet activation and to investigate any in-vivo role of platelet binding to neutrophils or other leukocytes.

The first investigation undertaken was to prepare blood as for free platelet flow cytometry (chapter 4) but with the addition of monoclonal antibodies against CD42b directly conjugated to PE (a component of the GpIb-V-IX platelet surface adhesion complex) and CD11b directly conjugated to FITC (the \( \alpha \) chain of the \( \beta 2 \)-integrin Mac-1 found on neutrophils). Flow cytometric analysis was performed on Becton Dickinson FACSan and FASCALibur. Data was then collected on FITC fluorescence at 515nm and PE fluorescence at 580nm.

When the leukocyte/erythrocyte population (figure 4.1) was examined there were distinct populations of cells visible which stained positive for CD11b
Platelet Neutrophil Interactions in Whole Blood

alone (neutrophils) and double positive for CD11b and CD42b (potential platelet neutrophil complexes) (figure 5.2).

Figure 5.2
Appearance of erythrocyte / leukocyte population after staining with CD11b:FITC and CD42b:PE. Distinct populations of neutrophils and potential platelet neutrophil complexes are visible. The events staining strongly for CD42b:PE are likely to represent homotypic platelet aggregates.
5.2.3 Refinement of Method

The use of whole blood diluted in buffer as in the previously described platelet method meant that relatively few neutrophils were present in the samples for analysis. Frequently those that were present were not always clearly visible due to the huge numbers of red cells swamping the flow cytometer leading to multiple simultaneous events which are automatically excluded from analysis by the flow cytometer. This process of 'aborting' simultaneous events which do not display a smooth peak in the parameter designated as 'threshold', (here forward scatter was used) acts to exclude from analysis chance coincident events, and to distinguish genuine heterotypic cell aggregates. Therefore a combination of the standard method of leukocyte flow cytometry (which employs red cell lysis and washing steps) and the previous platelet method was investigated. The commercially available FACSLyse (Becton Dickinson, UK) was employed to lyse the large red cell population by a pH dependent mechanism. In order to minimise sample preparation by avoiding a washing step (in a similar fashion to free platelet analysis) a minimum volume of FACSLyse required to achieve consistent lysis was used. Initial investigations showed that 200μl of FACSLyse consistently achieved excellent red cell lysis.

50 μl of blood was added to 5 μl of anti CD42b (PE) and 5 μl of anti CD11b (FITC) or isotype control antibodies within ten minutes of sampling. Following gentle mixing, the samples were left at room temperature for ten minutes before the addition of 200 μl of FACSLyse. Following gentle resuspension the samples were incubated for a further ten minutes before the addition of 500 μl of the platelet fix solution. Samples were analysed by flow cytometry within one hour of preparation.

Measurement of platelet-neutrophil complexes
Forward and side scatter measurements were made with gain setting in linear mode for the analysis of platelet / neutrophil interactions. The neutrophil
population was easily distinguished on this view (figure 5.3). The accuracy of the identification of neutrophils by forward and side scatter was confirmed by staining with an antibody to CD66b, a specific granulocyte surface marker. More than 98% of cells gated on these characteristics of size and granularity were CD66b positive. A total of 5000 - 10000 neutrophil events were collected from each sample. Results were compared to isotype matched (IgG1/IgG2a) antibody staining and considered positive if the fluorescence intensity exceeded that of 98% of the control antibodies (figure 5.4a). Events staining positive for both CD11b and CD42b were considered to represent PNCs and were distinguishable from events staining positive for CD11b alone (figure 5.4b). This method provided a flow cytometric profile that was relatively free from the unwanted cell intrusion into the gate for analysis. This profile was not improved by the addition of a single washing step to the preparation and so this was not adopted in order to preserve the approach of minimal sample preparation for both convenience and to limit artefactual activation.

Further attempts to refine the technique by varying the timing of fixation or the addition of azide (0.02%) as a metabolic inhibitor simply increased the amount of FACslyse or the time required for red cell lysis without altering the staining pattern seen.

In order to confirm that the occurrence of this double stained population did indeed represent platelet neutrophil complexes, confocal immunofluorescent microscopy was undertaken of a sample prepared in a similar manner. Anti-coagulated whole blood was stained with Propidium iodide (nuclear stain) and CD61:FITC, a component of platelet GpIIb/IIIa (figure 5.5), lysed with FACslyse solution and a single washing step undertaken to minimise the amount of cellular debris, red cell and free platelet 'contamination'. A single drop of the resuspended and fixed solution was then placed on a glass slide, covered and examined within one hour of preparation. Confocal rather than
standard immunofluorescent microscopy was attempted with the intention of defining the position of any adherent platelets to a neutrophil.

Figure 5.3.
Flow cytometer forward / side scatter profile for measurement of platelet / neutrophil complexes. This display shows the linear scales used for measurement of platelet / neutrophil complexes (c.f. figure 4.1). The neutrophil sized events are gated as shown. This sample is prepared by whole blood lysis without washing steps and therefore a large number of small events are seen corresponding to lysed red cell fragments.
5.2.4 Sample Collection

Blood was drawn via a 21G butterfly needle without the use of a tourniquet from healthy non-smoking adult volunteers who denied taking medication for at least two weeks. The first 2mls of blood was discarded, and a further 2mls taken and immediately added to 200μl of sodium citrate (3.8%).

5.2.5 Experiments

Multiple aliquots of blood taken from a single subject on each occasion were analysed after different conditions were applied. Investigations included assessment of %PNC in fresh whole blood, or following platelet stimulation with ADP, (final concentration of 10μM,), or thrombin (final concentration 0.4 units/ml in the presence of gly-pro-arg-pro 1mM to prevent fibrinogen cross-linking). The effect of neutrophil stimulation with 1μM fMLP (final conc.) was also investigated.

The influence of different anticoagulants on the numbers of PNCs at rest and following stimulation with ADP was investigated.

The effect of a delay in sample preparation was analysed at rest or following gentle mechanical agitation at 10 cycles per minute on a rotary shaker. The kinetics of PNC formation and free platelet CD62P expression following stimulation were investigated in the same manner.

Finally, the effects of blocking monoclonal antibodies to molecules known or suspected to be involved in the interactions between platelets and neutrophils were investigated. Purified anti-integrin antibodies were provided by Dr Nancy Hogg’s group (ICRF, London) which had been prepared from cell culture supernatants. The antibodies had been aliquotted and frozen and were defrosted immediately prior to use. Other studies in our laboratory had demonstrated activity of these blocking activities in several systems (Hogg et
al 1999). Blocking antibody direct against CD62P (G1) was a kind gift from Dr Rodger McEver (University of Oklahoma). The influence of prior incubation with these antibodies was investigated at rest and following the addition of ADP.

5.2.6 Statistics

All experiments were performed on at least three occasions. Data were analysed and expressed as mean, ± standard deviation and compared using paired t-tests or non-parametric tests as appropriate.
5.3. Results

5.3.1 Platelet-neutrophil complexes: Resting.

Analysis of samples from 27 healthy adults showed that 25%± 6% (mean, sd) of events within the neutrophil gate were positive for the platelet marker CD42b. These represent the percentage of neutrophils associated with platelets (%PNC). The effect of delay in sampling of freshly collected unstimulated blood was investigated in experiments in which %PNC was examined at intervals of up to 60 minutes after incubation on the bench at room temperature. No significant change in %PNC was detected until 45 min when an increase was apparent. Simultaneous examination of the same blood samples for CD62P on unbound platelet showed similar kinetics with significantly increased expression of CD62P by 60 minutes. In similar experiments in which the blood was agitated at room temperature throughout the incubation, little change was seen in CD62P expression despite an increase in %PNC formation (Data not shown). Although no significant changes were seen with a short delay in processing these initial investigations prompted subsequent experiments to be performed immediately on freshly sampled blood.

Figure 5.4. (overleaf)

Fluorescence profiles of a typical sample prepared for measurement of platelet / neutrophil complexes. Samples stained with (top panel) isotype control antibody (IgG1/IgG2a) and (middle panel) anti-CD11b (FITC) and anti-CD42b (PE). Events staining positive for both markers are those falling in the upper right quadrant which represent neutrophils with bound platelets (PNC), in this case 24% of the total neutrophils. The bottom panel shows the change in staining pattern one minute after the addition of ADP (10μM) when 43% of the total number of neutrophils are PNCs. Note that the PNC population is increased in total number following stimulation but also in mean CD42b expression (suggesting a greater number of platelets complexed to each neutrophil) and in mean CD11b expression.
Figure 5.4

- **PNC.001**
  - Control
  - IgG2a:PE vs. IgG1:FITC

- **PNC.002**
  - unstimulated
  - CD42b:PE vs. CD11b:FITC

- **PNC.003**
  - ADP 10uM
  - CD42b:PE vs. CD11b:FITC
The appearance of events staining positive for both neutrophil and platelet markers was confirmed to represent platelets in contact with the neutrophil surface on fluorescent microscopy (figure 5.5). Technical problems with the confocal microscopy software meant that plain immunofluorescent images only were available.

**Figure 5.5**
The appearance of platelet neutrophil complexes on confocal fluorescent microscopy. (43x) The polymorphous nucleus of the neutrophils stain orange (PI), and the smaller platelets green with CD61:FITC. Two platelets can be seen in close apposition to the neutrophil on the left and one to the neutrophil on the right. An unbound platelet is seen at the top of the image.
5.3.2 Platelet-neutrophil complexes: Platelet stimulation

Blood samples were collected as described and PBS or ADP (final conc. 10μM) was added to 'control' and 'stimulated' aliquots respectively. Samples were then analysed 1 and 30 minutes later after incubation at room temperature. In ADP stimulated samples there was a dramatic increase in the %PNC (figure 5.6). One minute after stimulation the mean percentage of neutrophils complexed to platelets rose to 45.3% ±14.2% (P<0.0001), and by 30 minutes had reached 67.9% ± 18.5%, (P<0.0001). Thrombin stimulation produced similar effects.

Previous studies (Rinder HM et al, 1991a) have indicated that there is a direct relationship between the intensity of expression of the platelet marker in the complex population and the mean number of platelets complexed to each neutrophil. This was therefore assessed by platelet CD42b MFI within PNCs. This revealed a significant increase in CD42b MFI in PNCs following stimulation with ADP compared to the resting state. At 1 min the CD42b MFI amongst PNCs after ADP stimulation was a mean of 3.5 times (95% confidence interval = 3.2 - 3.9) that amongst unstimulated PNCs. This remained elevated at 30 min (figure 5.7). The lack of discrete stepwise increments in the expression of PNC CD42b could be explained by adherence of platelet microparticles to neutrophils in addition to complete platelets. This is supported by recent observations (Forlow et al, 2000, Barry et al, 1999).

5.3.3 Platelet-neutrophil complexes: fMLP stimulated

Samples were also examined after stimulation with fMLP at a final concentration of 1μM. In contrast to ADP stimulation, there was no significant change in %PNC following incubation with fMLP (figure 5.6).
Figure 5.6
The changes in the percentage of neutrophils which are complexed to platelets after stimuli to platelet activation (ADP, thrombin) and neutrophil activation (fMLP). Values shown are the means and 95% confidence intervals (n =14 experiments ADP and fMLP stimulation and n =5 thrombin stimulation). The symbol * indicates statistical significance difference from unstimulated samples p<0.05, and ** indicates p<0.01.
Figure 5.7

Intensity of CD42b expression in PNC population. Mean values and 95% confidence intervals are shown. Previous studies in separated cell populations (Rinder HM, 1991a) have indicated that there is a direct relationship between the intensity of expression of the platelet marker and the mean number of platelets complexed to each neutrophil. Stimulation with ADP increased PNC CD42b MFI by factor of 3.5x control at 1 minute and 3x control at 30 minutes. fMLP causes a small but highly significant fall in CD42b MFI at 1 minute which is not apparent at 30 minutes. The symbol * indicates a statistically significant difference from unstimulated samples p<0.05, and ** indicates p<0.01. No symbol indicates no significant difference.
5.3.8 Kinetics of PNC formation and free platelet CD62P expression

Freshly sampled whole blood was prepared for examination for PNC and unbound platelet CD62P immediately (0 min) after collection. The same aliquot was then sampled at intervals following incubation at room temperature with ADP or thrombin for the analysis of PNC and CD62P.

The use of median fluorescence intensity to describe the pattern of CD62P staining has a theoretical advantage over the use of percentage positive fluorescence in that a separate population positive for CD62P was rarely seen. However, a number of reasons led us to adopt percentage positive fluorescence in this situation. Firstly, the literature to date overwhelmingly adopts this approach for assessment of platelet activation by expression of CD62P (but not by the level of expression of the active form of GpIIb/IIIa which is constitutionally expressed) including the original description of assessment of platelet activation by Shattil (Shattil et al, 1987) and in subsequent reviews of the subject from his group (Abrams et al 1991). This approach has subsequently been employed in many clinical studies and therefore use of %PF here allowed confirmation that our baseline data were similar to published data as well as increasing our confidence that artefactual platelet activation was not excessive in these experiments (Tschoepe et al, 1995 Collins et al, 1994, HM Rinder HM et al, 1994a, Rinder HM et al, 1994b, Janes et al, 1993, Warkentin et al, 1990). This would not be possible with MFI which is expressed in arbitrary units depending on the gain on each fluorescence detector. Secondly, there may be an increased risk of a type II error with MFI. The total platelet population is huge and the effect of an activated sub-population, e.g. 5-10% of the total circulating platelets, with a modest increase in intensity of staining for CD62P may approach the limit of detectability with the finite number of fluorescence detector channels on the flow cytometer. This may be a particular problem when a variable number of non-platelet events (e.g. cell fragments, dust) in the lowest fluorescence channels may inappropriately suppress the MFI to a greater extent than %PF.
This confounding factor can be limited by the use of freshly filtered solutions and careful setting of the platelet gate on the forward and side scatter and the co-staining with a platelet marker such as CD42b. In addition, it is interesting to consider that the level of CD62P expression is likely to be important in mediating adhesion to other cell types, particularly neutrophils. This is of particular interest in this experiment on the kinetics of platelet activation and PNC formation when the relationship between the numbers of platelets crossing this 'threshold' and the numbers of PNC is under investigation.

Figure 5.8
Kinetics of percentage platelet / neutrophil complex formation and % of platelets expressing CD62P following stimulation with ADP. The means of eight experiments and 95%ci are shown. In all cases there was a rapid rise in CD62P expression followed by a steady lower level of expression. In contrast, %PNC increased significantly by one minute and continued to rise throughout the study period in all cases.
The results are qualitatively similar if MFI CD62P is used rather than %PF. Unbound platelet CD62P expression increased significantly by 1 minute but plateaued thereafter (figure 5.8). This contrasts to the %PNC which continued to increase throughout the period of investigation. These experiments were performed with and without mechanical agitation. The difference between the profiles of CD62P expression and %PNC formation was exaggerated by mechanical agitation (figure 5.9 A-C).

![Graph showing CD62P expression and %PNC with time following ADP stimulation with agitation](image)

**Figure 5.9 A-C**

*The influence of mechanical agitation on the kinetics of percentage platelet-neutrophil complex formation and free platelet CD62P expression following stimulation.* Representative examples for each set of conditions is shown. Each experiment was performed on at least 3 occasions and gave qualitatively similar results. In all cases there is an early peak in CD62P expression at one min which is not sustained and tends to decrease subsequently. In all cases the %PNC increases by one min and continues to rise throughout the study period. The differences in the profiles of CD62P and
%PNC tended to be greater in the experiments which employed mechanical agitation (5.9 A,C) than those performed at rest (B, & Figure 5.7).

B) CD62P expression and %PNC with time following thrombin

C) CD62P expression and %PNC with time following thrombin stimulation with agitation
5.3.5 Mechanisms of Platelet / Neutrophil Interactions

During the development of the method the influence of different anticoagulants was investigated. Single blood samples were collected and aliquotted into the standard sodium citrate (final conc. 0.38%) as used for platelet flow cytometry, heparin (final conc. 1 to 10 units/ml) or EDTA (final conc. 1mM). The baseline levels of %PNC were recorded as was the response to ADP stimulation (figure 5.10.) When blood was collected into EDTA the increase in %PNC formation observed in response to ADP was greatly attenuated (n=6, mean increase = 2.6%, not significant).

![Figure 5.10](image)

**Figure 5.10**
The influence of anticoagulant on %PNC detected at rest and following ADP stimulation. Means and 95% ci of 5 experiments. ** indicates p<0.01

In citrate and heparin anticoagulants a significant increase from unstimulated level levels of %PNC following ADP stimulation. Note that EDTA anticoagulation completely prevents the increase in %PNC in response to ADP. Blood collected into sodium citrate with the addition of epoprostanol 10ng/ml which acts to limit platelet activation, behaved in a manner similar to EDTA. In unstimulated samples no change was apparent from samples anticoagulated with citrate alone, but no significant change in %PNCs was detectable following stimulation (data not shown).
Co-incubation with anti-CD62P antibody (G1), also inhibited new complex formation in a dose dependent manner (figure 5.11). Antibodies to CD11/CD18 had no demonstrable effect in this system. The efficacy of these antibodies against CD11a, CD11b and CD18 had been demonstrated previously in our laboratory in experiments investigating normal and abnormal β-2 integrin function (Hogg 1999). The resting percentage of PNC was not affected in these experiments.

Figure 5.11.
Influence of co-incubation with CD11a/CD11b/CD18 and CD62P blocking monoclonal antibodies. No significant effect on the baseline level of PNC formation was demonstrated with any antibody. Only G1 against CD62P influenced the formation of new PNCs in a dose dependent manner. A representative experiment is shown.
5.4 Discussion

In this chapter a novel method for analysing platelet activation and investigating platelet / neutrophil interactions in whole blood has been described.

Using this method, approximately 25% of neutrophils were shown to be complexed with platelets in 27 healthy adults at rest. This level of detectable complexes was consistent despite manipulations to minimise the potential for artefactual platelet activation such as collection of blood into epoprostanol.

Following specific platelet stimulation there was a rapid and sustained increase in complex formation as shown by both the percentage of total neutrophils in complexes and the intensity of CD42b staining in the PNC population (which may relate to the number of platelets adhering to each neutrophil). Experiments employing automated cell sorting and microscopy to support the suggestions made here concerning the variable number of platelets attaching to each neutrophil are planned. Such data would greatly increase the confidence with which these results can be interpreted.

The existence of platelet neutrophil complexes has been demonstrated in several studies, (Spangenberg, 1994; Rinder HM et al 1991) irrespective of the methodology employed. To date, studies examining neutrophil / platelet interactions have largely used separated cells or repeatedly washed cells and have recorded PNC values of between 3.6% (de Bruijne Admiraal et al 1992) and 34% (Rinder et al 1991a). In a previously described method for assessment of PNCs in whole blood, Rinder (Rinder HM et al 1991b) found a resting level of 39% PNC formation. This is of the same order of magnitude as that found in the present study. Following publication of the data included in this study (Peters et al 1997) a similar method described levels of PNCs in whole blood (Li et al 1997) which again were similar to those presented here.
The mechanisms of PNC formation have emerged in recent years and it is now known that platelet derived CD62P is a key molecule involved in platelet neutrophil adhesion (de Bruijne Admiraal et al. 1992; Larsen et al. 1989). Contained in the alpha granules of resting platelets, CD62P is translocated to the surface upon activation, allowing binding to counter-receptors on the surface of neutrophils. In agreement with previous work, we have shown using this system that CD62P is central to platelet neutrophil complex formation. This is also supported by the increase in PNC in response to platelet agonists and the inhibition of PNC in the absence of divalent cations (following EDTA anti-coagulation) which are essential for selectin adhesive function. It is of particular note that EDTA and CD62P blocking antibody prevented the formation of new complexes in response to ADP but did not reduce the baseline levels detected. This may reflect prior in-vivo formation of this baseline level of complexes (and perhaps stabilisation by additional adhesive mechanisms) rather than another mechanism being responsible for the initiation of these complexes. Further investigations with blocking antibodies against neutrophil PSGL-1 are planned to further understand the initial adhesion events in this system.

The role of neutrophils in these interactions is less clear. There is evidence that neutrophil stimulation can influence platelet neutrophil binding with reports of both increased (Rinder CS et al. 1992) and decreased (Lorant et al. 1995a; Rinder HM et al. 1994a; Evangelista et al. 1993a) complex formation. This may be due to the release of granule contents of both cell types in response to neutrophil stimulation. In this study we were unable to demonstrate a major role for neutrophils in the initiation of PNC formation. Neither neutrophil stimulation nor co-incubation with antibodies to CD11/CD18 caused a significant change in the percentage of neutrophils associated with platelets in this system. However neutrophil stimulation did result in a small but consistent decrease in the number of platelets bound to each neutrophil. In view of the recent evidence that neutrophil binding to (and transmigration across) immobilised platelets requires both CD62P and CD11b/CD18
(Diacovo et al 1996b) and that under shear stress conditions there is requirement for both CD62P and β2-integrin functions for the formation of stable PNCs, (Evangelista et al 1996) it is clear that the mechanisms involved in mediating platelet neutrophil interactions are affected by both the physical state and experimental conditions employed for examining these cells.

In studies using separated cells, activated neutrophils have been shown to activate platelets, causing the release of platelet granule contents (Evangelista et al 1993) and formation of platelet aggregates (Chignard & Renesto, 1994). There is also evidence that neutrophil release products such as proteases may influence platelet neutrophil binding (Chignard & Renesto, 1994). Such effects were not investigated in this chapter but of note is the observation that activation of neutrophils with fMLP did not affect %PNC in this system. Further studies are required to elucidate the reason for the discrepancies between the whole blood system described here and separated cell systems used in previous studies. Further investigations of the consequences of neutrophil activation on PNC formation are presented in chapter 8.

Platelets that are not adherent to other inflammatory cells and remain free in the circulation may not actually reflect the overall state of platelet activation. In experiments comparing free platelet expression of CD62P and %PNC formation over time following a stimulus to activation, there was a continued increase in %PNC following stimulation. In contrast, free platelet CD62P expression reached an early peak and then remained at the same level or fell for the remainder of the experiment in concordance with Rinder's (Rinder HM et al 1991b) previous studies. The experiments in which samples were continually agitated demonstrated the opposing trends in CD62P expression and %PNCs formation more clearly than the incubations at rest. This phenomenon can be explained if unbound activated platelets are randomly meeting and progressively binding to neutrophils over the time course of the experiment. This would have the effect of increasing the %PNCs whilst at the
same time removing the activated platelets from the unbound platelet population. As a result, unbound and therefore less activated platelets, analysed by conventional flow cytometry, would not necessarily reflect changes in the activation status of the platelet population overall.

Underestimation of platelet activation as a result of binding to other cells was considered in the original descriptions of platelet flow cytometry by Shattil (Shattil et al. 1987). This was discounted as the percentage of particles staining positive for platelet markers outside the unbound platelet gate did not change following stimulation. However, the method used in those studies ignored the possibility that multiple platelets may, and indeed are likely to, adhere to a single neutrophil (Rinder HM et al. 1991a). This complex (PNC) would then be recorded as a single event leading to an inevitable underestimation of the proportion of platelets falling outside the unbound platelet gate. The percentage of the total platelet number that are actually bound to other cells may therefore be considerably in excess of the 5% figure estimated by Shattil (Shattil et al. 1987). In addition, there may be in excess of 100 times more circulating platelets than neutrophils in blood. Therefore, a small change in the percentage of platelet events outside the free platelet gate may actually reflect an enormous change in % of PNC. This may correspond to a highly activated sub-population of the total platelet pool that may be of physiological importance.

The method described here is a simple but effective way of assessing platelet interactions with neutrophils. From each sample it is possible to measure the percentage of total neutrophils complexed with platelets, to estimate the relative number of platelets in association with each neutrophil (CD42b mean fluorescence amongst PNC complexes), and to elucidate neutrophil activation status (CD11b expression). Important further work includes the determination of the relationship between doses of agonists and PNC formation and the effect of combined stimulation of both platelets and neutrophils. These questions are addressed later in this thesis in chapter 8.
The assessment of PNCs may provide important additional information to supplement that available with free platelet flow cytometry alone. The technique is also ideally suited to the investigation of the molecular mechanisms involved in platelet / neutrophil interactions. The very small volumes of blood required allow for repeated sampling of individual patients to investigate the significance of platelet-neutrophil complexes in the pathogenesis of conditions such as acute sepsis, the acute respiratory distress syndrome and systemic inflammation following cardio-pulmonary bypass.
Chapter 6

Function of Platelet-Neutrophil Complexes

6.1 Introduction

6.2 Methods

6.3 Results

6.4 Discussion
6.1 Introduction

Evidence for the close links between the complex multi-cellular processes of haemostasis and inflammation has been presented in previous chapters. It has been established that inflammatory cytokines such as TNF-α and IL-1 can induce a pro-thrombotic state. Conversely, key components of the coagulation pathways, such as thrombin, have now been shown to contribute to inflammation by inducing IL-6 and IL-8 release from monocytes and endothelial cells (Johnson et al 1998).

The capacity for platelets to influence the haemostatic/inflammatory axis may rely upon direct contact with inflammatory cells. In chapter 5 heterotypic aggregation of platelets to neutrophils was described. Indirect evidence that these complexes may themselves have a physiological function is provided by studies which have shown changes in the numbers of, or capacity to form these heterotypic cell complexes in clinical conditions in which thrombosis and inflammation are prominent features (Peters et al 1998; Gawaz et al 1997; Serrano, Jr. et al 1997; Ott et al 1996). However, the functional consequences of platelet-neutrophil complex (PNC) formation have not been investigated.

This chapter employs the methods described in chapter 5, to investigate the function of PNCs.


6.2 Methods

6.2.1 Whole Blood Stimulation

PNCs were investigated as previously described in chapter 5. Monoclonal antibodies were purchased as described in chapter 2.2. Antibody 24 (Mab24) was a gift from Dr Nancy Hogg, ICRF, London and was conjugated to FITC.

Blood was collected as described in preceding chapters. Whole blood stimulation experiments were performed immediately after sampling as previously described. Blood was divided into aliquots of four ml and stimulated with ADP (final conc. 10µM,) fMLP (final conc. 1µM) or left as control. Samples were then analysed one and 30 min later after incubation at 37°C with gentle mechanical agitation on a rotary shaker at 10 cycles/minute.

Flow cytometric analysis of the neutrophil population was undertaken as previously described. Care was taken to minimise compensation errors by the analysis of samples stained with single fluorochromes and adjustment of compensation settings. Any overlap of green (FITC) emissions into the red (PE) wavelength detector (or vice versa) could be subtracted from the recorded value for each fluorescence intensity. This process was performed prior to each two colour analysis. In addition, weekly calibration checks of the flow cytometer were undertaken with latex beads with known amounts of fluorochromes (FITC, PE and PerCP) attached (CaliBrite3 Beads, Becton Dickinson, UK). The requirement for compensation for overlap could was therefore constantly monitored and faults in the filters, detectors or Cellquest software would be promptly detected. Median values for compensation required for colour overlap using these beads during the course of these studies were FL-1-%FL-2 0.9 (range 0.4-1.3), FI-2-%FL-1 21.7 (20.2-23.4) FL-2-%FL-3 0 (0-0) and
FL3-%FL-2 18.3 (17.0-19.7). All these calibrations performed during this study fell well within the ranges proscribed by the manufacturer.

The intensity of binding of antibodies to CD11b, CD62L and the activated binding site of Mac-1 (Mab24) were analysed in the whole neutrophil population and in the subset of neutrophils displaying binding of monoclonal antibodies against the specific platelet marker CD42b. The characteristics of this PNC population could then be compared to the unbound neutrophil population.

### 6.2.2 Phagocytosis Study

A suspension of killed *N. meningitidis* organisms in 0.5ml in RPMI without phenol red at an $A_{540}$ of 1 were mixed with an equal volume of FITC (1mg/ml in RPMI w/o phenol red) and incubated at 37°C for one hour as previously described (Klein *et al* 1996). The organisms were then spun at 9780g for one min, and washed twice with 0.5ml RPMI before resuspension in RPMI without phenol red at an $A_{540}$ of 1. 200 µl of this suspension was then added to four ml of anti-coagulated whole blood which was incubated with gentle mechanical agitation for 60 min. 50 µl samples were added to FACS tubes containing CD42b:PE and prepared as above. Analysis was performed as above and the relative percentage of neutrophils phagocytosing bacteria assessed in the PNC and unbound neutrophil populations.

### 6.2.3 Oxidative Burst

Oxidative burst was assessed by examining the capacity of neutrophils to oxidise dihydrorhodamine 123 (DHR) to the fluorescent dye rhodamine 123 as previously described (Vowells *et al* 1995). Briefly, 300 µl of heparinised whole blood was added to a number of FACS tubes to each of which 4ml of warmed (37°C) red cell lysis buffer were added (150mM
NH₄Cl, 20mM NaHCO₃, 1mM EDTA, pH 7.2). After gentle mixing, and five min incubation all erythrocytes were completely lysed. The tubes were centrifuged at 2200 rpm, the supernatants removed, and the pellets resuspended in HBSS +5% FCS. A further washing step wash performed before resuspension of the pellet in 400μl of HBSS + 5% FCS. To this 1.8μl of DHR (29mM solution in DMSO) was added and 20μl of PerCP conjugated monoclonal antibody against CD61 which is a component of the platelet adhesion molecule IIb/IIIa (or isotype matched control) and the samples incubated for 10 min at room temperature. Samples were then stimulated with ADP (final conc. 10μM) or left as controls and analysed without fixation on the flow cytometer. Rhodamine fluorescence was recorded at 515nm and PerCP fluorescence above 650nm.
6.3 Results

6.3.1 Adhesion molecule expression

At rest, all neutrophils expressed CD11b and CD62L. Neutrophil activation with fMLP induced a significant increase in CD11b expression, and decrease in CD62L expression. Following incubation of whole blood with the platelet agonist ADP, there were also significant increases in neutrophil CD11b expression detected after both 1 and 30 min. Similarly, ADP stimulation led to a fall in neutrophil CD62L that was significant after 30 min (figure 6.1A). These data indicate that a specific stimulus to platelet activation such as ADP induces changes in neutrophil activation that are detectable in the whole neutrophil population.

As described above, examination of the neutrophils from whole blood stained with CD42b:PE and CD11b:FITC allowed the detection of two populations of cells: free neutrophils and platelet-neutrophil complexes (PNCs). fMLP stimulation increased the intensity of CD11b staining in both PNCs and free neutrophils (ratio PNC CD11b MFI / free neutrophil CD11b MFI =1.0). However CD11b was expressed at higher levels in PNCs than free neutrophils in unstimulated samples (1 min: ratio =1.12 p<0.05), and following ADP stimulation (1 min ratio =1.18 p<0.05) (figure 6.1 and 6.2).

Following whole blood stimulation with fMLP for 30 min, CD62L (L-selectin) was only just detectable, and therefore no comparison could be made between PNCs and free neutrophil MFI. However, less CD62L was expressed on PNCs than free neutrophils in unstimulated (30 min, ratio =0.87, p<0.01,) or ADP stimulated samples (30 min, ratio =0.85 p<0.01), (figure 6.1 and 6.2). This finding of decreased CD62L expression in the CD42b positive population could not be attributable to under-compensation for overlapping fluorochrome emission spectra.
Figure 6.1

Neutrophil adhesion molecule expression. A The specific platelet agonist ADP causes a significant increase in CD11b expression and a significant fall in CD62L that is detectable when the whole neutrophil population is assessed. B Ratios of the level of CD11b or CD62L in PNC to free neutrophils are shown. PNCs express more CD11b, and less CD62L than unbound neutrophils at rest and after ADP stimulation. Means of at least 3 experiments and 95% ci are shown.

◊ = CD62L MFI after 30 min stimulation with fMLP was only just detectable. (*=p<0.05, **=p<0.01 compared to control in A, or compared to unity, i.e. no difference between N and PNC, in B)
Figure 6.2.
Example of fluorescence profiles for adhesion molecules in free and PNC neutrophils. Events displaying positive CD42b staining in the neutrophil population on forward and side scatter are considered to represent PNCs. The profiles shown are following ADP stimulation of whole blood and the figures are the MFI for the respective antibodies and populations. There is more CD11b A) and Mab24 C) and less CD62L B) binding in PNCs than free neutrophils. These changes are most marked in the populations that are most strongly positive for CD42b. A small population that are CD42b positive but CD62L negative do not contribute the significantly to the recorded values.
ADP is not reported to influence neutrophil adhesion molecule expression directly and ADP receptors are not reported on neutrophils (Gachet 1997). Simple experiments were performed to exclude this possibility of ADP causing direct neutrophil activation in this study. A comparison of the changes in neutrophil CD11b expression seen with ADP stimulation in whole blood (as described above) and in separated neutrophils (see chapter 2) was undertaken (figure 6.3). These experiments were performed on 3 occasions, and qualitatively similar results were obtained. As with the studies presented above in figure 6.1, ADP (or the potent platelet agonist thrombin) caused an increase in CD11b staining in whole blood. In separated cells, unstimulated CD11b levels are somewhat higher than in whole blood as a result of inevitable neutrophil activation during separation, (CD11b MFI = 184 at 30 min), but neither ADP (CD11b MFI = 170 at 30 min) or thrombin (CD11b MFI = 120 at 30 min) caused an increase in CD11b expression. A positive control was provided by fMLP stimulation which did increase CD11b above the unstimulated level (CD11b MFI = 248 at 30 min).

Another potential artefactual cause of these results was considered. Each PNC, as detected by the flow cytometer, must include at least one neutrophil and one platelet. We and others believe that many platelets can bind to a single neutrophil. However it is also possible that some PNCs could contain more than one neutrophil and these would still be counted by the cytometer as a single event. Such complexes would be likely to demonstrate higher levels of adhesion molecule expression (as well as other neutrophil functions) by virtue of the two or more neutrophil functions or surface antigens being assessed as a single event. This possibility was addressed by reviewing the forward scatter profile (related to particle size) plotted against the parameters under investigation. We did not observe a distinct population with high forward scatter and fluorescence staining. This was confirmed by comparing the CD11b expression in the top half of the forward scatter population with the bottom
half. No significant difference in CD11b expression was detectable between the different forward scatter populations (mean difference high forward scatter-low forward scatter =3.9 units, p=0.10, paired t-test, n=16).

Furthermore, the observation of lower levels of CD62L amongst PNCs also suggests that any contribution made by larger aggregates must be small.
Figure 6.3
Comparison of the changes in CD11b expression with platelet agonists in whole blood and separated neutrophils. A significant change in neutrophil CD11b expression is apparent within 1 min of ADP, thrombin or fMLP stimulation in whole blood. These changes persist to 30 min. There is no change in neutrophil CD11b above control levels with platelet agonists in separated neutrophils though the response to fMLP remains intact.
6.3.2 Adhesion molecule activation

The monoclonal antibody, Mab24, directed against the activation dependent binding site of CD11b/CD18, binds to unstimulated neutrophils at very low levels. Neutrophil activation with fMLP increased median fluorescence intensity compared to unstimulated samples (mean increase 2.6 fold, \( p<0.001 \)) after 1 min and further by 30 min (3.6 fold, \( p<0.001 \)) (figure 6.4A). Incubation with ADP also increased Mab24 binding (1 min, 1.4 fold \( p<0.05 \)). As observed with the levels of CD11b, Mab24 binding was greater on PNCs than on free neutrophils (figure 6.2 and 6.4B) in both unstimulated (ratio PNC/N Mab24 MFI = 1.13, \( p<0.05 \)) and ADP stimulated (1 min ratio = 1.3 \( p<0.05 \)) whole blood. There was no difference between Mab24 binding to PNCs or free neutrophils following fMLP stimulation (1 min ratio = 1.0, \( p=0.86 \))

A

B

Figure 6.4

Neutrophil Mac-1 activation. There is a rapid and significant increase in Mab24 binding in the whole neutrophil population A after ADP stimulation. B PNCs bind more Mab24 than unbound neutrophils at rest and following ADP stimulation. Means of at least 3 experiments and 95\% ci are shown. (\( *=p<0.05, **=p<0.01 \) compared to control in A, or compared to unity , i.e. no difference between N and PNC, in B).
6.3.3 Influence of CD62P Blockade

The changes in adhesion molecules of the whole neutrophil population observed with ADP stimulation were assessed in the presence of the blocking monoclonal antibody CD62P (G1) (figure 6.5) or isotype matched control. Data are presented for the 30 min time point, as the number of conditions being examined with the addition of G1 inhibition meant that small differences in the timing of sampling may have occurred at a one min time point. A 10 second variation in the time of sampling is likely to be insignificant in the context of a 30 min stimulation but may be a very important error in 60 seconds. G1 prevents the formation of PNCs as previously described Chapter 5. A significant reduction but not abolition of the ADP-induced changes in CD11b expression was observed (figure 6.5). The ADP induced changes in Mab24 binding were completely inhibited by the presence of G1 antibody but the changes in CD62L expression were not altered significantly.

Figure 6.5. (overleaf)
Influence of platelet-neutrophil contact on platelet-induced changes in neutrophil adhesion molecules. The changes in adhesion molecules of the whole neutrophil population with ADP stimulation in the presence of the CD62P blocking antibody (G1) were assessed. The ADP-induced increase in CD11b expression was significantly attenuated but remained greater than control (A). The ADP-induced changes in Mab24 binding were completely inhibited by the presence of G1 antibody (B) but the changes in CD62L expression were not altered significantly (C). Means and 95% confidence intervals of 4 or more experiments are shown. (*=p<0.05, **=p<0.01 by paired t-tests). The 30 min time point is shown in order to reduce the possible contribution of any small effects of differences in timing that might contribute to changes observed at 1 minute.
Figure 6.5

A

Unstimulated

Unstimulated + G1

ADP

ADP + G1

30 min: CD11b MFI Sample / Unstimulated

B

Unstimulated

Unstimulated + G1

ADP

ADP + G1

30 min: Mab24 MFI Sample / Unstimulated

C

Unstimulated

Unstimulated + G1

ADP

ADP + G1

30 min: CD62L MFI Sample / Unstimulated
6.3.4 Phagocytosis

In order to investigate the phagocytic capacity of PNCs, an existing model of whole blood phagocytosis utilising *Neisseria meningitidis* was employed (Heyderman *et al* 1999). After 60 min incubation of freshly sampled whole blood with FITC labelled killed *Neisseria meningitidis Group B*, neutrophil phagocytosis could be clearly detected. We observed greater phagocytosis of the micro-organism amongst PNCs than in the free neutrophil population (ratio at 60 min, 1.5x, 95%ci =1.38-1.62 p<0.001,n=34) (figure 6.6).

As with the changes seen in adhesion molecules, ADP led to increased phagocytosis (mean % phagocytosis after ADP =1.5x control 95% ci 1.2-1.8, p<0.01). This significant increase was not inhibited by co-incubation with G1 blocking monoclonal antibody directed against CD62P (figure 6.6).
Comparison of PNC and free neutrophil phagocytosis. Representative examples of PNC/N phagocytosis of *Neisseria meningitidis* after 30 min are shown. Bacteria are stained with FITC. A higher proportion of PNCs phagocytose bacteria than unbound neutrophils (ratio PNC/N =1.4). Total phagocytosis is increased following ADP stimulation (32% vs. 23%) B compared to A unstimulated. Co-incubation with G1 antibody had no consistent effect on the total amount of phagocytosis in unstimulated C or ADP stimulated samples D, despite decreases in the %PNC detected.
6.3.5 Neutrophil Superoxide activity

As previously described, PMA caused approximately a ten fold increase in fluorescence intensity reflecting the oxidation of DHR to fluorescent rhodamine 123 by reactive oxygen species (data not shown).

In unstimulated samples, greater fluorescence was detected in PNCs than in free neutrophils (ratio DHR MFI PNC/N =1.69, p<0.001). This effect was further enhanced by platelet stimulation with ADP (ratio =1.74, p<0.001) (figure 6.7A, C). Indeed, the fluorescence intensity of the whole neutrophil population was significantly increased by co-incubation with ADP (figure 6.7B). G1 monoclonal blocking antibody against CD62P reduced PNC formation and also reduced rhodamine 123 fluorescence in both unstimulated and ADP stimulated samples (figure 6.7B,C).

In order to investigate the possibility that these observations may be the result of toxic oxygen metabolite production by platelets (Yeaman, 1997) purified platelets were studied in this assay. PMA induced changes in auto-fluorescence but did not induce a detectable oxidative burst (data not shown).
Oxidative burst activity.
A) The DHR median fluorescence for the whole neutrophil population was significantly increased by ADP stimulation. (*=p<0.01 vs. unstimulated control) This effect is completely abolished by the presence of the G1 blocking monoclonal antibody against CD62P (**=p<0.01 vs. no G1). G1 inhibition of control levels of DHR MFI suggests that background platelet activation was present and contributing to oxidative burst activity before ADP was added.

B) There is significantly greater oxidative burst activity in PNCs than unbound neutrophils. (Ratio PNC/N DHR MFI >1.0 at rest and following ADP stimulation, P<0.01). The addition of ADP increases PNC oxidative burst further. (means and 95%ci of 3 experiments are shown).
C). Example of neutrophil DHR fluorescence.

The DHR fluorescence is greater amongst those neutrophil which also stain positive for the platelet marker CD61:PerCP. The second panel displays the intensity of DHR fluorescence in a sample incubated with the G1 blocking monoclonal antibody against CD62P. There are fewer PNCs and less total DHR fluorescence.
Figure 6.8

The influence of G1 blocking antibody on %PNC and neutrophil and PNC DHR fluorescence. The results of a single representative experiment of three performed with 3 replicates are shown, 30 min after stimulation with ADP. (A) It can be seen that G1 causes a marked decrease in the %PNC in both control and stimulated samples. (B) The DHR fluorescence of unbound neutrophils is not altered by the presence of G1 but (C) there is a decrease in PNC DHR fluorescence towards the values seen in free neutrophils.

6.4 Discussion

Data describing the potential for soluble platelet factors or membrane components to induce neutrophil activation (Nagata et al 1993; Del Maschio et al 1989) led to the hypothesis that PNCs represent a circulating population of activated or primed inflammatory cells. The data presented in this chapter are consistent with this hypothesis and indicate that neutrophils within platelet-neutrophil complexes appear to be more activated than unbound neutrophils. In unstimulated whole blood, neutrophils with platelets attached displayed a more 'activated' pattern of adhesion molecule expression, produced more superoxide and ingested...
more bacteria than neutrophils which were not associated with platelets. These findings are indicative that neutrophils within platelet-neutrophil complexes represent an activated sub-set of cells. In addition, it can be inferred that the platelet component of PNCs must also represent an activated sub-population of platelets, as CD62P expression is an essential pre-requisite for PNC formation (Evangelista et al 1993; de Bruijne Admiraal et al 1992).

To investigate the effect of platelet stimulation on neutrophil phenotype and function, experiments were performed in which whole blood was incubated with ADP. Platelet stimulation led to an exaggeration of the profile observed in unstimulated blood for all of the parameters studied. Therefore following ADP stimulation, the entire neutrophil population was found to express slightly more CD11b/CD18, less CD62L, more antibody 24 binding sites, produce more superoxide and phagocytose more N. meningitidis. These results extend the findings from previous studies, which show that platelet activation is a potent stimulus for both PNC formation and neutrophil function (Chignard & Renesto, 1994; Hamburger & McEver, 1990). However, the observed changes in the expression of CD11b or activation of Mac-1 (Mab24 binding) are small and the functional consequences of changes of this size is uncertain. Previous studies have not shown differences in the levels of CD11b/CD18 expression on adherent and non-adherent neutrophils (Klein et al, 1993)(Finn & Rebuck, 1994) and suggest that the activation status of this molecule may be a more important factor.

One important factor that is apparent from inspection of the scatterplots is that the most marked increase in CD11b and Mab24 staining (and capacity for oxidative burst) was seen in the highest CD42b staining sub-populations (figure 6.2 and 6.7). One explanation would be inadequate fluorescence overlap compensation, despite the measures taken to avoid this (although this would not explain the DHR fluorescence profile as this
does not overlap with the emission spectrum of PerCP). In addition, the appearance of the more activated neutrophils with lower CD62L expression in the highest CD42b staining population cannot be explained by inadequate compensation. An alternative explanation is that the more activated neutrophil profile occurs to the greatest extent in those neutrophils that have the greatest level of co-staining for CD42b. We have discussed in chapter 5 that there is some evidence for the PNCs that stain most strongly for CD42b containing the greatest number of platelets per neutrophil. Therefore, this appearance could also represent a 'dose effect' whereby the neutrophils complexed to more platelets have the most activated profiles.

In both resting and ADP stimulated blood, the observed phenotypic and functional changes were associated with an increased number of PNCs. It is tempting to postulate that heterotypic adhesion between platelets and neutrophils is a pre-requisite for the neutrophil changes seen in this study. However, experiments using the CD62P blocking antibody, G1, indicate that the relationship between PNC formation and neutrophil activation is more complex. Incubation with G1 antibody not only markedly diminished the number of PNCs formed in response to stimulation, but also completely inhibited the platelet induced changes in Mab24 binding and superoxide production. For these parameters, it would appear that CD62P is absolutely necessary. This is consistent with previous studies in which platelets have been shown to increase neutrophil superoxide anion production (Ruf & Patscheke, 1995), via soluble CD62P (Nagata et al 1993).

Platelet-induced changes in CD11b and CD62L expression were only partially inhibited by G1. These findings indicate that in addition to CD62P, other factors also contribute to platelet mediated neutrophil adhesion molecule expression in whole blood. Platelet derived platelet activating factor (PAF) (Chignard & Renesto, 1994), β-thromboglobulin (Walz &
Baggiolini, 1989) and IL-1 (Hawrylowicz et al 1991) have all been shown to induce inflammatory cell activation. These and/or other mediators are likely to be involved in the changes observed in neutrophil adhesion molecule expression and would explain how platelet activation could effect the entire neutrophil population and not just those complexed to platelets.

ADP stimulation of blood increased the capacity of neutrophils to phagocytose *N. meningitidis* by 50%. This was associated with a similar increase in the ratio of PNCs containing bacteria compared to uncomplexed neutrophils. Purified P-selectin has been shown to modulate CD11/CD18 function to promote washed neutrophil phagocytosis of labelled zymosan particles (Cooper et al 1994) and platelets have been reported to increase mouse macrophage phagocytosis of Salmonella (Mandell & Hook, 1969). However, we could not detect any effect of G1 on the percentage of neutrophils which had phagocytosed the bacteria, indicating that platelet - neutrophil heterotypic adhesion is not required for platelet enhancement of phagocytosis. This raises the question as to why there was such a dramatic over-representation of phagocytosis within the PNCs.

This may be explained by considering the role of neutrophils integrins in complex formation. A number of groups have observed the capacity of immobilised platelets to bind leukocytes (including neutrophils) via a multi-step selectin and β2 integrin dependent mechanism (Kuijper et al 1997; Diacovo et al 1996; Kuijper et al 1996; Buttrum et al 1993) analogous to that which occurs between activated endothelium and leukocytes. Platelet CD62P, platelet derived PAF and neutrophil β2-integrin activation have recently been described as requirements for maximal adhesion of neutrophils on immobilised platelets (Evangelista et al 1996). This requirement for selectin and integrin involvement in PNC formation has also been demonstrated in mixed cell conjugates under dynamic conditions (Ostrovsky et al 1998).
*Neisseria meningitidis* has previously been shown to be a potent stimulus to increased \( \beta_2 \) integrin expression (Klein *et al* 1996). Furthermore, exogenous stimuli including fMLP and endotoxin further enhance neutrophil integrin expression and phagocytosis in whole blood (Heyderman *et al* 1999). Therefore, it seems probable that soluble platelet release products may act in a similar fashion and are responsible for the ADP induced increase in phagocytosis seen in this study. In this situation, the over-representation of phagocytosis in PNCs may simply reflect the stabilisation of platelet-neutrophil complexes which occurs in the presence of increased \( \beta_2 \) integrin activation as a result of neutrophil/bacterial contact. This suggests that neutrophil adhesion molecules may be important in the formation of PNCs.

These observations in whole blood are consistent with previous studies using separated cells and suggest a functional role for PNCs in vivo. Irrespective of the requirement of heterotypic adhesion for enhancing neutrophil function, the fact that PNCs contain the most activated neutrophils and platelets within the blood may facilitate both cell types to perform their respective functions in response to inflammatory stress or changes in local blood flow. These results indicate that PNCs could be amongst the most adhesive cells in the circulation. As such, they may also have the greatest capacity to bind to activated vascular endothelium. This may explain the recent data from adults with severe endothelial dysfunction associated with multi-organ failure in whom low circulating PNCs were found to be correlated with a poor outcome (Gawaz *et al* 1997). This study provides further support for a role for PNCs in both health and disease. It is tempting to consider these activated sub-populations of inflammatory cells as targets for immunotherapy.
Chapter 7

Platelets and the Vascular Endothelium.

7.1 Introduction

7.2 Methods

7.3 Results

7.4 Discussion
7.1 Introduction

In chapter 4 the interactions of the most activated platelets with other cells was raised as a possible explanation for the lack of platelet activation detectable with free platelet flow cytometry. This possibility was further investigated in chapter 5 with the observations of platelet neutrophil complex formation. Interactions of activated platelets with the vascular endothelium or sub-endothelial matrix particularly in the pulmonary micro-vasculature of ARDS patients was also proposed as an explanation for the results of the studies in chapter 4. The capacity of platelets to interact with damaged vascular endothelium is well described (Roth, 1992). The mechanisms by which platelets roll and firmly adhere to exposed adhesive proteins in the sub-endothelial matrix as a primary event in haemostasis are complex and are reviewed in chapter 1. Recent studies have demonstrated that activated platelets may also bind to the intact vascular endothelium, principally via the action of activated GpIIb/IIIa complex binding to adhesive proteins which are also bound to endothelial adhesion molecules (figure 1.10) (Bombeli et al 1998; Li et al 1996). Apoptotic endothelial cells have now been shown to bind even resting platelets (Bombeli et al 1999).

In order to test the original hypothesis that platelets contribute to the processes of acute inflammation such as are seen in ARDS, therefore, the consequences of platelet binding to or co-incubation with the vascular endothelium was further investigated. A direct contribution of platelets to changes in endothelial function in inflammation is supported by observations that activated platelets express IL-1 (Hawrylowicz et al 1989) which can induce endothelial adhesion molecule expression (Hawrylowicz et al 1991) and IL-8 production (Kaplanski et al 1993). Direct platelet-endothelial interactions suggest that platelets may be able to accelerate recruitment of the well recognised effector cells of the early inflammatory response, neutrophils and monocytes.
This chapter aims to investigate the capacity of platelets to influence directly the role of the endothelium in controlling leukocyte adhesion and recruitment. The hypothesis investigated is that activated platelets accelerate the recruitment of leukocytes to the vascular endothelium.

This hypothesis was tested in four ways. Firstly the capacity of washed platelets to influence human umbilical vein endothelial cell adhesion molecule expression was investigated in an established laboratory model of endothelial activation and injury. The capacity of platelets to promote washed neutrophil adhesion during co-incubation was then investigated in the same model. In chapter 6 the more activated adhesion molecule profile of neutrophils in platelet neutrophil complexes was described. The cultured human umbilical vein endothelial cell model was manipulated to mirror the activated vascular endothelium seen in cases of sepsis (chapter 1) and the propensity for PNC adhesion in comparison to free neutrophil adhesion assessed. Finally %PNC was estimated in a population of type 1 diabetic patients in whom a simultaneous non-invasive assessment of vascular endothelial function by high resolution ultrasound was undertaken.
7.2 Methods

7.2.1. Platelet induced endothelial adhesion molecule expression.

The capacity of platelets to induce human umbilical vein endothelial cell adhesion molecule expression was measured in an established laboratory model of endothelial activation and injury (Klein et al 1992). Human umbilical vein endothelial cells were cultured as described in chapter 2. Single donor cord HUVECs were used for each experiment. Experiments were performed on HUVECs which had formed a confluent monolayer in secondary culture on 24 well plates.

Platelets were separated from whole blood as described in chapter 2 or recovered from clinical platelet transfusion packs and washed twice in HBSS without Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. Washed platelets were resuspended in MCDB131 culture medium containing 20% fetal calf serum and the platelet count determined on a Coulter counter.

Platelet concentration was adjusted to 5 x 10\textsuperscript{9}/ml or 1 x 10\textsuperscript{7}/ml. HUVECs were washed and one ml of control medium, platelets or the supernatant from the first wash of platelet concentrates or platelet rich plasma were added to the culture wells. Positive controls were provided by the addition of TNF-\alpha to a final concentration of 1\mu g/ml. The platelet agonist ADP (final concentration 5\mu M) was added to half the wells containing medium alone or platelets. The co-cultures were incubated at 37\textdegree C, 5% CO\textsubscript{2} on a rotary shaker at ~15Hz for between one and four hours before all wells were washed with medium. Analysis for HUVEC CD62E expression was undertaken at 4-6 hours and for VCAM and ICAM-1 expression at 24 hours.

Adherent HUVECs were removed from the culture plates by gentle mechanical action after washing twice with warmed PBS and incubated with Pucks' Saline A solution (containing EDTA). HUVECs were washed with
PBS+5% FCS and 0.02% azide and incubated for 20 min with monoclonal antibodies against or isotype matched control. After a further washing step, FITC labelled goat anti-mouse immunoglobulin monoclonal antibody (Serotec, UK) was added and the cells incubated for a further 20 min. After a final wash, cells were resuspended in 250μl PBS, 5% FCS and azide and analysed by flow cytometry. HUVECs were analysed with forward scatter measurement gain setting in logarithmic mode and side scatter measurements in linear mode. The HUVEC population could easily be distinguished from cellular debris on forward and side scatter characteristics (figure 7.1). 5000-10000 HUVEC events were collected and the data analysed as median fluorescence intensity.

**Figure 7.1**

**Characteristic forward and side scatter appearance of HUVECs.** The HUVECs are distinguishable as a separate population of large granular cells which is then selected by the definition of a region as shown.
7.2.2. Platelet induced neutrophil adhesion.

Blood was collected from a healthy donor and both platelets and neutrophils were separated as described in Chapter 2. As with the above experiment, single donor confluent HUVEC cultures in 24-well plates were taken. After a washing step, control medium, medium containing $2.5 \times 10^7$ platelets or medium containing a final conc. of 5ng/ml of TNF-$\alpha$ was added to the wells. The culture plate was then incubated for 60 min at 37°C, 5% CO$_2$ on a rotary shaker at ~15 cycles/min. A further washing step was undertaken and $1 \times 10^6$ neutrophils added to each well. After a further 30 min incubation as above, the supernatant from each well was removed and the HUVECs washed twice with medium. Inspection of the wells at this stage demonstrated that only neutrophils attached to HUVECs remained in the well. Pucks' Saline A solution was then added to each well which caused detachment of HUVECs from the plastic and the neutrophils from the HUVECs. Any residual cells were removed by gentle mechanical action. The cells were washed in PBS+5% FCS and 0.02% azide before the pellet was stained with a neutrophil marker (CD15:FITC, or CD66b:FITC) or an isotype matched control antibody. In some experiments the capacity of platelets to enhance adhesion to activated endothelium (prior incubation with TNF-$\alpha$ at 5ng/ml for 4 hours) was investigated.

The stained cells were then analysed by flow cytometry as above. The percentage of events staining positive for the neutrophil marker was considered to represent a measure of the neutrophils which had adhered to the HUVEC/platelet surface.

7.2.3 Adhesion of platelet neutrophil complexes to HUVECs

Single donor HUVECs were prepared and confirmed as confluent in secondary culture on surface modified polystyrene 48 well plates by inspection. A proportion of wells were prepared (denoted 'blanks') to which
HUVECs had not been added to provide controls for possible neutrophil or platelet adhesion to plastic. In this way the contribution of HUVECs vs. activated HUVECs could be assessed.

At time zero all wells were washed with MCDB131 culture medium containing 20% FCS. Lipopolysaccharide endotoxin (final conc. 1μg/ml) was added to half the wells and incubated for at least four hours. Subsequently all wells were washed thoroughly with RPMI + 5%FCS and heparinised whole blood diluted 1:1 with RPMI + 5% FCS added. Initial studies (not shown) indicated that this dilution did not significantly alter the number or behaviour of PNCs. Dilution was performed in order to increase the ratio of endothelial cells to neutrophils to improve the ability of this model to detect differential neutrophil adhesion. In some experiments the blood was also stimulated with LPS (final conc. 10ng/ml) prior to exposure to HUVECs. The mixed cell cultures were then incubated for 1 hour while being gently agitated on a rotary shaker (~15 cycles/min, 37°C, 5% CO₂). After this period the supernatants were sampled and 100μl examined as for measurement of PNCs (chapter 5). 5000 neutrophil events were collected for each sample. The time required to collect 5000 events varied considerably, which limited the analysis of results to the relative proportion of neutrophils which were unbound in suspension to those that were observed in PNC, but did not allow assessment of absolute numbers of cells in these two populations.

7.2.4 Platelet neutrophil complexes in Type 1 diabetic patients

Patients with type 1 diabetes who were taking part in a clinical trial of cholesterol reduction and L-arginine therapy conducted by the Vascular Physiology Unit at the Institute of Child Health (principal investigator Dr. M. Mullen , Head of Unit Prof. J. Deanfield) were assessed for the level of circulating PNCs. Endothelial-dependent vasodilator function was also assessed by means of an established technique, which employs high resolution ultrasound imaging of the brachial artery (Clarkson et al 1997;
Leeson et al. 1997). The baseline diameter of the brachial artery in cross section was recorded at a fixed point in the cardiac cycle by software which links to the electrocardiogram. A pressure cuff was inflated around the lower forearm to occlude distal arterial flow for 4.5 min. When this cuff was then released, physiological vasodilatation of the brachial artery occurs in response to the increased blood flow. This 'flow-mediated dilatation' is known to be mediated by endothelial production of nitric oxide and is markedly attenuated or absent following a variety of acute or chronic insults to vascular endothelial function (Mullen et al. 1997). Flow-mediated dilatation is a sensitive and specific measure of conduit artery NO bioavailability (Mullen et al. 1997) and hence is a measure of the metabolic function of the vascular endothelium. Sub-lingual glyceryl tri-nitrate (a pharmacological vasodilator) is used to demonstrate endothelial independent vasodilatation to provide a positive control.

Subjects were 64 adults, aged 18 - 45 years, with type 1 diabetes of > 2 years duration. They were non-smokers, had resting supine blood pressure <150/90 mmHg, had no clinical evidence of large vessel atherosclerosis, and were not taking vasoactive or cholesterol lowering medication. All subjects gave informed written consent and the local research ethics committee approved the protocol. Subjects were studied in a fasting state before randomisation or the administration of any study medications. Subjects lay at rest for at least 10 minutes prior to the first ultrasound scan and remained supine throughout the procedure. The right brachial artery was imaged in longitudinal section 2-10 cm above the elbow using a standard 7 MHz linear array transducer supported by a flexible stereotactic clamp and an Acuson 128XP/10 ultrasound system (Acuson Ltd., Mountain View, Ca, USA). The image was magnified using a resolution box function and the operating parameters were set to optimise the lumen/arterial wall interface. Blood flow velocity was estimated using pulsed wave Doppler with the cursor set at 70° to the longitudinal axis of the artery and the range gate [1.5 mm] in the centre of the artery. A segment of the artery was selected and once a
stable, clear image was achieved, 5 seconds of radio-frequency signal from this segment was routed to an A-mode tracking device (Ingenious Systems, The Netherlands) where the data was stored for later analysis of vessel diameter. Brachial artery diameter was determined at rest and 55 to 65 seconds after a brief period of reactive hyperaemia induced by inflating a pneumatic tourniquet placed around the forearm to 300mmHg and its rapid release after 4.5 minutes. The increase in brachial artery blood flow velocity over the first 20 seconds of reactive hyperaemia was determined using pulsed wave Doppler. After a further 10-15 minutes rest to allow vessel recovery, sublingual glyceryl trinitrate (GTN) 400 μg was administered and the response to this endothelium-independent dilator assessed after 3 minutes. Vessel dilatation in response to flow (FMD) and GTN were expressed as the percentage increase in vessel diameter from baseline.
7.3. Results

7.3.1. Platelet induced endothelial adhesion molecule expression.

Co-incubation of HUVECs with platelets had no influence on the expression of the HUVEC adhesion molecules CD62E, VCAM or ICAM-1 (figure 7.2). Activation of platelets with ADP did not alter adhesion molecule expression. The use of platelets which had been freshly separated from whole blood as described in chapter 2 or prepared from clinical transfusion concentrates had no influence on these observations. The supernatant from the initial platelet washing step also had no detectable influence on endothelial adhesion molecule expression. The capacity of the HUVECs to upregulate adhesion molecules in this system was consistently demonstrated in response to TNF-α which induced a 10 fold increase in CD62E, a 4 fold increase in ICAM-1 and an 6 fold increase in VCAM compared to control MFI. Each experiment was performed on five occasions and a representative example is shown (figure 7.2).

Figure 7.2 (overleaf)
Platelet induced endothelial adhesion molecule expression.
Expression (MFI) of the three principle vascular endothelial adhesion molecules following incubation with medium, supernatant of separated platelets, TNF-α (positive control) and resting and ADP stimulated platelets at a range of concentrations. There is no significant difference from control expression for any of adhesion molecules with incubation with resting or activated platelets while TNF-α causes very marked increases in CD62E, ICAM-1 and VCAM expression.
Figure 7.2 (legend on previous page)
7.3.2. Platelet induced neutrophil adhesion.

Data was expressed as the proportion of flow cytometer events which stained positive for a neutrophil marker. As the HUVEC monolayers were confluent and from a single donor, a consistent number of HUVECs could be anticipated in each well. Therefore the percentage of events staining positive for the neutrophil marker could be used to indicate the number of neutrophils which remained in the well despite the vigorous washing steps by virtue of adhesion to the endothelium.

Positive control for these experiments was provided by prior incubation of HUVECs with TNF-α (5ng/ml) for four hours. This dose and interval is known to induce a significant increase in endothelial CD62E (see chapter 1.2.2). As anticipated TNF-α stimulation caused a significant increase in the number of neutrophils bound to endothelium in this model (figure 7.3).

The prior addition of platelets to unstimulated endothelial cell monolayers did not significantly increase the number of neutrophils which were adherent after 30 min above that adherent to endothelium alone (figure 7.3A). Although there was a consistent trend towards greater neutrophil adhesion in the presence of platelets previously exposed to ADP this did not achieve significance (p=0.24, ADP stimulated platelets vs. endothelium alone).

However, the presence of platelets did cause a small but significant increase in neutrophil adhesion to HUVECs that had been exposed to TNF-α for four hours prior to the addition of platelets (figure 7.3B), (p=0.03 TNF-α stimulated endothelium & platelets vs. TNF-α stimulated endothelium).
Figure 7.3

Platelet mediated neutrophil adhesion to HUVECs. There is no significant increase in the adhesion of neutrophils after 30 min of co-incubation to unstimulated endothelium in the presence of platelets that were at rest or had received a prior stimulus to activation with ADP A. However, platelets did increase neutrophil adhesion to pre-activated endothelium after 30 min (following 4 hours of TNF-α stimulation) B. The results of two typical experiments are shown with data points representing multiple wells examined on a single occasion. Each experiment was performed on three occasions. Median values and interquartile ranges are shown.
7.3.3 Adhesion of platelet neutrophil complexes to HUVECs

Platelet neutrophil complexes are expressed as a proportion of the total neutrophil population as described in chapter 5. Multiple replicates of the same experiment are shown in figure 7.4. The %PNC observed in the supernatant of control culture wells containing no HUVECs was greater than that seen in freshly examined whole blood as described previously. This increase is likely to be related to artefactual platelet activation induced during the time on the rotary shaker. Although an influence of the plastic increasing PNCs cannot be excluded in the control wells cannot be excluded, this seems unlikely over this time course in view of the experiments presented in chapter 5. One interpretation of these results is that the presence of HUVECs caused a significant fall in %PNC in the supernatant compared to that seen above control wells containing no endothelium (figure 7.4). It is not possible in this system to distinguish between preferential adhesion of PNC to HUVEC or an influence of HUVEC which decreases the tendency to form PNC in the cell suspension in close proximity.

The observed fall in %PNCs was even greater in the presence of activated endothelium (following exposure to LPS for 4 hours prior to experiment) and blood which had been exposed to LPS (figure 7.4B). Similar experiments which employed exposure to Neisseria meningitidis in place of LPS demonstrated the same pattern of lower %PNCs in the presence of pre-activated endothelium (data not shown).
Figure 7.4.

**PNCs adherence to HUVECs** 'Blank' denotes control wells containing no HUVECs. In unstimulated A and LPS stimulated blood B, %PNCs are significantly lower in the presence of endothelial cells (*=p<0.01 vs. blank) after 60 min of incubation. PNCs are lower still (**=p<0.05 vs. unstim.) in the presence of unstimulated endothelium in B.
7.3.4 Platelet neutrophil complexes in Type 1 diabetic patients

The sample population consisted of 64 young adults (70% male) (mean age 34.3 years, range 20-45 years) type 1 diabetics who had been diagnosed for a mean of 14.9 years. The %PNCs were recorded and flow mediated dilatation assessed as described. A significant relationship between low flow mediated dilation (corresponding to attenuated endothelial vasodilator function by virtue of decreased nitric oxide production) and lower %PNC was identified on univariate regression (p=0.018 see table 7.1). No other factor including biochemical markers of the quality of glycaemic control (e.g. fructosamine), circulating neutrophil and platelet counts or factors which are purported to relate to endothelial function (plasminogen activator inhibitor-1 or VWF levels) was significantly associated with %PNC.

The relationship between %PNC and flow-mediated dilatation was then examined in a multiple linear regression model (table 7.1). Correction for other biochemical or haematological factors (including circulating platelet and neutrophil counts) did not eliminate the statistical significance of the relationship.
Figure 7.5

Relationship between flow mediated vasodilatation (FMD) and %PNCs. The %PNCs (mean and SE) for each quintile of FMD are shown. Abnormally low FMD corresponds to the quintiles 1 & 2. There is a clear reduction in the level of %PNC seen in patients with abnormally low FMD. At levels of normal FMD there is no significant change in %PNC.
Table 7.1
Relation between FMD and PNCs after adjustment for other risk factors.
The regression co-efficient for the model is shown with FMD as the dependent variable and PNC alone or in combination with the other factors as the independent variable. The association between FMD and PNCs was not confounded by any demographic or diabetic characteristics of the subjects or any of the measured biochemical or haematological risk factors.

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<th>P value</th>
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<td>0.018</td>
</tr>
<tr>
<td>Age</td>
<td>1.12 (0.27 - 1.97)</td>
<td>0.01</td>
</tr>
<tr>
<td>Gender</td>
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<td>0.021</td>
</tr>
<tr>
<td>Total cholesterol</td>
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<td>0.014</td>
</tr>
<tr>
<td>HDL cholesterol</td>
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</tr>
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<td>Triglycerides</td>
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<td>Fibrinogen</td>
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<tr>
<td>PAI-1</td>
<td>1.28 (0.39 - 2.16)</td>
<td>0.006</td>
</tr>
<tr>
<td>vWF</td>
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</tr>
<tr>
<td>Duration of diabetes</td>
<td>1.13 (0.26 - 2.00)</td>
<td>0.012</td>
</tr>
</tbody>
</table>
7.4 Discussion

7.4.1. Platelet induced endothelial adhesion molecule expression.

Expression of endothelial adhesion molecules was unchanged by co-incubation with resting or activated platelets or the supernatant from their preparation. This contrasts with data from Hawrylowicz et al who observed IL-1 expression on activated platelets (Hawrylowicz et al 1989) and increased endothelial adhesion molecule expression following co-incubation for 6-24 hours (Hawrylowicz et al 1991). Recently, thrombin activated platelets have been reported to express the ligand for CD40 (CD40L, CD154) and to increase adhesion molecule expression via this mechanism (Henn et al 1998).

These data are difficult to reconcile with our results. HUVEC adhesion molecule expression following a stimulus to activation was clearly demonstrated in response to TNF-α. The platelets used in our experiments were from a variety of donors either freshly prepared from whole blood or from clinical transfusion packs. Although epoprostenol was used to limit artefactual platelet activation in preparation, this does not appear to have reduced platelet responsiveness to agonists subsequently as in experiments in which it was omitted from the method, HUVEC adhesion molecule expression remained unchanged (data not shown). The number of platelets added (0.5-1.0x10^7/ml) was less than that used in some studies (2x10^7/ml (Hawrylowicz et al 1991) or 3x10^9/ml (Henn et al 1998)). This sub-physiological concentration was chosen on the basis of the previous studies and the knowledge that these same cells would be incubated under conditions of relative stasis. A single experiment with platelets at a concentration of 1x10^9/ml revealed no change. The observation of CD154-dependent expression of endothelial adhesion molecules was made after activated platelets were centrifuged into close apposition with HUVEC, a method which may bear little relationship to in-vivo interactions. The use of
more potent platelet agonists; thrombin (Henn et al 1998; Hawrylowicz et al 1991) or ADP and adrenaline combined (Kaplanski et al 1993) may also have contributed to increased activation seen following co-incubation in these studies. Thrombin has recently been reported to activate endothelial cells directly and hence we avoided its use (Kaplanski et al 1997). These experiments did not demonstrate any induction of HUVEC adhesion molecule expression by platelets under these conditions.

These experiments were designed to investigate the effect of isolated platelet activation on endothelial adhesion molecule expression. It can be argued that this question is of limited physiological relevance as isolated platelet activation is likely to be a rare event in-vivo. Further experiments investigating the capacity of platelets to potentiate the endothelial response to factors known to induce activation (e.g. TNF-α, LPS or activated neutrophils) would be of importance.

7.4.2. Platelet induced neutrophil adhesion.

When washed platelets were co-cultured with unstimulated endothelium they did not alter subsequent neutrophil adhesion. This is consistent with the findings of unchanged endothelial adhesion molecule profiles reported above. However, in the presence of pre-activated endothelium (after exposure to TNF-α for four hours) a small increase in neutrophil adhesion was seen.

While these experiments were being performed, data describing the capacity of immobilised platelets to support multi-step leukocyte adhesion in sophisticated models were published (Diacovo et al 1996b; Nash, 1994b). Intra-vital microscopy in mice suggested that activated platelets could increase lymphocyte rolling adhesion via a CD62P dependent and CD62L independent mechanism (Diacovo et al 1996a). Subsequent work employing a monolayer of immobilised platelets in a flow chamber indicates that
neutrophils can adhere to and transmigrate across this monolayer. This process is mediated via multi-step adhesion molecule-ligand interactions as have been previously documented to occur between activated endothelium and neutrophils (Diacovo et al 1996b). The importance of platelet CD62P was highlighted, as was the contribution of the neutrophil $\beta_2$-integrin CD11b/CD18. Further work has now supported these original observations and indicated that platelet GPIIb/IIIa binding of fibrinogen provides the ligand for CD11b/CD18 mediated adhesion (Kuijper et al 1997; Weber & Springer, 1997).

The observations made in this chapter are consistent with these previous studies. A possible mechanism by which platelets may contribute to neutrophil recruitment to activated endothelium but not to resting endothelium is apparent. The principle mechanism for platelet adhesion to neutrophils is CD62P-PSGL-1. This interaction on a tethered platelet would lead to neutrophil rolling and the stimulation of CD11b/CD18 expression and activation (chapter 6) which prepares the neutrophil for firm adhesion to endothelial immunoglobulin superfamily adhesion molecules (principally ICAM-1) or indeed to platelet ICAM-2 (Diacovo et al 1994). When the endothelium has been exposed to TNF-$\alpha$, there is a greatly increased concentration of ICAM-1 (as well as VCAM) available on the endothelial surface (figure 7.2). Therefore, the probability of neutrophil firm adhesion may be increased by virtue of the CD62P mediated binding to platelets. This is consistent with the only study to date which has examined these interactions with all three cell types in an in-vitro model (Kuijper et al 1997). The relative importance of neutrophil-endothelial interactions and neutrophil-platelet interactions in this model of combined inflammation and haemostasis has yet to be defined.

The investigation of the binding of neutrophils seen in these conditions of relative stasis could be criticised but relative stasis is a characteristic of the micro-vasculature in severe sepsis (which in the presence of disseminated
intra-vascular coagulopathy and fibrin deposition may be very severe) (Hinshaw, 1996). It is interesting to speculate that the contribution of platelets may be exaggerated in the absence of anti-coagulation as fibrin/fibrinogen binding to activated platelet GpIIb/IIIa acts not only as an adhesive bridge to neutrophil integrins (Weber & Springer, 1997) but also to a range of endothelial adhesion molecules (figure 1.10) (Bombeli et al 1998). Further work is planned to investigate the contribution of platelets to neutrophil adhesion under variable flow conditions in models of septic endothelial activation. Attempts will be made to perform these studies in the presence and absence of adhesive proteins such as fibrinogen.

7.4.3 Adhesion of platelet neutrophil complexes to HUVECs.

The fall in %PNC detectable in the supernatant in the presence of endothelium is consistent with the observations made in chapter 6 that PNCs display a more activated adhesion molecule profile than do free neutrophils.

The capacity of a neutrophil in a PNC to bind to the endothelium may be greater than a free neutrophil by virtue of the changes in β2-integrin expression and activation reported above. However a PNC also contains one or more activated platelets and hence has an increased range of potential ligands on the endothelium or sub-endothelial matrix because of the additional adhesion molecules (activated GpIIb/IIIa) 'grafted' onto the neutrophil surface. Therefore a PNC which is primed for adhesion is more likely to be removed from the supernatant than is an unbound neutrophil (figure 7.6) This effect is exaggerated by the prior activation of the endothelium which induces increased neutrophil adhesion.
7.4.4 Platelet neutrophil complexes in Type 1 diabetic patients

In this collaborative study young subjects with type 1 diabetes were studied because they represent a population with a major risk factor for vascular disease, in which endothelial dysfunction has been demonstrated from an early age (Krolewski et al 1987). This study demonstrated a reduction in circulating %PNC in patients with evidence of attenuated endothelium-dependent vasodilator function as assessed by flow-mediated dilatation.

Flow-mediated dilatation requires endothelial nitric oxide production and is abolished by the administration of nitric oxide synthetase inhibitors. Endothelial nitric oxide is known to act to limit local platelet adhesion and can attenuate CD62P expression (Vallance et al 1997). The overlap between endothelial functions is reviewed in chapter 1.2.5, but it is apparent that the adhesive characteristics of endothelium with impaired NO metabolism will be altered.

As suggested above, if the endothelium is less able to resist adhesion, then PNCs may adhere more rapidly than free neutrophils (figure 7.6).

These data support the hypothesis that levels of circulating PNCs are largely determined by their rate of adhesion to activated vascular endothelium, and provide in vivo evidence of a link between endothelial dysfunction and impaired NO bioavailability in young diabetic subjects. These data could also be interpreted as an effect of activated vascular endothelium lessening the tendency to form PNC via the production of soluble factors such as NO.

However, the overall level of circulating PNCs is likely to represent the balance between their formation (largely determined by platelet activation), and their adhesion to the vascular endothelium. There was no suggestion that patients with lower PNCs were less able to form complexes. Indeed, in in-vitro stimulation experiments with ADP those with lower levels of PNCs
responded by forming more PNCs than did those with initially normal levels (data not shown). However, the very short half-life of NO (and PGI₂) mean that these observations cannot exclude the influence of soluble factors released from activated endothelium which are not apparent when samples are stimulated ex-vivo. Theoretically, this question could be answered by infusing a platelet agonist into volunteers before analysis for PNC.

Whatever mechanism is responsible for this association, the measurement of PNCs might represent a simple, novel method for the \textit{in vivo} assessment of dynamic interactions between inflammatory complexes and vascular endothelium and provide insight into the pathogenesis of chronic inflammatory processes such as atherosclerosis as well as acute severe inflammation that is the subject of this thesis.
Figure 7.6

Are platelet neutrophil complexes are primed for endothelial adhesion?

The increasingly activated adhesion molecule profile displayed by PNC compared to free neutrophil and the wider range of potential ligands by virtue of platelet adhesion may mean that a PNC will selectively adhere to endothelium in preference to unbound neutrophils. The %PNC assessed from patients therefore represents a function of the degree of in-vivo platelet, neutrophil and endothelial activation. An alternative explanation is that activated endothelium reduces the tendency to form PNC in the immediate surrounding by alteration production of soluble factors such as NO and prostacyclin (PGI₂).
7.5 Conclusions and Future Work

The data presented in this chapter support a contribution of platelets in the acceleration of neutrophil recruitment to endothelium. A direct effect of platelets on the endothelium was not demonstrated although an indirect effect that requires multiple interactions between platelets, neutrophils and the endothelium is supported. The laboratory and clinical data appear consistent in suggesting that PNCs are influenced by activated endothelium.

Future work will be aimed initially at examining the endothelium in the models of adhesion described above to determine if platelets and neutrophil do adhere together to activated HUVECs.

The effect adding shear stress to the laboratory model of these multi-cellular interactions and investigating the influence of anti-adhesion therapies on inflammatory cell recruitment. This contribution of PNCs to inflammation may represent a target for immunomodulation that allows slight attenuation of adhesion rather than the abolition seen with less specific targets.

Further collaborative work with the Vascular Physiology Unit at The Institute of Child Health is planned to attempt to confirm the relationship between endothelial nitric oxide production and circulating %PNC by administration of short acting inhibitors of endothelial nitric oxide production to healthy volunteers. %PNC can be determined in the same subject with and without intact endothelial NO production. Estimation of %PNC will then be applied more widely to determine the impact of therapeutic manipulation (e.g. the administration of fibrinolytic agents) on this proposed marker of inflammatory cell adhesion in subjects with established endothelial dysfunction (e.g. unstable angina) in a clinical setting.
Chapter 8

Platelet-Neutrophil Complexes in Sepsis

8.1 Introduction

8.2 Methods

8.3 Results

8.4 Discussion
8.1 Introduction

The investigation of levels of platelet activation by free platelets flow cytometry was discussed in chapter 4. In chapter 5 the capacity of activated platelets to bind neutrophils was described and the possible advantages of assessing platelet-neutrophil complex formation in addition to free platelet studies was discussed. In chapter 6 it was shown that neutrophils in platelet neutrophil complexes are more activated than free neutrophils. Whilst this was shown to be mediated by CD62P in some situations, there are some neutrophil functions (e.g. phagocytosis) which were not inhibited by the presence of anti-CD62P antibody suggesting that neutrophil activation may also contribute to PNC formation.

This chapter describes experiments designed to investigate the contribution that neutrophil activation makes to the formation of platelet neutrophil complexes. In-vitro stimulation of whole blood with lipopolysaccharide endotoxin (as a model of Gram negative sepsis) as well as more specific neutrophil agonists are employed. Further studies are described which investigate the potential mechanisms by which neutrophil activation may influence the formation of PNCs.

Widespread neutrophil activation is a well documented feature of the acute systemic inflammatory response to any severe insult. Therefore the suggestion that neutrophil activation contributes to PNC formation is likely to have particular relevance to the levels of PNC recorded in clinical severe sepsis and septic shock. The final part of this chapter reports the changes seen in platelet-neutrophil complexes in children with clinical meningococcal disease.
8.2 Methods

8.2.1 Whole blood stimulation

The monoclonal antibodies used are detailed in chapter 2.2. Stimulation was performed in freshly sampled whole blood added to sodium citrate anticoagulant (final conc. 0.38%) in a similar manner to that described in previous chapters (sections 5.2.5., 6.2.1) In experiments in which more than one agonist was used the potential neutrophil agonist was added first followed immediately by the platelet agonist ADP (10μM). Incubations were performed in a minimum of 4 ml of blood which was placed on a rotary shaker at 8-10 cycles/min at 37°C.

The neutrophil agonists employed were lipopolysaccharide endotoxin (LPS) at a final concentration of 10ng/ml unless otherwise stated, fMLP at 10^{-6}M or KIM127 (20μg/ml). KIM127 is a direct and specific activator of the β2-integrins, which binds to and alters the CD18 component thereby exposing the active binding site in the absence of other activation signals to the cell expressing the integrin (Andrew et al 1993; Robinson et al 1992). Initial experiments were performed to assess the response of neutrophil β-2 activation to KIM127 in different anticoagulants. A much greater increase in β-2 integrin activation as assessed by binding of the ‘activation reporter’ monoclonal antibody Mab24 (see chapter 6) was detected in heparin than citrate anticoagulated blood. Therefore for experiments employing KIM127, heparin at a final concentration of 10 unit/ml was preferred to citrate as the anticoagulant.
8.2.2 Time course of neutrophil and platelet parameters in clinical Meningococcal septicaemia

The study protocol was approved by the local research ethics committee and written informed consent obtained. The Paediatric Intensive Care Unit Emergency Transport Service informed a member of our laboratory staff (Dr Garth Dixon or myself) via a radiopager upon referral of cases of suspected meningococcal septicaemia. We then accompanied the transfer team in order to perform these and other studies on these cases. Samples were collected as soon as possible in the course of the disease from indwelling intra-arterial catheters into a final concentration of 0.38% sodium citrate. The sample time (to the nearest hour) was recorded from the first dose of parenteral antibiotics used to treat this episode.

Figure 8.1
A 10 month infant with severe meningococcal septicaemia. This case presented with a Glasgow meningococcal septicaemia prognostic score (GMSPS) of 15, neutropenia, thrombocytopenia and profound hypotension. (Clinical photograph reproduced with parents' consent).
Samples were collected throughout the PICU stay whilst intra-arterial access was available and repeated at the same time as routine clinical venous samples were collected during out-patient follow-up of survivors. Neutrophil and platelet counts, taken as part of routine clinical care, were recorded. Neutrophils were analysed for %PNC and CD11b, CD62L expression and Mab24 binding by flow cytometry as previously described. Platelets were investigated for levels of the activated form of GpIib/IIIa by the binding of PAC-1 monoclonal antibody. All monoclonal antibodies used are detailed in section 2.2. Samples were analysed immediately after collection except those that were obtained during stabilisation prior to transfer from the referring hospital to Great Ormond Street Paediatric Intensive Care Unit.

Patients were recruited for this study from November 1996 onwards and two of the antibodies (Mab24 and PAC-1) were only available from Summer 1998. Therefore these are reported on fewer patients. Clinical details including the Glasgow Meningococcal Sepsis Prognostic Score (GMSPS) (Thomson et al. 1991) (tables 8.1 and 8.2) were also recorded, as was the outcome. Control levels of CD11b and PNC in single system failure PICU patients were provided by the studies in chapter 4. Levels of CD62L and Mab24 binding are compared to healthy adult laboratory controls.
Table 8.1
The Glasgow Meningococcal Sepsis Prognostic Score.

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<th>Parameter</th>
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<tr>
<td>Age &gt;4 years: BP &lt;85 Systolic</td>
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<tr>
<td>Skin/rectal temp difference &gt;3°C</td>
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</tr>
<tr>
<td>Modified coma score &lt;8 OR deterioration of &gt; 3 points in 1 hour</td>
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</tr>
<tr>
<td>Deterioration in hour before scoring</td>
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</tr>
<tr>
<td>Absence of meningism</td>
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<tr>
<td>Extending purpuric or widespread ecchymoses</td>
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</tr>
<tr>
<td>Base deficit (capillary or arterial) &gt;8</td>
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<td>maximum score</td>
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Table 8.2
Modified Coma Score.

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<th>A Eyes Open:</th>
<th>B Best verbal response</th>
<th>C Best motor response:</th>
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<td>Spontaneously</td>
<td>Orientated 6</td>
<td>Obeys commands 6</td>
</tr>
<tr>
<td>to speech</td>
<td>words 4</td>
<td>Localises pain 3</td>
</tr>
<tr>
<td>to pain</td>
<td>vocal sounds 3</td>
<td>moves to pain 3</td>
</tr>
<tr>
<td>eyes not open</td>
<td>cries 2</td>
<td>(flexion / extension)2</td>
</tr>
<tr>
<td></td>
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<td>none 1</td>
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</tbody>
</table>

Total Modified Coma score: A+B+C max 16
8.3 Results

8.3.1 Effect of Lipopolysaccharide endotoxin on PNC

The influence of *E. coli* LPS on the number of platelet neutrophil complexes detected in freshly sampled whole blood was investigated across a wide range of doses. There were no significant changes in PNC or free platelet CD62P expression although a trend towards increased PNC was seen at doses of 1-100ng/ml. Figure 8.2 shows the responses to LPS. The confidence intervals reflect the observed wide variability in PNC formation.

**Figure 8.2**

Lipopolysaccharide Endotoxin stimulation of whole blood at 60 min. Means and 95% CI are shown of 5 experiments. Incubation of freshly sampled whole blood with lipopolysaccharide endotoxin had no impact on free platelet CD62P expression over 60 min at a wide range of doses. A similar pattern was evident at 30 min. The wide variability in response to LPS may have been caused by samples with differing levels of prior activation. Therefore further investigations
were performed using the authors' blood on every occasion as venesection was always straightforward. The 10ng/ml dose of LPS was further investigated as this showed the response that most closely approached significance in the initial studies and because this approximates to levels observed in clinical Gram negative sepsis (figure 8.3). In ten experiments, no significant difference was detected between PNC detected in unstimulated samples and following exposure to LPS at 10ng/ml.

**Figure 8.3**

Single donor response PNCs to LPS stimulation at 10ng/ml. 10 experiments are shown. There is no significant difference between %PNC detected in stimulated and unstimulated blood (paired t-test, p=0.41). The possibility that the apparent wide variability in response to LPS in the initial experiments may have reflected variable artefactual prior platelet activation in those cases that in which LPS appeared to increase the %PNC was further investigated by exploring the effect of co-stimulation with the specific platelet agonist ADP in addition to LPS.
There was a highly significant accentuation of the response to ADP by co-stimulation with LPS at when measured at 30 min (figure 8.4).

![Figure 8.4](image.png)

**Figure 8.4**

The influence of co-stimulation of whole blood with LPS and ADP on %PNC. The %PNC are shown at 1 and 30 min following stimulation with ADP or LPS or both. There is a significant increase in %PNC at 30 min following co-stimulation with LPS & ADP compared to ADP alone (Wilcoxon signed ranks test, n=10, p=0.005). Individual data points are shown as blue circles, and means and 95% ci are shown as boxes and error bars. Data points for individual experiments are not linked for clarity.
One explanation for this response could be a direct effect of LPS acting to increase platelet activation and inducing increased PNC formation solely through increased levels of CD62P expression. The same samples were therefore also analysed for levels of free platelet activation (figure 8.5). No significant differences were seen in platelet CD62P expression or GpIIb/IIla activation as demonstrated by binding of PAC-1 antibody between samples exposed to ADP alone or LPS/ADP. Therefore a direct effect of LPS on platelet activation was not detectable. However, as discussed in chapter 5 (5.3.8) free platelet analysis may underestimate the state of activation of the platelet population in whole blood. Therefore the same co-stimulation experiments were performed with washed platelet suspensions prepared as described in chapter 2. No accentuation of the response to ADP was seen with CD62P or activated GpIIb/IIla expression with the addition of LPS (data not shown).

Therefore in the context of platelet activation with ADP, LPS enhances PNC formation. This effect does not appear to be the result of effects of LPS on platelets because free platelet activation markers were not increased by LPS.

These results indicate that LPS stimulation of neutrophils potentiates the formation of PNCs. However neutrophil activation alone is not sufficient to cause PNC formation in the absence of a stimulus to platelet activation.
Figure 8.5
The influence of co-stimulation of whole blood with LPS and ADP on Free Platelet Activation. The platelet agonist ADP induced significant activation of platelets as shown by changes in CD62P (1 and 30 min) A and B PAC-1 binding (at 1 min). No potentiation of this effect was apparent with co-incubation with LPS.
8.3.2 Effect of fMLP on platelet-neutrophil complex formation

In order to investigate if this capacity to accentuate PNC formation in response to platelet stimulation is a specific property of LPS, similar co-stimulation experiments were performed with fMLP (final conc. 10^(-6)M) and ADP. The same pattern was demonstrated with a significantly greater formation of PNC 30 min after exposure to fMLP and ADP than with ADP alone. There was a trend towards a significant increase with co-stimulation at one minute which was not apparent with LPS. This is consistent with the observations reported in chapter 6 (figure 6.1A and 6.4A) that fMLP has a very rapid influence on neutrophil activation as measured by increased β-2 integrin expression and activation and CD62L shedding.

Figure 8.6
Platelet neutrophil complexes after ADP and ADP/fMLP. A) A representative experiment is shown. fMLP alone did not increase %PNC at 1 or 30 min. Increases in the response to ADP are seen with fMLP in a similar manner to LPS. B) Five experiments are shown. There is a significant (Wilcoxon p=0.03) increase in %PNC following ADP/fMLP as compared to ADP alone at 30 min.
8.3.3 Mechanisms of Platelet-neutrophil complex formation

These data suggest that neutrophil agonists are able to contribute to the formation of PNCs. Several mechanisms were considered by which this might occur: A) changes in the expression or affinity of the principle ligand for activated platelet CD62P on neutrophils (PSGL-1, see chapter 1, figure 1.6), B) decreased neutrophil expression of CD39 which has activity in attenuating platelet responsiveness to ADP (an ecto-ADPase) or C) altered levels of β-2 integrin activation or expression.

The expression of CD11b, CD39 and PSGL-1 and the level of Mab24 binding were assessed on neutrophils from citrated whole blood with and without LPS stimulation (figure 8.7). As expected there were marked changes in β-2 integrin expression and activation but no significant changes in CD39 or PSGL-1 expression.

In order to determine if this change in β-2 integrin expression activity was responsible for the accentuation of PNC formation in the presence of activated platelets, the influence of the monoclonal antibody KIM127 was investigated. KIM127 has the property of being a direct activator of the β2-integrins by inducing a conformational change in the CD18 component of the heterodimer (Andrew et al 1993; Robinson et al 1992). KIM127 at 20μg/ml final conc. was found to be highly effective at inducing the activated form of CD11b/CD18 on neutrophils as determined by Mab24 binding in preliminary experiments (n=6, Mab24 MFI pre vs. post KIM127, p=0.028 Wilcoxon signed ranks test, data not shown).

In co-stimulation experiments similar to those described above with LPS and fMLP, KIM127 antibody caused a highly significant increase in PNC formation compared to ADP alone that was apparent by 1 min (figure 8.8). A control antibody, Mouse IgG1 at 20μg/ml, had no influence on PNC formation.
From these experiments it would appear that neutrophil β-2 integrin activation increases PNC formation in response to platelet activation.

Figure 8.7
Expression of candidate molecules for neutrophil influence on PNC formation after stimulation with LPS. A representative experiment is shown. There are marked increases in CD11b expression and Mab24 binding following LPS stimulation. There were no significant changes in CD39 expression or PSGL-1 expression as assessed by the binding of PL-1 and PL-2 monoclonal antibodies.
Figure 8.8

%PNC after stimulation with ADP and co-stimulation ADP/KIM127.

A) %PNC at 30 min after ADP alone and the same samples stimulated with KIM127 at 20µg/ml and ADP (n=12, mean increase =8.2%, paired t-test p=0.013). 

B) Representative example of time course of response to KIM127 and ADP. Note that KIM127 causes no increase in %PNC in the absence of ADP and that an increased response to ADP with KIM127 is already apparent a 1 min.
8.3.4 Neutrophil and platelet parameters in Meningococcal septicaemia

Twenty-three patients with acute meningococcal disease were investigated. There were six deaths (26%) in this group, all occurring within 48 hours of admission. The Glasgow Meningococcal Septicaemia Prognostic Scores ranged from 1-15, with 13 cases being high risk (score 12-15, mortality 46%) and 9 low risk (8-11 mortality 0%). Only one case was investigated with a GMSPS of <8. First samples for analysis of platelet and neutrophil counts were taken a maximum of one hour after the first dose of parental antibiotic whereas samples for assessment of neutrophil and platelet activation markers were taken at a median of 8 hours (interquartile range 5-15 hr) after the first dose of parental antibiotic.

Median neutrophil count on presentation amongst survivors was 10.9 (range 1-36.6) compared to 0.50 (0.1 to 9.7) amongst non-survivors (Mann-Whitney test p=0.001). A similar pattern was observed with the median platelet count on presentation (survivors 102 (14-236), non-survivors 21.5 (6-81) p=0.002). When the product of the initial platelet and neutrophil counts were analysed the difference between groups become more marked: non-survivors 17.8 (0.6-261.9), survivors 915 (46-5192) (p<0.001) (table 8.3). In this series a product of platelet and neutrophil counts <40 on the first blood count provided a highly sensitive (83%) and specific (100%) predictor of outcome with a positive predictive value of 100%. The performance of a factor as a useful predictor is usefully expressed as the area under a curve of sensitivity plotted against 1-specificity for a series of threshold values – called a receiver operating curve. The closer the area to 1.0 the greater the performance of the predictor –representing a balance between specificity and sensitivity. Multi-factorial clinical prediction tools widely used in paediatric intensive care such as the paediatric index of mortality score (Shann 1997) an area under the receiver operating curves
of ~0.89. The total area under the receiver operating curve for the platelet neutrophil product in this population was =0.986.

Neutrophil CD62L was significantly lower in non-survivors (p=0.019). There was a non-significant trend towards greater expression of CD11b and β-2 integrin activation (Mab24 binding) in non-survivors. Of particular note is the observation that there was a trend (p=0.09) towards lower levels of the active form of free platelet GpIIb/IIIa (as assessed by PAC-1 MFI) in non-survivors than survivors.

Although %PNC were not significantly different between survivors and non-survivors (table 8.3), there was a significant negative correlation (Pearson correlation coefficient-0.59, p=0.013) between presentation %PNC and clinical disease severity as assessed by the GMSPS. Total neutrophil counts were also significantly correlated to GMSPS (-0.47, p=0.047), but no significant correlations were seen with platelet count, CD11b, CD62L, Mab24 and PAC-1 MFI.
Table 8.3

Univariate analysis of neutrophil and platelet parameters on the presentation blood count in clinical meningococcal disease. The median values and range are given and comparison between survivors and non-survivors made with the Mann-Whitney Test.

<table>
<thead>
<tr>
<th></th>
<th>Non-Survivors</th>
<th>Survivors</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>neutrophil count</td>
<td>0.5 (0.1-9.7)</td>
<td>10.9 (1.0-36.63)</td>
<td>0.001</td>
</tr>
<tr>
<td>platelet count</td>
<td>21.5 (6-81)</td>
<td>102 (14-236)</td>
<td>0.002</td>
</tr>
<tr>
<td>Neutrophil platelet product</td>
<td>17.75 (0.6-261)</td>
<td>915 (46-5192)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD11b MFI</td>
<td>153.3 (78.86-183.7)</td>
<td>89.94 (48.9-217)</td>
<td>0.052</td>
</tr>
<tr>
<td>CD62L MFI</td>
<td>20.50 (13.98-28.65)</td>
<td>54.26 (14.83-134.78)</td>
<td>0.019</td>
</tr>
<tr>
<td>Mab24 MFI</td>
<td>49.71 (33-134.4)</td>
<td>29.8 (17-58.82)</td>
<td>0.083</td>
</tr>
<tr>
<td>%PNC</td>
<td>13.6 (0.16-28.4)</td>
<td>14.3 (6.4-60)</td>
<td>0.49</td>
</tr>
<tr>
<td>Platelet PAC-1 binding MFI</td>
<td>7.2 (4.2-19.6)</td>
<td>19.6 (4.8-58.9)</td>
<td>0.09</td>
</tr>
</tbody>
</table>
8.3.5 Time course of changes in neutrophil and platelet parameters in meningococcal septicaemia

There are many difficulties inherent in interpreting the analysis of these samples that were collected from a range of patient ages, ethnicity, at different times of day with variable intervals between sample collections and analysis at differing time points in the natural history of the disease. Therefore, all the changes seen might simply represent viability in these factors. However, this type of study cannot be performed under ideal conditions due to the unpredictable nature of the acute clinical situation. This must be borne in mind with respect to the following observations.

The initial samples demonstrated markedly increased levels of the activation markers CD11b, and Mab24 binding in comparison to healthy laboratory controls or single system failure PICU patients. The percentage of platelet neutrophil complexes on the initial samples was lower than control levels. CD11b MFI, and the %PNC fell rapidly following admission (figs 8.9 & 8.10). Mab24 MFI also tended to fall but this trend failed to achieve significance with a second sample available in seven cases only. Neutrophil CD62L expression was less than control on presentation but shown a variable time course in reverting to normal. In cases in which continued sequential measurements were possible including out-patient review, (n=6), values of CD11b, Mab24 binding, %PNC and CD62L had returned to control levels by one month (fig 8.9).
The time course of free platelet activation was difficult to interpret (fig 8.11). As noted above there was a trend for higher levels of GpIIb/IIIa expression in eventual survivors on the first sample, but the subsequent course is very variable. This may be attributable to the frequent use of platelet transfusions in patients with disseminated intra-vascular coagulopathy and clinical bleeding. Therefore analysis on later samples is likely to be displaying a combination of the activation status of stored and endogenous platelets.

**Figure 8.9**

Time course of changes in neutrophil and platelet parameters in a case of severe meningococcal disease. A representative example of moderately severe disease (GMSPS =11) is shown. OPD = outpatient follow-up visit 28 days after first admission.
Figure 8.10

Time course of Neutrophil CD11b, Mab24 CD62L and %PNC in severe meningococcal disease. MFI are presented in arbitrary fluorescence units and PNC as a % of the neutrophil population. There are significant falls in CD11b Mab24 and PNC with time (Mann-Whitney tests). CD62L expression varies widely. Open circles represent eventual PICU survivors and filled circles represent samples taken from non-survivors. All deaths were attributable to overwhelming sepsis rather than brain stem herniation complicating meningitis. Control values reflect single system failure PICU cases examined concurrently and mean and 95% ci are shown.
Figure 8.11

Time course of Platelet activated GpIbb/IIIa expression in severe meningococcal disease. MFI is presented in arbitrary fluorescence units. As with figure 8.10 open circles represent PICU survivors and filled circles PICU deaths. No clear time course can be observed, perhaps due to the frequency with which therapeutic platelet transfusion is used to aid haemostasis.
8.4 Discussion

This chapter describes the investigation of the contribution that activated neutrophils make to the formation of platelet neutrophil complexes. These studies show that the expression of the activated form of the β-2 integrin CD11b/CD18 on neutrophils increases the likelihood of platelet neutrophil complex formation following contact with an activated platelet expressing surface CD62P. This effect is directly dependent on the CD11b/CD18 activation and not on other changes that might been seen with generalised neutrophil activation (e.g. CD62L loss) because it was seen following the use of specific agonists which cause direct ('outside-in') changes in β-2 integrin activation (KIM127).

These findings are consistent with the data presented in chapter 6 that neutrophils within platelet neutrophil complexes are a more activated sub-population. The current studies indicate that it is the more activated neutrophils which are more likely to bind to the activated platelets. The relative contribution of this selection effect and the influence of platelet derived activation of neutrophils is also addressed in chapter 6 with the use of the CD62P blocking monoclonal antibody G1 (figure 6.5). The importance of platelet derived neutrophil activation is apparent in that the increased Mab24 binding to neutrophils (figure 6.5B) seen in blood stimulated with ADP is dependent on CD62P function as it is completely abolished by G1 antibody. However ADP induced changes in CD11b are only partially inhibited by the presence of the G1 antibody. The complexity of these observations indicates that both mechanisms (if not also undefined processes) contribute to the formation of PNCs as an activated sub-population.

The findings presented here are consistent with studies of platelets in different in-vitro systems. Recent work in washed cell suspensions under dynamic shear stress indicate that platelet CD62P and the activated form
of neutrophil CD11b/CD18 contribute to PNC formation (Evangelista et al 1996). The platelet ligand for activated CD11b/CD18 may be fibrinogen bound to activated platelet GpIIb/IIIa (Kuijper et al 1997; Weber & Springer, 1997). The term 'cross-talk' was used to describe this series of interactions between platelets and neutrophils that occur in the formation of these complexes under these conditions (Evangelista et al 1996). These observations have not previously been made in whole blood. Studies of the binding of washed neutrophils to immobilised platelet monolayers have been reviewed in chapter 7 but again suggest a multi-step adhesion process requiring platelet CD62P and neutrophil CD11b/CD18 (Diacovo et al 1996b; Kuijper et al 1996b; Buttrum et al 1993b).

The studies described in cases of meningococcal disease revealed very high levels of neutrophil activation markers within a few hours of the first dose of parenteral antibiotics. As might have been anticipated there were greater levels of neutrophil activation (significantly lower CD62L, and trends towards increased CD11b and Mab24) amongst non-survivors than survivors. Of particular interest are the rapid alterations of the levels of all of these indicators towards normal with time. After 24 hours CD11b and Mab24 were approaching normal while %PNC fell from an initially wide range to low levels. This is consistent with the only published study of platelet neutrophil complexes in sepsis (Gawaz et al 1997). These changes were particularly clear in individuals in which multiple observations were possible (figure 8.9).

There is inevitably some difficulty in interpreting these data as clinical interventions such as platelet transfusions may have a significant influence on the parameters measured. However the observations made are consistent with our understanding of the processes involved. Early in the course of the disease there are high levels of circulating neutrophil activation from exposure to bacterial endotoxin. This level falls rapidly
with antibiotic therapy and fluid administration but also continuing endothelial activation will promote recruitment of those neutrophils with the more activated adhesion molecule profile.

Platelet neutrophil complexes may be seen at a wide range of levels initially as there is both widespread platelet activation as part of disseminated intra-vascular coagulopathy and neutrophil activation. However with time the adhesive properties of these PNC will mean that they are selectively recruited (see chapter 7, figure 7.6), in preference to unbound platelets and neutrophils, until endothelial adhesive function returns towards normal.

The observations that very early platelet and neutrophil counts relate very closely to outcome has been well described previously (Mok & Butt, 1996; Algren et al 1993; Busund et al 1993; Kornelisse et al 1997). The high performance of platelet and neutrophil counts as predictors of outcome in this series merits further attention. The usefulness of the GMSPS may be declining with more modern intensive care, for example in this small series the highest risk patients (GMSPS 12-15) exhibited only a 46% mortality. Collaborative work with two other tertiary PICUs is underway to refine clinical scoring by the addition of simple laboratory data such as the initial haematology in a population of 180 cases of meningococcal sepsis requiring intensive care in the last two years.

The trend towards lower levels of the active form of free platelet GpIIb/IIIa in non-survivors than survivors is interesting as again it suggests that the more activated platelets may be have been removed from the circulation by adhesion to other cells or adhesive proteins. These observations underline the potential difficulties of examining platelets in isolation that has been highlighted previously. A study of platelet parameters in an adult ICU population showed no relationship between GpIIb/IIIa activity and disease severity (Gawaz et al 1997).
In summary the work presented in this chapter demonstrates that platelet neutrophil complexes are the result of interactions between platelets, neutrophils and the vascular endothelium. Activation of adhesive functions on each cell type will influence the rate of formation, stabilisation and recruitment of PNCs. The observation that neutrophil $\beta$-2 integrin activation advances platelet binding in whole blood provides another mechanism by which platelets may be recruited into inflammatory processes such as acute sepsis. The studies in clinical meningococcal disease provide evidence of the rapid changes in circulating cell populations which reflect the rate at which the endothelium adjusts its adhesive functions. These studies are consistent with the hypothesis generated in chapter 7 that reduced PNCs reflect altered endothelial adhesive function. Future work to model the three-way interactions between these cell types in suspensions and under variable flow conditions is planned.
Chapter 9

Final Discussion

9.1 Platelets in Acute Inflammation

9.2 Questions addressed in this thesis

9.3 Assessing the validity and significance of this project

9.4 Conclusions and Future Work
9.1 Platelets in Acute Inflammation

The overlap between haemostasis and thrombosis is becoming apparent in clinical conditions. For example in ischaemic heart disease, where abnormal haemostasis has long been considered to be the primary pathology, inflammatory components are now recognised (Niebauer et al. 1999; Ridker et al. 1998; Vallance et al. 1997). As a consequence of this greater understanding, therapies with primarily anti-coagulant or fibrinolytic activity are being widely proposed in acute inflammatory conditions such as SIRS (Eisele et al. 1998; Zenz & Muntean, 1998; Smith et al. 1997).

The dual role of platelets in haemostasis and inflammation has been the subject of an increasingly large body of research over the last five years. Three major areas have been investigated 1) the mechanisms by which platelets may have pro-inflammatory roles involving adhesive mechanisms (Bombeli et al. 1998; Weber & Springer, 1997; Diacovo et al. 1996; Diacovo et al. 1994), 2) the production and effects of soluble or surface molecules (Henn et al. 1998; Kaplanski et al. 1993; Hawrylowicz et al. 1991; Hawrylowicz et al. 1989) and 3) the nature of interactions with other inflammatory cells (Evangelista et al. 1999; Cerletti et al. 1995; Chignard & Renesto, 1994). The work presented in this thesis has relevance to each of these three areas and has provided the basis for future studies addressing the interactions between platelets, neutrophils and the vascular endothelium in patients and laboratory models.

9.2 Questions addressed in this thesis

Is the host response important in the pathophysiology of ARDS?

The audit of acute hypoxaemic respiratory failure on paediatric intensive described in chapter 3 suggested that the nature of the host response to an inflammatory insult has a greater bearing on the outcome than the cause of
the insult. This can clearly been seen in the excellent prognosis for very severe respiratory failure in a previously well child and the very poor prognosis for even mild respiratory failure in the immunodeficient child.

\textbf{Can platelet activation be detected in the circulation of patients with ARDS?}

Circulating markers of inflammation and neutrophil activation (neutrophil elastase and soluble CD62L) (Donnelly \textit{et al} 1995 and Donnelly \textit{et al} 1994) have previously been demonstrated in cases of ARDS that have occurred as a result of a direct insult to the lungs (aspiration or infection) or a more generalised insult (trauma or burns). Therefore it was anticipated that circulating platelet activation might be detectable even in cases when inflammation was localised to the lung, especially in the context of severe immunodeficiency when platelets were hypothesised to play a role of particular significance.

The initial studies performed in this project on children with acute inflammation in the form of acute respiratory distress syndrome did not demonstrate significant changes in levels of circulating platelet activation. While there were a number of problems with this study because of the inevitable heterogeneity of the patient population and the therapies, one explanation may have been that many of the detectable platelets were those that had been transfused to aid haemostasis, while the most activated platelets had been recruited to inflammatory sites such as the pulmonary vasculature. Other explanations include lack of sensivity of the technique, an underpowered study, or that platelets are not significantly activated in this condition.

\textit{How could platelets be contributing to inflammatory processes?}

\textbf{A. Platelet interactions with neutrophils.}

The capacity of activated platelets to adhere to neutrophils was assessed and a flow cytometric method for investigation of platelet binding to neutrophils was developed. This method demonstrated that free platelet
flow cytometry underestimated the true extent of platelet activation (chapter 5). This technique of flow cytometric assessment of platelet neutrophil complexes was then used to investigate the properties of the neutrophils contained in these complexes. It appears that PNCs represent an activated sub-population of neutrophils and that part of this more activated profile is a consequence of contact with an activated platelet (chapter 6). A number of authors have suggested that neutrophil activation contributes to the formation or breakdown of platelet neutrophil complexes (Evangelista et al 1996; Chignard & Renesto, 1994). The contribution of neutrophil activation to platelet neutrophil complexes was investigated in chapter 8 when activated neutrophils were shown to be more likely to bind to an activated platelet via β-2 integrin function.

B. Platelet interactions with the vascular endothelium.
Recent data have revealed a range of interactions that occur between resting and activated platelets and vascular endothelial cells that were not previously appreciated (Bombeli et al 1999, Bombeli et al 1998). The interactions of platelets with cultured human umbilical vein endothelial cells were assessed in chapter 7. Platelets were not shown to induce endothelial adhesion molecule expression but did accelerate activated neutrophil binding to activated endothelium.

C Platelet modulation of neutrophil interactions with endothelium.
Work published during the preparation of this thesis demonstrated that immobilised platelets can support neutrophil rolling and firm adherence via a multi-step process (Weber & Springer, 1997; Diacovo et al 1996b). In addition to the observation that activated neutrophil adhesion to activated endothelium was increased in the presence of platelets, fewer platelet neutrophil complexes were detectable in the supernatant above activated than resting endothelium. One interpretation of this is that the platelet-neutrophil complexes adhere to the endothelium more readily than free neutrophils. This was anticipated from the more activated adhesion
molecule profile demonstrated in chapter 6. This interpretation is also consistent with the observation that the levels of circulating PNC in-vivo were directly related to vascular endothelial vasodilator function in a population of diabetic patients. The data presented in this thesis cannot distinguish between an effect of activated or injured endothelium which reduces the PNC in the immediate environment (e.g. via a soluble mediator), or selective recruitment of these PNC to adhere to the activated endothelium.

**How do platelets, neutrophils and endothelial cells interact in severe inflammation?**

Activated endothelium and activated neutrophils are both present in the acute systemic inflammatory response syndrome of meningococcal septicaemia. Assessment of PNCs in the acute and convalescent stages of this condition provided an insight into the dynamic interactions that are occurring between the three cell types. The relationship of poor endothelial function and low PNCs that had observed in the diabetic patients in chapter 7 was also seen in this complex scenario.

These findings may be interpreted in several ways. Firstly, the measured PNCs may reflect the state of endothelial adhesive function in-vivo. This may provide a method for assessing endothelial adhesive function alongside non-invasive testing of endothelial vasodilator function for the investigation of risk factors and therapies for ischaemic heart disease and related conditions. More interesting perhaps is the idea that platelet neutrophil complexes are not just chance associations of activated cells that passively reflect the state of endothelial function but that they contribute significantly to the pathophysiology of acute inflammation. One can imagine that PNCs provide an alternative method for rapid neutrophil recruitment to sites of endothelial injury, especially when the sub-endothelial matrix is exposed, that does not require the classical steps of endothelial activation (chemokine production and ICAM, VCAM
Discussion 219

upregulation). Such a concept may offer further support for the investigation of anti-platelet and fibrinolytic agents as therapies for acute inflammatory conditions.

9.3 Assessing the validity and significance of this project

The use of new and previously unvalidated methods has to be considered when interpreting the data presented in this project. The assessment of platelet neutrophil complexes in whole blood was a novel technique. However, the reproducibility of the findings presented in a number of different systems including in-vitro stimulation as well as different patient populations supports the validity of these investigations. Further support is provided by work from other institutions published during the course of this project proposing similar techniques (Li et al 1997) and observing similar patterns amongst related patient populations (Gawaz et al 1997; Ott et al 1996). In addition the investigations of neutrophil functions in platelet neutrophil complexes is consistent with work using soluble CD62P (Cooper et al 1994; Nagata et al 1993) and mouse macrophage phagocytosis (Mandell & Hook, 1969). The observations that neutrophil β-2 integrins contribute to platelet binding to neutrophils again is consistent with recently published data (Evangelista et al 1999; Weber & Springer, 1997) which employed different physical conditions including variable shear stresses and binding of flowing cells.

Human umbilical endothelial cells monolayers have been widely used (Kaplanski et al 1998b; Wang et al 1997b; Lorant et al 1995b; Bevilacqua et al 1989b) in many laboratories. It is difficult to be certain of the significance of findings generated using such an in-vitro model for pathophysiology. Indeed, we found that some of our results were not consistent with published data suggesting that activated platelets alone can induce changes in endothelial adhesion molecule expression (Henn et al 1998;
Hawrylowicz et al. (1991) but methodological differences may explain these variations. Further experiments are underway to investigate these effects.

9.4 Conclusions and Future Work

There is increasing evidence of the importance of platelets as inflammatory cells. In-vitro and in-vivo studies have suggested that a major mechanism for these effects is the direct binding of platelets to neutrophils via platelet CD62P and neutrophil β-2 integrin and that these complexes represent activated sub-populations of both cell types. Assessment of platelet neutrophil complexes may inform on the state of in-vivo endothelial adhesive functions. Future work is planned to assess further the relationship between vascular endothelial function and circulating platelet neutrophil complexes by the use of specific inhibitors of endothelial vasodilator function and in-vivo models of sepsis in human volunteers. The mechanisms of platelet-neutrophil-endothelial interactions are to be assessed using cell suspensions of all three cell types under dynamic conditions and flow systems employing platelet and endothelial cell monolayers.
References


Beekhuizen, H., Verdegaal, E.M., Blokland, I. & van Furth, R. (1992) Contribution of ICAM-1 and VCAM-1 to the morphological changes in monocytes bound to human venous endothelial cells stimulated with recombinant interleukin-4 (rIL-4) or rIL-1 alpha. *Immunology*, 77, 469-472.


controlled, double-blind trials with antithrombin III in severe sepsis .


References


Kaplanski, G., Marin, V., Fabrigoule, M., Boulay, V., Benoliel, A.M., Bongrand, P., Kaplanski, S. & Farnarier, C. (1998) Thrombin-activated human endothelial cells support monocyte adhesion in vitro following expression of intercellular adhesion molecule-1 (ICAM-1; CD54) and


the damaged vessel wall: cooperative substrates for neutrophil adhesion under flow conditions. *Blood, 89*, 166-175.


COMMENT

THE ROLE OF THE VASCULAR ENDOTHELium IN SEPSIS

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INTRODUCTION

Sepsis, the systemic response to infection, is defined by changes in body temperature, heart rate, respiratory rate and peripheral white cell counts. Severe sepsis, which can progress to multiple-organ dysfunction syndrome (MODS), is a major cause of morbidity and is the most common cause of death in intensive care units. Sepsis, and the similar process, systemic inflammatory response syndrome (SIRS) [1,2], which follows major insults such as trauma, pancreatitis or burns, is always associated with dysfunction of the vascular endothelium.

ENDOTHELIAL FUNCTION IN HEALTH AND DISEASE

In health, the vascular endothelium provides a thromboresistant surface, regulates local blood flow, controls the adhesion and migration of leukocytes and provides a semipermeable membrane which is essential for sustaining intravascular protein and fluid composition. Each of these functions of the endothelium are altered in SIRS and MODS. Disseminated intravascular coagulation (DIC), hypotension, leukopenia and 'capillary leak' represent the clinical consequences of severe disruption of each of these functions, and each is consistently associated with a poor outcome [3,4].

This review will describe the current understanding of the causes and nature of these changes in endothelial function. We will also describe recent attempts to manipulate endothelial functions for therapeutic benefit.

Leukocyte adhesion

The regulation of leukocyte traffic is critical to the control of infection and wound healing. Many of the molecular mechanisms that are involved in controlling leukocyte adhesion and migration have now been characterized. Three main stages have been identified involving three principal families of adhesion molecules: the selectins, the integrins and the immunoglobulin superfamily (Figure 1).

Leukocyte rolling

The first stage of 'leukocyte rolling' is principally mediated by the selectins: E-selectin (CD62E) and P-selectin (CD62P) present on endothelial cells and L-selectin (CD62L) present on leukocytes [5]. These molecules form transient bonds with their ligands resulting in leukocyte slowing. Leukocytes then have the opportunity to sample the endothelial microenvironment. In sepsis, this endothelial microenvironment will contain pro-inflammatory cytokines [e.g. interleukin (IL)-1, tumour necrosis factor (TNF)-α, IL-6] and chemokines (e.g. IL-8), which provide stimuli for the leukocyte to prepare for firmer adhesion [6,7].

Firm adhesion and transmigration

Firm adhesion and subsequent transendothelial migration of inflammatory cells is largely mediated by the leukocyte β₂-integrin molecules (principally LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) and by endothelial expression of the immunoglobulin superfamily [ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), VCAM-1 (CD106), PECAM-1 (CD31) and MadCAM-1] [5,8].

The selective recruitment of leukocyte subsets to sites of inflammation is orchestrated by the controlled production and interaction of different inflammatory mediators which are able to direct expression of adhesion molecules and mediator release at the endothelial cell surface. The differences in the kinetics...
Rolling adhesion  
Selectin mediated  
Leukocyte samples for activated endothelium; if endothelium provides a pro-inflammatory stimulus then adhesion progresses

Firm adhesion and transmigration  
Leukocyte integrin mediated  
Endothelial expression of immunoglobulin superfamily determines pattern of leukocyte recruitment

Figure 1. Stages of leukocyte adhesion to the endothelium. The endothelium controls; rolling adhesion via selectin expression, progression to firm adhesion via production of local pro-inflammatory stimuli, and the subset of leukocytes transmigrating via the pattern of immunoglobulin superfamily expressed.

of endothelial adhesion molecule expression, such as E-selectin, ICAM-1 and VCAM-1, induced by these mediators may contribute to the selective recruitment of leukocyte subsets to sites of inflammation [6,8,9].

Pro-inflammatory cytokines IL-1 and TNF-α induce expression of all three major adhesion molecules, E-selectin, ICAM-1 and VCAM-1, on endothelium. E-selectin is expressed early and transiently, peaking within 6 hours, and leads to preferential attraction of neutrophils. ICAM-1 and VCAM-1 expression occurs later (12-24 hours after stimulation) and is more sustained, resulting in attraction of lymphocytes and monocytes [10,11].

IL-4 is also an important regulator of endothelial cell function and recruitment of leukocyte subsets. Alone, it selectively enhances VCAM-1 [10,12], leading to adhesion of lymphocytes, monocytes, eosinophils and basophils but not neutrophils [13-16]. In combination with other cytokines, it modulates adhesion molecule expression and leukocyte subsets attracted to sites of inflammation. IL-4 inhibits TNF-α and IL-1-induced E-selectin and ICAM-1 but enhances VCAM-1, favouring the attraction of lymphocytes over neutrophils [10,17,18]. In contrast, interferon (IFN)-γ augments TNF-α-induced E-selectin expression and prolongs its half-life on the cell surface, thus favouring neutrophil attraction [19,20].

Recent evidence indicates that platelets may also regulate leukocyte-endothelial interactions directly [21] and indirectly via activated platelets binding to leukocytes [22]. Once bound to leukocytes, platelets can modulate both functional and adhesive properties [22]. Platelet-neutrophil complexes (PNCs) contain activated sub-populations of both cell types. Neutrophils in these complexes express more CD11b [22] and possess an increased capacity to produce toxic oxygen metabolites essential for microbial killing [23]. Platelets in these complexes express more CD62P and have an increased capacity to bind fibrinogen. In sepsis, these PNCs may be important in inflammatory cell recruitment, enhancing leukocyte and platelet adhesion to the vascular endothelium.
It is now clear that adherent platelets can support the processes of inflammatory cell adhesion and transmigration in similar fashion to endothelial cells [25,26].

Microorganisms may themselves be potent inducers of adhesion molecule expression. There are data to suggest that bacterial adherence is particularly important in regulating the levels of adhesion molecules on inflammatory cells. It is probably that leukocytes and platelets attracted to bacteria bound to the endothelium may contribute to the endothelial injury [27].

In summary, endothelial adhesion molecules are regulated by individual, or combinations of, cytokines, inflammatory cells and mediators, which may be additive, synergistic or antagonistic. This complex regulatory mechanism allows for precise expression of molecules needed for the endothelium to recruit the appropriate leukocyte to sites of inflammation and the maintenance of an inflammatory response.

HROMBORESISTANCE

The healthy vascular endothelium is thromboreistant [28,29]. This is disrupted in acute sepsis, when there is a reduction in anticoagulant and an increase in pro-coagulant properties (Table 1). This is apparent clinically as DIC, which is associated with a high mortality [3,4].

The mechanisms leading to DIC are complex but involve the activation of both fibrinolysis and coagulation [30]. The vascular endothelium is directly involved in both of these processes and markers of endothelial activation correlate with the severity of the DIC [31]. In vivo evidence of the sequence of events in the pathogenesis of DIC has been obtained from experiments in which endotoxin was administered to healthy volunteers [30,32-34]. Endotoxin induces an initial increase in fibrinolytic activity within 2 hours, principally via tissue plasminogen activator (t-PA). This is rapidly superseded by suppression of fibrinolysis mediated by raised levels of plasminogen activator inhibitor type 1 (PAI-1) [30]. Both t-PA and PAI-1 are produced by the vascular endothelium [5]. Following simple bolus endotoxin administration, abnormal fibrinolytic activity can persist for up to 24 hours [36].

The next detectable stage in DIC pathogenesis is the activation of the 'extrinsic' or tissue factor (TF) coagulation pathway [30]. TF is a potent pro-coagulant, small membrane protein which binds and activates factor VII leading to thrombin generation and clot formation. TF is expressed by endothelial cells and by monocytes adherent to activated endothelium [39]. The primary inhibitor of TF activity, TF pathway inhibitor (TFPI), is synthesized by healthy endothelium and expressed on the cell surface bound to glycosaminoglycans (GAGs) [38]. Bound TFPI levels are decreased in sepsis [38]. Clinical sepsis is a vastly more complicated picture than the response to a single dose of endotoxin, but observational studies have demonstrated similar processes in respect of the fibrinolytic [39] and TF [40] pathways in SIRS and MODS. Indeed, the importance of TF has been demonstrated in primate models of sepsis in which anti-TF or anti-factor VIIa blocking monoclonal antibodies were able to completely inhibit thrombin and fibrin production [41].

Diffuse, widespread clotting factor activation may present a serious clinical problem in the context of reduced fibrinolytic activity mediated by PAI-1. This process may be exacerbated by pro-inflammatory cytokines that downregulate constitutive endothelial anticoagulant molecules, including thrombomodulin [42-44] and GAGs [45] (Table 1).

The contact or 'intrinsic' coagulation pathway is also activated in acute systemic inflammation via factor XII, following contact with activated platelets, the sub-endothelial matrix at sites of endothelial damage, or Gram-negative bacterial surfaces [46]. Blockade of this pathway in a primate model did not alter DIC but attenuated the hypotension induced by experimental septicaemia (presumably by decreasing production of the vasodilator kallikrein which accompanies factor XII activation) [47].

The healthy endothelium has three principal mechanisms by which it resists platelet activation: endothelial cell surface expression of an ecto-ADPase (CD39) [48], constitutive endothelial nitric oxide (NO) production [49] and prostacyclin (PGI₂) production [50]. Endothelial activation may shift the balance towards promotion of activated platelet binding to fibrin, sub-endothelial matrix, other platelets and other inflammatory cells [49].
Table 1. Endothelium-derived factors which contribute to altered haemostasis in SIRS.

GAGs = glycosaminoglycans; iNOS = inducible nitric oxide synthetase. These changes in the haemostatic balance lead to intravascular thrombosis.

<table>
<thead>
<tr>
<th><strong>COAGULATION SYSTEM</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue factor (VIIa)</td>
<td>Increased on endothelial cells and endothelial bound monocytes</td>
</tr>
<tr>
<td>Factor XII</td>
<td>Activated by exposed sub-endothelium</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor</td>
<td>Primarily endothelium derived</td>
</tr>
<tr>
<td></td>
<td>Expressed on surface bound to GAGs</td>
</tr>
<tr>
<td></td>
<td>Surface bound TFPI decreased in sepsis</td>
</tr>
<tr>
<td>GAGs and associated molecules</td>
<td>Anti-thrombin III and heparin co-factor II</td>
</tr>
<tr>
<td></td>
<td>Lost from endothelial surface</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>Decreased endothelial expression</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th><strong>FIBRINOLYTIC SYSTEM</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue plasminogen activator</td>
<td>Initial increase in activity then suppressed</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>Raised</td>
</tr>
<tr>
<td>Protein C &amp; S</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>PLATELETS</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD39 (ecto-ADPase)</td>
<td>Endothelial inhibitor of platelet activation; decreased expression following activation</td>
</tr>
<tr>
<td>Prostacyclin (PGI\textsubscript{2})</td>
<td>Decreased production</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Change to iNOS dependent production. ?resultant effect on haemostasis</td>
</tr>
</tbody>
</table>

**LOCAL BLOOD FLOW**

The normal metabolic activity of the vascular endothelium exerts a vasodilator influence. Although many factors influence vascular tone, endothelium-dependent nitric oxide (NO) production is principally responsible for basal vasodilatation. Endothelial cyclo-oxygenase activity, producing PGI\textsubscript{2}, thrombox-

\[ \text{ane A}_2 \] and similar molecules (prostanoids), further contributes to vasoactive and antiplatelet effects. These basal effects can be massively exaggerated in sepsis, when pro-inflammatory cytokines induce a widespread increase in NO synthetase [51] and cyclo-oxygenase activity [52]. These changes contribute to a dysregulated microcirculation with redistribution of organ blood flow, a generalized reduction.
in vascular resistance and intravascular pooling [53]. The consequences of these effects are clear in SIRS. Excessive vasodilatation is a principal component of the pathogenesis of shock. In addition, the decreased local blood flow accelerates clotting factor activation and intravascular thrombus formation, which contributes to organ hypoperfusion and MODS [53].

The endothelium does not recover promptly from an inflammatory insult and low levels of vasodilator production persist well into recovery, a process that has been termed 'endothelial stunning' [49,54]. The concept that endothelium-dependent vasodilatation and platelet inhibition may be attenuated following sepsis has been proposed as an explanation for the apparent increase in risk for acute cardiovascular events after systemic insult [49].

Endothelial permeability

Normal vascular permeability is maintained by a combination of endothelial cell integrity and basement membrane characteristics, which allows the free passage of water and small molecules while confining larger molecules to the intravascular compartment [55].

The ability of the endothelium to regulate permeability is dictated by the presence of cell-surface binding proteins, the inherent leakiness of intracellular junctions, the composition of subendothelial matrix proteins and endothelial cell membrane surface charge [55,56]. The specific contribution of each of these components to vascular permeability varies with different blood vessels and the presence of inflammatory stimuli. However, the presence of negatively charged molecules, such as GAGs, on the luminal surface appears to be of importance [56,60].

Patients with septic shock invariably have a profound 'capillary leak' and may require resuscitation with as much as three times their circulating volume in 24 hours. The pro-inflammatory cytokines IL-1, TNF-α and IFN-γ directly alter the endothelial permeability [45]. These effects are compounded by release products from adherent inflammatory cells.

Integration of endothelial functions

The principal functions of the vascular endothelium as described in this review are highly integrated. This is particularly apparent in sepsis, when all functions are disrupted. For example, loss or saturation of platelet activation inhibition contributes to fibrinogen binding and clotting factor consumption. However, as the same receptors are also involved in platelet and neutrophil adhesion, inflammatory cell recruitment will be affected [25]. A further example of integration of function occurs with the release of endogenous vasodilators, which contribute to intravascular pooling and may encourage inflammatory cell recruitment. This effect would be exaggerated by colloid loss increasing plasma viscosity [53]. The extent of this overlap between different endothelial functions explains the rapidity with which deficiency in one area leads to the familiar spiral of MODS.

Therapeutic potential

A range of soluble factors have been investigated in phase II or III clinical trials, which have been designed to attenuate SIRS. Antibodies to TNF-α, soluble TNF-α receptors, anti-endotoxin antibodies and IL-1 receptor antagonists have all been disappointing [61]. Therapies aimed at endothelium-derived mediators, including anti-prostaglandin treatments [62], NO synthetase inhibitors [63-65] and blocking antibodies against adhesion molecules, have yet to demonstrate any efficacy in the clinical situation. Early studies targeting the fibrinolytic pathways by administration of recombinant t-PA [64,66] or protein C [67-69] appear promising. The future may well see a complex cocktail of therapies to modulate adhesion, promote fibrinolysis, replace clotting factors and limit NO production while balancing immune functions with appropriate cytokine administration [2,70].

Conclusion

The vascular endothelium may be considered as the tissue responsible for many of the clinical consequences of SIRS. Disruption of its normal function by circulating mediators and inflammatory cell adhesion alters its ability to maintain coagulation homeostasis, control local blood flow, maintain fluid balance and regulate inflammatory cell adhesion.
REFERENCES


[12] Beekhuizen H, Verdegai EM, Blokland I, van Furth R. Contribution of ICAM-1 and VCAM-1 to the morphological changes in monocytes bound to human venous endothelial cells stimulated with recombinant interleukin-4 (rIL-4) or rIL-1 alpha. Immunology 1992; 77: 469-72.


- CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells.

[22] Peters M, Heyderman RS, Hatcher DJ, Klein NJ.
- Investigation of neutrophil-platelet interactions in whole blood by flow cytometry.

[23] Nagata K, Tsuji T, Todoroki N et al.
- Activated platelets induce superoxide anion release by monocytes and neutrophils through P-selectin (CD62).

- Platelet function in septic multiple organ dysfunction syndrome.

- Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin GDIb/CD18.

- Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to α5β3 and stimulated by platelet-activating factor.
  J Clin Invest 1997; 100: 2085-93.

- The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by Neisseria meningitidis.

[28] Heyderman RS.
- Sepsis and intravascular thrombosis.

[29] Levi M, ten Cate H, van der Poll T, van Deventer SJ.
- Pathogenesis of disseminated intravascular coagulation in sepsis.

- Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways.

- Plasma levels of soluble E-selectin in patients with disseminated intravascular coagulation.

- Activation of the contact and fibrinolytic systems after intravenous administration of endotoxin to normal human volunteers: correlation with the cytokine profile.

[33] Suffredini AF, Harpel PC, Parrillo JE.
- Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects.

[34] Pajkrt D, van der Poll T, Levi M et al.
- Interleukin-10 inhibits activation of coagulation and fibrinolysis during human endotoxemia.
  Blood 1997; 89: 2701-5.

- Activated protein C stimulates the fibrinolytic activity of cultured endothelial cells and decreases antiactivator activity.

- Inhibition of endotoxin-induced activation of the coagulation and fibrinolytic pathways using a recombinant endotoxin-binding protein (rBPI23).

[37] Collins PW, Noble KE, Reittie JR, Hoffbrand AV, Pasi KJ, Yong KL.
- Induction of tissue factor expression in human monocyte/endothelial cocultures.

[38] Bajaj MS, Bajaj SP.
- Tissue factor pathway inhibitor: potential therapeutic applications.

- Plasminogen activator inhibitor 1 and 2, alpha-2-antiplasmin, plasminogen, and endotoxin levels in systemic meningococcal disease.

[40] Shimura M, Wada H, Waki Y et al.
- Plasma tissue factor and tissue factor pathway inhibitor levels in patients with disseminated intravascular coagulation.
Investigation of platelet-neutrophil interactions in whole blood by flow cytometry

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Abstract

Evidence is increasing that platelets can initiate and propagate inflammatory processes by interacting with leucocytes and the vascular endothelium. Platelets have been shown to bind to neutrophils, existing as platelet/neutrophil complexes (PNC) within the circulation. We describe a simple flow cytometric method for assessing and investigating platelet interactions with neutrophils in small volumes of whole blood. Twenty-five percent (sd 6%) of circulating neutrophils from healthy adults were associated with platelets. Formation of these platelet-neutrophil complexes was CD62P (P-selectin) and divalent cation dependent. Platelet activation (with ADP or thrombin) caused a rapid and sustained rise in %PNC which differed from the pattern of free platelet activation as assessed by CD62P expression. F-met-leu-phe induced neutrophil activation but did not increase the percentage PNC. Platelet activation also caused increased neutrophil CD11b/CD18 expression which was most marked on neutrophils complexed with platelets. This straightforward technique is simple, reproducible, and allows assessment of platelet-neutrophil interactions and activation of neutrophils. It may also provide a method for estimating platelet activation in whole blood. © 1997 Elsevier Science B.V.

Keywords: Platelets; Neutrophils; Adhesion molecules; Flow cytometry

1. Introduction

Platelets have the capacity to initiate and propagate inflammatory processes through the release of cytokines and growth factors (Hawrylowicz et al., 1989; Chignard and Renesto, 1994) and by interacting directly with leucocytes (de Bruijne-Admiraal et al., 1992; Palabrica et al., 1992; Nash, 1994) and the vascular endothelium (Roth, 1992; Diacovo et al., 1996a,b). While the role of platelets in inflammation has been analysed extensively in-vitro, studies in patients have been limited by the propensity for platelets to become activated during preparation for analysis (Shattil et al., 1987; Abrams and Shattil, 1991). Despite these difficulties, a number of methods exist for the assessment of platelet activation including the measurement of soluble release products (β-thromboglobulin and thromboxane A2, Kaplan, 1980; Reilly et al., 1986) functional assays...
126

(platelet aggregometry, Wu and Hoak, 1974), radio-
labelled fibrinogen binding assays (George et al.,
1986), and platelet density estimations (van Oost et
al., 1983). Over the last five years flow cytometric
analysis, with monoclonal antibodies to the platelet
surface glycoproteins CD42b (GpIb), the activated
form of GpIIb/IIIa and the surface bound α-granule
membrane protein CD62P (P-selectin, Shattil et al.,
1987; Warkentin et al., 1990) has been widely used.
This technique requires little preparation and pro-
vides reproducible data. While this method has many
theoretical advantages, its value in the clinical setting
is still under evaluation.

Recently there has been considerable interest in
platelet interactions with other cells including neu-
trophils, monocytes, lymphocytes and endothelial
cells (Rinder et al., 1991a,b). The relevance of these
interactions has yet to be fully determined, but com-
unication between platelets and these inflammatory
cells is likely to provide an important link between
thrombosis and inflammation. The demonstration of
increased platelet–neutrophil complexes in experi-
mental models of systemic inflammation (Rinder et
al., 1992) suggests that analysis of such complexes
may provide an additional means of assessing platelet
activation.

We have developed a simple and reproducible
method for the measurement of platelet–neutrophil
complexes (PNC) in whole blood. This technique
also allows simultaneous measurement of platelet
and neutrophil antigens, providing a means of exam-
ing platelet/neutrophil activation in the early stages
of severe inflammation.

2. Materials and methods

2.1. Materials

Monoclonal antibodies were purchased as fol-
loows: fluorescein isothiocyanate (FITC) labelled IgGl
irrelevant control antibody and phycoerythrin (PE)
labelled IgG2a anti-CD42b from DAKO, High
Wycombe. FITC labelled IgGl anti-CD11b and FITC
labelled IgG1 anti CD62P from Serotec, Oxford.
FITC labelled IgG1 anti CD66b from Pharmingen,
Cambridge, and double-stained (FITC/PE) IgG1 and
IgG2a negative control from Becton Dickinson, Ox-
ford. Adenosine diphosphate (ADP), thrombin, gly-
pro–arg–pro and F–met–leu–phe (FMLP) were pur-
chased from Sigma, Poole, and FACSlyse solution
from Becton Dickinson. CD11 and CD18 antibodies
were kindly provided by Dr. N. Hogg, ICRF, Lon-
don (H52, CD11a), and Dr. M. Robinson, Celltech
Therapeutics, Slough (KIM 247, CD11b and 6.5E;
CD18).

2.2. Samples

Blood was drawn from healthy volunteers via a
21G butterfly needle without the use of a tourniquet.
The first 2 ml of blood were discarded, and a further
2 ml taken and immediately added to 200 μl of
sodium citrate (3.8%).

2.2.1. Measurement of free platelet CD62P

Sample preparation for the assessment of free
platelet CD62P expression was as previously de-
scribed (Shattil et al., 1987; Warkentin et al., 1990).
Briefly, 5 μl of blood were added to 50 μl of
platelet buffer (10 mmol/l HEPES, 145 mmol/l
NaCl, 5 mmol/l, 1 mmol/l MgSO4, pH 7.4), con-
taining 5 μl of anti CD62P or control IgG1 antibody.
Following gentle resuspension, the sample was incu-
bated at room temperature for 20 min before the
addition of 250 μl of fixative (0.2% formaldehyde in
phosphate buffered saline (PBS) pH 7.4). Flow cyto-
metric analysis was performed within 1 h of fixation.

2.2.2. Measurement of platelet–neutrophil complexes

50 μl of blood were added to 5 μl of anti CD42b
(PE) and 5 μl of anti CD11b (FITC) or isotype
control antibodies in a polypropylene FACS tube,
within 10 min of sampling. Following gentle mix-
ing, the samples were left at room temperature for 10 min
before the addition of 200 μl of FACSlyse (containing
a final concentration of ~ 1.5%, formaldehyde, and
~ 5% diethylene glycol). Following gentle re-
suspension the samples were incubated for a further
10 min at room temperature before the addition of
500 μl of the platelet fix solution. Samples were
analysed by flow cytometry within 1 h of prepara-
tion.

2.3. Antibody concentration

All the antibody concentrations used were those
that achieved saturation under appropriate condi-
Fig. 1. Fluorescence profiles of a typical sample prepared for measurement of platelet/neutrophil complexes stained with (A) isotype control antibody and (B) anti-CD11b (HTC1 FL1) and anti-CD42b (PE) FL2. Events staining positive for both markers are those falling in the upper right quadrant which represent neutrophils with bound platelets (PNC), in this case 24% of the total neutrophils. (C) Shows the change in staining pattern one minute after the addition of ADP (10 μM) when 43% of the total number of neutrophils are PNC. Note that the PNC population is increased in total number following stimulation (C) but also in mean CD42b expression (suggesting a greater number of platelets complexed to each neutrophil) and in mean CD11b expression.
Fig. 2. The responses of platelet–neutrophil complexes to stimuli to platelet activation (ADP, thrombin) and neutrophil activation (FMLP). Values shown are the means and 95% confidence intervals (n = 14 experiments ADP and FMLP stimulation and n = 5 thrombin stimulation). In (A)–(C) the symbol ' indicates statistical significance difference from unstimulated samples p < 0.05, and ** indicates p < 0.01. (A) % PNC rapidly rises following ADP or thrombin stimulation but is not significantly changed by FMLP. (B) The mean number of platelets complexed to each neutrophil (estimated by the CD42b mean fluorescence intensity amongst PNC) is increased by ADP (MFI increased by factor of 3.5 × control at 1 min and 3 × control at 30 min). (C) Both ADP and FMLP cause a rapid and sustained increase in PNC CD11b expression compared to control. (D) CD11b expression is significantly greater in neutrophils complexed to platelets both at rest and following ADP stimulation. FMLP stimulation increases CD11b expression in bound and unbound neutrophils and the ratio of bound to unbound becomes non-significant. The symbols ' and ** indicate the same p values described above but in comparison to unity (i.e., no difference between complex and free CD11b expression).
tions. The antibody used to detect CD11b was titrated against FMLP activated neutrophils, and saturating concentrations were achieved using the volume of antibody recommended by the suppliers. The antibody against CD42b was tested on resting platelets and was used at a final concentration of 7.5 μg/ml. The antibody against CD62P was titrated against thrombin stimulated platelets and was used at half the volume recommended by the suppliers.

2.4. Flow cytometric analysis

Flow cytometric analysis was performed on Beckton Dickinson FACSan and FASCalibur. Data was then collected on FITC fluorescence at 515 nm and phycoerythrin fluorescence at 580 nm.

2.4.1. Measurement of free platelet CD62P

Forward and side scatter measurements were made with gain settings in logarithmic mode and the platelet population easily identified on forward and side scatter characteristics (Fig. 1a). Platelet sized events were selected on the forward/side scatter profile and the accuracy of the gating confirmed by staining with CD42b (> 98%). CD62P fluorescence was compared to background staining with IgG1 control
antibody and expressed as percentage of antibody-positive platelets, defined as those with a fluorescence intensity exceeding that of 98% of the platelets stained with control antibody.

2.4.2. Measurement of platelet–neutrophil complexes

Forward and side scatter measurements were made with gain setting in linear mode for the analysis of platelet/neutrophil interactions. The neutrophil population was easily distinguished on this view. Results were compared to isotype matched (IgG1 and IgG2a) antibody staining and considered positive if the fluorescence intensity exceeded that of 98% of the control antibodies (Fig. 1a). Events staining positive for both CD11b and CD42b were considered to represent PNCs and were distinguishable from events staining positive for CD11b alone (Fig. 1b).

The mean fluorescence intensity (MFI) of CD42b expression in the population staining positive for both CD11b and CD42b (Fig. 1b) has been reported to reflect the number of platelets complexed with each neutrophil (Rinder et al., 1991a). We compared the MFI of CD42b amongst PNCs in the resting state, with that amongst PNCs following stimulation.

CD11b expression on neutrophils increases with activation. We were able to compare the level of CD11b expression by the free and bound neutrophils and examine responses to exogenous stimuli.

2.5. Experiments

Analysis was performed on the blood of apparently healthy, non-smoking adults. Investigations included assessment of %PNC in fresh whole blood or following platelet stimulation with ADP, to a final concentration of 10 µM, or thrombin (final concentration 0.4 units/ml in the presence of gly-pro-arg-pro 1 mM to prevent fibrinogen cross-linking), and neutrophil stimulation with 1 µM FMLP. The effect of a delay in sample preparation was analysed at rest or following gentle mechanical agitation at 10 Hz on a rotary shaker. The kinetics of PNC formation and CD62P expression following stimulation were investigated in the same manner. Finally, the effects of the addition of blocking monoclonal antibodies to molecules known or suspected to be involved in these interactions were investigated.

2.6. Statistics

All experiments were performed on at least four occasions. Data were analysed and expressed as mean ± standard deviation and compared using paired Student’s t-tests. Analysis and graphical preparation was performed using Microsoft Excel 7.0 software.

3. Results

3.1. Platelet–neutrophil complexes: resting

The accuracy of the identification of neutrophils by forward and side scatter was confirmed by staining with an antibody to CD66b, a specific granulocyte surface marker. More than 98% of cells gated on these characteristics of size and granularity were CD66b positive. Analysis of 27 healthy adults showed that 25% ± 6% (mean, sd) of neutrophils within this gate were also positive for the platelet marker CD42b. These represent the percentage of neutrophils associated with platelets (%PNC).

This figure of 25% PNCs was not altered by the collection of blood into prostacyclin (final conc. 2 ng/ml) to exclude an ex-vivo platelet activation artefact.

In experiments in which %PNC was examined in freshly collected blood at intervals of up to 60 min after incubation on the bench at room temperature, there was a significant increase in the %PNC by 45 min. Simultaneous examination of the same blood samples for unbound platelet CD62P showed similar kinetics with a significantly increased expression of CD62P by 60 min. In similar experiments in which the blood was agitated at room temperature throughout the incubation, little change was seen in CD62P expression despite an increase in %PNC formation.

3.2. Platelet–neutrophil complexes: Platelet stimulation

Blood samples were collected as described and one aliquot was left as a control with ADP added to the second. Samples were then analysed 1 and 30 min later after incubation at room temperature. In ADP stimulated samples there was a dramatic increase in the %PNC. One minute after stimulation
the mean percentage of neutrophils complexed to platelets rose to 45.3% ± 14.2% (P < 0.0001, Fig. 1c and Fig. 2a) and by 30 min had reached 67.9% ± 18.5% (P < 0.0001, Fig. 2a). Thrombin stimulation produced similar effects (Fig. 2a).

Estimation of the number of platelets complexed with each neutrophil, as assessed by platelet CD42b MFI within PNC, revealed a significant increase in platelets following stimulation with ADP compared to the resting state. At 1 min the mean CD42b MFI amongst PNCs after ADP stimulation was 3.52 (95% confidence interval = 3.2–3.9) times that amongst unstimulated PNCs. This remained elevated at 30 min (Fig. 2b).

CD11b MFI expression was recorded in the individual populations of unbound neutrophils and those complexed with platelets. ADP stimulation caused a significant increase in CD11b expression at 1 and 30 min amongst bound neutrophils. At 1 min after addition of ADP the mean PNC CD11b expression was 1.74 (95% c.i. = 1.53–1.95) times the control (P < 0.0001), and at 30 min 1.36 (1.22–1.50) times the control (P < 0.0001, Fig. 2c). Mean CD11b expression was greater on those cells complexed to platelets than on unbound neutrophils both at rest and following ADP stimulation (Fig. 2d). This pattern persisted at 30 min.

3.3. Platelet–neutrophil complexes: FMLP stimulated

Samples were also examined after stimulation with FMLP at a final concentration of 1 μM. As expected, FMLP caused a significant mean increase in PNC CD11b expression, to more than double that seen on unstimulated cells at 1 and 30 min (Fig. 2c). In contrast to ADP stimulation (Fig. 2d), FMLP stimulation induced similar levels of CD11b in both bound and unbound. There was no significant change in %PNC following incubation with FMLP (Fig. 2a).

3.4. Kinetics of PNC formation and free platelet CD62P expression following stimulation

Freshly sampled whole blood was prepared for examination for PNC and unbound platelet CD62P immediately (0 min) or sampled at intervals following incubation at room temperature with ADP or thrombin. These experiments were performed with and without mechanical agitation. Unbound platelet CD62P expression increased significantly by 1 min

![Fig. 3. Kinetics of percentage platelet–neutrophil complex formation and free platelet CD62P expression in unagitated sample following stimulation with ADP. The means of eight experiments with 95% confidence intervals are shown. In all cases there was a rapid rise in CD62P expression within one minute followed by a steady lower level of expression. In contrast, the %PNC increased significantly by one minute and continued to rise throughout the study period in all cases.](image-url)
Fig. 4 Influence of co-incubation CD11a/CD11b/CD18 and CD62P blocking monoclonal antibodies. No significant effect on the baseline level of PNC formation was demonstrated with any antibody. Only G1 against CD62P influenced the formation of new PNCs in a dose dependent manner. A representative experiment is shown.

but plateaued (unagitated, Fig. 3) or fell (agitated, data not shown) thereafter. This contrasts to the %PNC which continued to increase throughout the period of investigation.

3.5. Mechanisms of platelet/neutrophil interactions

When blood was collected into EDTA the increase in %PNC formation observed in response to ADP was greatly attenuated (n = 6, mean increase = 2.6%, not significant). Co-incubation with anti-CD62P antibody (clone G1), also inhibited new complex formation in a dose dependent manner (Fig. 4). Antibodies to CD11a/CD18 had no demonstrable effect in this system (Fig. 4). The resting percentage of platelet-neutrophil complexes was not affected in these experiments.

4. Discussion

In this study we present a novel method of analysing platelet activation and investigating platelet/neutrophil interactions in whole blood. We found consistently that at rest approximately 25% of the neutrophils were complexed with platelets in 27 healthy adults. Following platelet stimulation there was a rapid and sustained increase in complex formation as shown by both the percentage of total neutrophils in complexes and the number of platelets adhering to each neutrophil.

The existence of platelet neutrophil complexes has been demonstrated in several studies (Rinder et al., 1991a,b; Spangenberg, 1994), irrespective of the methodology employed. To date, studies examining neutrophil/platelet interactions have largely used separated cells or repeatedly washed cells and have recorded PNC values of between 3.6% (de Bruijne-Admiraal et al., 1992) and 34% (Rinder et al., 1991a). In the only other method described for assessment of PNCs in whole blood, Rinder et al. (1991b) found a resting level of 39% PNC formation. This is of the same order of magnitude as that found in the present study. However the sample preparation employed required initial fixation for 60 min and three subsequent washing steps, a process which would have proven prohibitively long for our intended uses. The method we describe avoids cell separation, fixation prior to staining and multiple washing steps. This method follows the principles of the Shattil et al. (1987) method for the analysis of free platelets with
minimal sample processing prior to analysis. All the studies to date have employed a specific platelet marker for identification of the platelet component of PNCs. It is possible that some events so identified do not represent intact platelets but platelet microparticles. This may explain the presence of the small population of events which are positive for CD42b when compared to the isotype control, but remain distinct from the major population of CD42b positive cells. We are currently investigating the nature of this population in more detail.

The mechanisms of PNC formation have emerged in recent years and it is now known that platelet derived CD62P is a key molecule involved in platelet neutrophil adhesion (Larsen and Celi, 1989; de Bruijne-Admiraal et al., 1992). Contained in the alpha granules of resting platelets, CD62P is translocated to the surface upon activation, allowing binding to counter-receptors on the surface of neutrophils. In agreement with previous work we have shown using this system that CD62P is central to platelet neutrophil complex formation. This is also supported by the increase in PNC in response to platelet agonists and the inhibition of PNC in the absence of divalent cations.

The role of neutrophils in these interactions is less clear. There is evidence that neutrophil stimulation can influence platelet neutrophil binding with reports of both increased (Rinder et al., 1992) and decreased (Evangelista et al., 1993; Rinder et al., 1994; Lorant et al., 1995) complex formation. This may be due to the release of granule contents of both cell types in response to neutrophil stimulation. In this study we were unable to demonstrate a major role for neutrophils in the initiation of PNC formation. Neither neutrophil stimulation nor co-incubation with antibodies to CD11/CD18 caused a significant change in the percentage of neutrophils associated with platelets. However neutrophil stimulation did result in a small but consistent decrease in the number of platelets bound to each neutrophil. In view of the recent evidence that neutrophil binding to (and transmigration across) immobilised platelets requires both CD62P and CD11b/CD18 (Diacovo et al., 1996a) it is clear that the mechanisms involved in mediating platelet neutrophil interactions are affected by both the physical state and experimental conditions employed for examining these cells.

In studies using separated cells, activated neutrophils have been shown to activate platelets, causing the release of platelet granule contents (Evangelista et al., 1993) and formation of platelet aggregates (Chignard and Renesto, 1994). There is also evidence that neutrophil release products such as proteases may influence platelet neutrophil binding (Chignard and Renesto, 1994). Such effects were not apparent in our system. Further studies are required to elucidate the reason for the discrepancies between the whole blood system described here and separated cell systems used in previous studies.

Interestingly, while antibodies to β2 integrins were not shown to influence PNC in this study, there was a significant increase in neutrophil CD11b expression in response to platelet stimulation. Activated platelets are known to be able to stimulate neutrophil superoxide anion production (Nagata et al., 1993), chemiluminescence (Ruf et al., 1992) and the release of granule products. Platelet induced expression of neutrophil adhesion molecules however, has not been previously described. CD11b expression was greatest in those neutrophils which were complexed with platelets, confirming the importance of cell to cell contact noted in previous studies. In view of the importance of CD11b in neutrophil--endothelial interactions these observations further support the view that platelets are likely to have a role in the earliest stages of inflammatory cell adhesion (Palabrica et al., 1992; Diacovo et al., 1996a,b).

Our results in conjunction with other studies suggest that PNCs are of physiological importance. Indeed platelet interactions with endothelial cells, neutrophils and monocytes are likely to be essential events in inflammation. Platelets that are not adherent to other inflammatory cells and remain free in the circulation may not actually reflect the overall state of platelet activation. In experiments comparing free platelet expression of CD62P and %PNC formation over time following a stimulus to activation, we observed a continued increase in %PNC following stimulation. In contrast, free platelet CD62P expression reached an early peak and then remained at the same level or fell for the remainder of the experiment in accordance with previous studies of Rinder et al. (1991b). The experiments in which samples that were continually agitated demonstrated the op-
posing trends in CD62P expression and %PNCs formation more clearly than the experiments with incubations at rest. This phenomenon can be explained if unbound activated platelets are randomly contacting and progressively binding to neutrophils over the time course of the experiment. This would have the effect of increasing the %PNCs whilst at the same time removing the activated platelets from the unbound platelet population. As a result, unbound and therefore less activated platelets, analysed by conventional flow cytometry, would not necessarily reflect changes in the activation status of the platelet population overall.

Underestimation of platelet activation as a result of binding to other cells was considered in the original descriptions of platelet flow cytometry by Shattil et al. (1987). This was discounted since the percentage of particles staining positive for platelet markers outside the unbound platelet gate did not change following stimulation. However, the method used in these studies ignores the possibility that multiple platelets may, and indeed are likely to, adhere to a single neutrophil (Rinder et al., 1991a). This complex (PNC) would then be recorded as a single event leading to an inevitable underestimation of the proportion of platelets falling outside the unbound platelet gate. The percentage of the total platelet number that are actually bound to other cells may therefore be considerably in excess of the 5% figure estimated by Shattil et al. (1987). In addition, there may be an excess of 100 times more circulating platelets than neutrophils in blood. Therefore a small change in the percentage of platelet events outside the free platelet gate may actually reflect an enormous change in % of PNC. This may correspond to a highly activated sub-population of the total platelet pool which may be of physiological importance.

The method described here is a simple but effective way of assessing platelet interactions with neutrophils. From each sample it is possible to measure the percentage of total neutrophils complexed with platelets, to estimate the relative number of platelets in association with each neutrophil (CD42b mean fluorescence amongst PNC complexes), to elucidate neutrophil activation status (CD11b expression) and to determine the differential states of neutrophil activation between bound and unbound neutrophils. We believe that the assessment of PNCs provides important additional information to supplement that available with free platelet flow cytometry alone. The technique is also ideally suited to the investigation of the molecular mechanisms involved in platelet/neutrophil interactions. The very small volumes of blood required permits repeated sampling of individual patients to investigate the significance of platelet–neutrophil complexes in the pathogenesis of conditions such as acute sepsis, the acute respiratory distress syndrome and systemic inflammation following cardio-pulmonary bypass.

Acknowledgements

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References


Acute hypoxemic respiratory failure in children: case mix and the utility of respiratory severity indices

Abstract

Objective: Acute hypoxemic respiratory failure (AHRF) is a common reason for emergency pediatric intensive care. An objective assessment of disease severity from acute physiological parameters would be of value in clinical practice and in the design of clinical trials. We hypothesised that there was a difference in the best early respiratory indices in those who died compared with those who survived.

Design: A prospective observational study of 118 consecutive AHRF admissions with data analysis incorporating all blood gases.

Setting: A pediatric intensive care unit in a national children's hospital.

Interventions: None.

Results: Mortality was 26/118, 22% (95% confidence interval 18–26%). There were no significant differences in the best alveolar-arterial oxygen tension gradient (A-aDO₂, torr), oxygenation index (OI), ventilation index (VI), or PaO₂/FIO₂ during the first 2 days of intensive care between the survivors and non-survivors. Only the mean airway pressure (MAP, cm H₂O) used for supportive care was significantly different on days 0 and 1 (p ≤ 0.05) with higher pressure being used in non-survivors. Multiple logistic regression analysis did not identify any gas exchange or ventilator parameter independently associated with mortality. Rather, all deaths were associated with coincident pathology or multi-organ system failure, or perceived treatment futility due to pre-existing diagnoses instead of unsupportable respiratory failure. When using previously published predictors of outcome (VI > 40 and OI > 40; A-aDO₂ > 450 for 24 h; A-aDO₂ > 470 or MAP > 23; or A-aDO₂ > 420) the risk of mortality was overestimated significantly in the current population.

Conclusion: The original hypothesis was refuted. It appears that the outcome of AHRF in present day pediatric critical care is principally related to the severity of associated pathology and now no longer solely to the severity of respiratory failure. Further studies in larger series are needed to confirm these findings.

Key words Respiratory failure · Mechanical ventilation · Lung disease · Pediatrics

Introduction

Acute hypoxemic respiratory failure (AHRF) remains a common reason for emergency pediatric intensive care. Even with currently available ventilatory support it retains a significant morbidity and mortality with one subgroup, those with acute respiratory distress syndrome (ARDS), having a mortality rate of 40–75% [1–6]. However, it has also been suggested that much lower mortalities (< 10%) may occur in specific conditions.
such as respiratory syncytial virus-related ARDS [7]. In this setting, promising new interventions, including permissive hypercarbia, exogenous surfactant, inhaled nitric oxide, high frequency oscillatory ventilation, extracorporeal membrane oxygenation and perfluorocarbon-assisted gas exchange have been described with the purpose of ‘rescuing’ potential survivors from severe pathology. In order to evaluate effectively such therapies, clinical trials have adopted assessments of disease severity for patient recruitment. Such an approach is based on the hypothesis that the initial magnitude of acute severe physiological derangement equates with subsequent mortality. Thereby warranting the experimental intervention as well as providing a measure for verifying the similarity between treatment patients and control patients.

Our experience from a retrospective study of children with severe AHRF [8] questioned the usefulness of the previously reported respiratory predictors of outcome [2-5]. Rather, in common with Sarmaik and colleagues [9], who found in their pediatric study that response to an intervention better predicted intensive care outcome, we reported that greater improvement in oxygenation to an intervention (a standard dose of inhaled nitric oxide) was associated with improved outcome [8]. This suggests that with current clinical expertise, a test of potential ventilation-perfusion mismatch, shunt and lung injury reversibility is a more appropriate predictor of outcome than the status prior to any intervention. In an individual patient, such a response to intervention should be indicated by their best, rather than worst, measure of gas exchange. Therefore, the purpose of the present prospective, single institution study of AHRF in children was to assess whether the best, early respiratory indices in non-survivors were significantly different from those who survived.

Patients and methods

Approval for this observational study was obtained from our institution’s Ethics Committee and patient data was stored according to the requirements of the Data Protection Act. Between August 1 1995 and March 31 1997, all children older than 1 month and less than 16 years of age admitted to our Pediatric Intensive Care Unit (PICU) were eligible for inclusion in this prospective study. Inclusion criteria were modified from the American-European Consensus Conference diagnostic criteria for ARDS [10]: a) acute onset of respiratory failure over less than 48 h, b) evidence of a severe defect in oxygenation (arterial oxygen tension to fraction of inspired oxygen ratio (PaO2/FiO2) of less than 200 mm Hg) for at least six consecutive hours on the day of PICU admission, c) no evidence of left atrial hypertension and d) four quadrant interstitial shadowing on chest X-ray. Children without the characteristic chest X-ray appearances of ARDS, but meeting the other criteria for ARDS, were described as cases of AHRF.

Details of the patients’ acute diagnoses as well as any underlying conditions were recorded. An electronic patient charting system (CareVue, Hewlett Packard) was reviewed daily and ventilator and physiological parameters recorded and stored on a separate data base. Every blood gas analysis performed throughout the patients’ admissions was reviewed and the oxygenation index (OI = mean airway pressure (MAP) x FiO2 /PaO2) and alveolar-arterial oxygen tension gradient (A-aDO2), PaO2/FiO2 ratio and ventilation index (VI = respiratory rate x PaCO2 x peak inspiratory pressure / 1000) were calculated for each blood gas measurement. Analyses carried out used the best value obtained over the period under assessment. For the comparison with previously reported studies [2-5] every blood gas was reviewed and the respective study criteria applied for patient selection.

The ventilatory strategy employed in these patients was one of permissive hypercarbia (target PaCO2 ≤ 8 Kpa, provided pH > 7.25) with limitation of peak inspiratory pressure (< 35 cmH2O) while employing high MAPs to ensure maximum lung volume recruitment via the use of peak end expiratory pressure and inverse inspiratory:expiratory ratios. High frequency oscillatory ventilation was employed if oxygenation was inadequate with a MAP of 20 cmH2O or greater. The use of inhaled nitric oxide therapy throughout the last 12 months of the study was controlled by an institution approved multi-center randomisation protocol. Extracorporeal membrane oxygenation was employed when no stability could be achieved with the above techniques. Death or survival to discharge from the PICU were the end points of the study. In children who died, the mode of death was recorded: failed resuscitation, limitation or withdrawal of support or brain death. The clinical course of patients with AHRF was categorised according to the severity of disrupted gas exchange and whether or not improvement occurred within 3 days.

The data were stored in a Microsoft Access 2.0 data base and analysed with Microsoft Excel 7.0 and statistical software (Statistical Package for Social Sciences 6.13, SPSS Inc.), Comparisons between non-survivor and survivor data were performed with an independent sample t-test after transformation to normality if required. Multiple logistic regression analysis was performed against survival for a range of respiratory parameters from days 0, 1 and 2 in those patients with available data for those days. Parameters found to be significant in the univariate analysis were tested as well as those indices previously suggested to be associated with outcome from ARDS or AHRF [2-5]. In addition, age, weight, multi-organ system failure (MOSF) score [11] and the presence of underlying immunodeficiency were also tested in the model. Beta coefficients from significant independent predictors were converted to adjusted odds ratios with 95% confidence intervals. Comparison with published series [2-5] included a meta-analysis of reported results, calculation of the likelihood ratio for a positive test result, and the two-sample test for proportions.

Results

Out of 850 admissions to the PICU, 118 patients were admitted with AHRF over the 20 months of the study. The median age was 9 months (range 1-167 months), and weight 4.3 kg (1-53 kg). The median length of PICU stay was 8 days (range 0-80 days). The PICU mortality was 39/332, 5% (95% confidence interval 4-7%) in the non-AHRF cases, and significantly greater in the AHRF cases, 26/118, 22% (14-30%, p<0.001). One patient with AHRF died within 6 h of admission, having been inappropriately intubated and re-suscitated: since treatment was limited from the time of
admission, his respiratory indices data were excluded from analysis. Fifty-two children who fulfilled the full criteria for ARDS in addition to AHRF had a significantly higher mortality in comparison to the non-ARDS, AHRF patients: 36.5% (19/52) and 10.6% (7/66), respectively \( (p < 0.001) \). The ventilatory strategy in these patients is reflected in the group median \( \text{PaCO}_2 \) and MAP for survivors and non-survivors for each day of the study (Fig. 1). Non-conventional or specialised intensive care treatments included extra-corporeal membrane oxygenation (4 cases, 1 death), high frequency oscillatory ventilation (25 cases, 9 deaths), artificial surfactant (15 cases, 4 deaths) and intention to use nitric oxide (38 cases, 12 deaths).

**Outcome and acute physiological disturbance**

The range of physiological parameters most widely suggested to be associated with outcome are shown as a univariate analysis (Table 1) for the day of admission (day 0), and the subsequent two complete days on the PICU. On days 0 and 1 the eventual survivors do not differ significantly from non-survivors in terms of early A-a\( \text{DO}_2 \), \( \text{PaO}_2/\text{FIO}_2 \), OI or VI (when using the best values from all blood gases over the particular time period). By day 2, however, the A-a\( \text{DO}_2 \) just reached significance \( (p = 0.05) \). Although it should be noted that up to this time there had been significant attrition in patient numbers with nine of the patients present on day 0 not surviving and seven others improving to extubation, and therefore beyond the need for blood gas monitoring. In relation to the ventilatory parameters, there were significant differences between survivors and non-survivors in the maximum MAP employed on day 0 and day 1, and the highest peak inspiratory pressures employed on day 0.

In stepwise multiple logistic regression analysis, best and worst gas exchange parameters (A-a\( \text{DO}_2 \), \( \text{PaO}_2/\text{FIO}_2 \), OI, VI, \( \text{PaCO}_2 \), peak inspiratory pressure, positive end expiratory pressure and MAP) were not independently associated with poor outcome on days 0, 1 or 2 of admission. The findings were not altered by correction for age, weight, MOSF score or the presence of immunodeficiency. The association between ARDS and increased mortality noted on univariate analysis was not significant when corrected for the presence of MOSF score \( \geq 2 \).

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**Table 1** Univariate analysis of respiratory parameters and survival. The best daily respiratory parameters are analysed for each of the first 3 days of admission and values are expressed as mean (95% confidence interval). (S survived, D died, A-a\( \text{DO}_2 \) alveolar arterial oxygen gradient (mm Hg), OI oxygenation index, VI ventilation index, PIP peak inspiratory pressure (cm\( \text{H}_2\text{O} \)), PEEP positive end expiratory pressure (cm\( \text{H}_2\text{O} \)), MAP mean airway pressure (cm\( \text{H}_2\text{O} \))

<table>
<thead>
<tr>
<th>Day</th>
<th>Outcome</th>
<th>S</th>
<th>D</th>
<th>( p ) value</th>
<th>S</th>
<th>D</th>
<th>( p ) value</th>
<th>S</th>
<th>D</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A-a( \text{DO}_2 )</td>
<td>234 (159-335)</td>
<td>268 (197-234)</td>
<td>0.42</td>
<td>146 (94-243)</td>
<td>207 (137-324)</td>
<td>0.25</td>
<td>151 (75-233)</td>
<td>205 (137-362)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>( \text{PaO}_2/\text{FIO}_2 )</td>
<td>170 (112-240)</td>
<td>145 (99-272)</td>
<td>0.60</td>
<td>203 (148-300)</td>
<td>147 (105-303)</td>
<td>0.70</td>
<td>217 (134-297)</td>
<td>178 (86-281)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>OI</td>
<td>6 (6-18)</td>
<td>9 (5-15)</td>
<td>0.33</td>
<td>6 (4-9)</td>
<td>9 (5-13)</td>
<td>0.28</td>
<td>6 (4-9)</td>
<td>12 (6-14)</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>23 (16-31)</td>
<td>24 (19-40)</td>
<td>0.84</td>
<td>18 (11-27)</td>
<td>21 (14-37)</td>
<td>0.13</td>
<td>22 (10-27)</td>
<td>22 (19-32)</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>PIP</td>
<td>30 (26-34)</td>
<td>34 (30-38)</td>
<td>0.01</td>
<td>29 (25-32)</td>
<td>31 (27-34)</td>
<td>0.35</td>
<td>28 (23-31)</td>
<td>27 (25-30)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>PEEP</td>
<td>6 (5-8)</td>
<td>8 (5-10)</td>
<td>0.20</td>
<td>7 (6-8)</td>
<td>6 (6-10)</td>
<td>0.18</td>
<td>7 (6-8)</td>
<td>6 (5-9)</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>14 (11-16)</td>
<td>18 (15-21)</td>
<td>0.05</td>
<td>15 (12-18)</td>
<td>17 (16-20)</td>
<td>0.05</td>
<td>14 (11-17)</td>
<td>15 (12-19)</td>
<td>0.15</td>
</tr>
</tbody>
</table>
### Outcome and clinical course

The clinical course of patients with AHRF was categorised according to the severity of the disruption of gas exchange. Using the best daily $A-aD_O_2$ (but the classification is identical if $PaO_2/FI_O_2$ or $OI$ are used), five distinct patterns of AHRF were identified (Fig. 3). Survivors follow one of three clinical patterns: a course of mild disease only (pattern A: $n = 45$), early improvement within 3 days (pattern B: $n = 23$) or later improvement longer than 3 days (pattern C: $n = 24$). Non-survivors die with persistent, severe, hypoxemic, respiratory failure (pattern D: $n = 17$) or during or following resolution of AHRF (pattern E: $n = 9$).

The majority of deaths occur in children with persistently severe abnormal gas exchange (pattern D). Of these children, none were previously healthy (ten were immunodeficient, three were ex-premature infants and the others had inherited metabolic disorders or major chromosomal abnormalities). Further, only six of these patients died whilst receiving full supportive treatment including cardiopulmonary resuscitation, the remainder had either support withdrawn (4/17) or a limitation of intensive care therapy (7/17) because of the severity of the associated conditions or co-incident organ failure. Therefore only six cases with AHRF reached a point of unsupportable respiratory failure, and none of these were previously normal children. The other children who died (pattern E) most frequently did so from severe cerebral injury (5/9 from brain death including the three previously normal children who died) with the other four cases having supportive treatment withdrawn or limited because of the severity of associated diseases.

### Discussion

The principal observation in this preliminary report of AHRF in children is that associated or underlying diagnoses - case mix - have significant bearing on population outcomes. Not surprisingly, children meeting criteria for ARDS had a poorer outcome. Further, when using a defined ventilatory strategy, which in our practice emphasises permissive hypercarbia and lung volume recruitment, severity of ventilatory parameters (i.e., high $MAP$) rather than indices of gas exchange, reflected better the likelihood of poor outcome. Most importantly, where the acute physiological parameters fail to differ between good and poor outcome patients, we propose that the presence of severe pre-existing disease or associated pathology, rather than severity of respiratory failure alone is associated with outcome in modern pediatric practice. This hypothesis should be tested in a larger series since, although we recruited 118 patients, there were only 26 deaths on which many of our conclusions are based.
Comparative assessment of respiratory indices

Comparisons with previously published respiratory predictors of outcome from studies of AHRF and ARDS in childhood are shown in Table 2. None of the proposed physiological correlates of outcome were applicable to our series. Reviewing every blood gas and applying the published criteria we found in each case, the predictor overestimated our patients' risk of mortality, excepting the very severe criteria from the Melbourne study in 1991, which used a peak inspiratory pressure greater than 40 cm H₂O and A-aDO₂ more than 580 mm Hg [4]. These were rarely achieved in our population (7 cases) and hence the confidence intervals remain so wide (18-90%) that no useful conclusion could be drawn.

The largest study of pediatric AHRF [6], a multi-center retrospective study including 470 cases from 1991, identified an association between acute respiratory physiological disturbance and outcome. However, children who became brain dead or had treatment withdrawn because of perceived treatment futility — in the setting of severe neurological insult — were excluded from the subsequent analysis. Such an approach (included because the study was principally designed to identify extra-corpooreal membrane oxygenation candidates) would have excluded from our analysis all the normal children who died. Since brain injury is a possible complication of severe hypoxemia or the disease processes that initiated hypoxemia, our view was that these patients should be included in our attempt to identify factors associated with outcome.

The difference between our current findings and those of scores or predictors identified in the late 1980s and early 1990s may, in fact, relate to a fundamental change in ventilatory strategy. As shown in Fig. 1, our median PaCO₂ was 6–8 kPa instead of the 5.3–6 kPa reported in the Pediatric Critical Care Study Group multi-center retrospective study of children managed in 1991 [6].
The institutions in our series exhibited one of five patterns in their clinical course. Deaths amongst children admitted in AHFR can occur with active and progressive lung disease (pattern D) or in spite of resolving lung disease (pattern E). In children who survive, recovery may be slow or fairly rapid. On inspecting the data, it is apparent that there are similarities in the initial respiratory indices in children who survive despite severe, prolonged gas exchange disruption (pattern C) and those who die despite improving or improved gas exchange parameters (pattern E). The relative proportion of patients with these patterns in a population being studied will clearly determine the utility of gas exchange parameters in predicting survival; conversely, as is our experience when including patients with underlying immunodeficiency or other associated diseases, these proportions may confound their use. Of further note is the mode of death in children with persistently, severely abnormal gas exchange (pattern D): cases rarely reached a level of respiratory failure which was unsupportable by current techniques. Instead, in the majority of cases (11/17) treatment was discontinued or limited as a result of non-pulmonary factors. Thus, it has been suggested that mechanical ventilation should now be considered less a form of treatment than a form of organ support during disease resolution [14].

Secondly, can severity systems that solely employ acute pulmonary physiological parameters and do not incorporate underlying etiology be used to good effect, specifically in pediatric AHFR? Perhaps the reported value of such pulmonary physiological predictors, with their institution specificity, are more a reflection of physician behavior, i.e., patient selection and local ventilatory strategy employed, than patient pulmonary pathology. In this context, it is of interest that we found the presence or absence of AHFR in all PICU patients to be a discriminator. More recently developed non-linear, multiple logistic regression models that predict the risk of death for children less than 16 years of age (e.g., the ‘Pediatric Risk of Mortality III’ - PRISM III [15] and the ‘Paediatric Index of Mortality’ - PIM [16]) may improve the outcome prediction since they incorporate both diagnostic and disease categories as well as acute physiological respiratory parameters. However, these two severity scoring systems do differ: not least in their strategy employed. PRISM III utilizes pH, PaCO\(_2\), PaO\(_2\) and FIO\(_2\) and respiratory rate, whereas PIM utilizes PaO\(_2\) and FIO\(_2\), and whether or not mechanical ventilation is being used.

Observations from adult intensive care studies of lung injury have indicated that outcome is not necessarily related to the level of arterial oxygenation [12, 13]. In contrast, many previous pediatric studies in defined populations have supported a contrary notion [1, 2-6]. In the present pediatric study, we have observed that mortality from respiratory failure appears to be related to associated disease rather than the severity of initial gas exchange per se. The implications of such a hypothesis are wide. Firstly, is there much to be gained by refining further the techniques of respiratory support when mortality is frequently determined by non-pulmonary factors? Indeed, it has been suggested that mechanical ventilation should now be considered less a form of treatment than a form of organ support during disease resolution [14].

Table 2 Comparison of previously published [2-5] respiratory severity parameters with the present series (PPV positive predictive value for mortality, VI ventilation index, OI oxygenation index, PIP peak inspiratory pressure (cmH\(_2\)O), A-aDO\(_2\); alveolar arterial oxygen gradient (mm Hg), MAP mean airway pressure (cmH\(_2\)O), LR + the likelihood ratio for a positive test result, i.e. the ratio of finding the predictor in non-survivors to finding it in survivors) * indicates intermediate to high diagnostic impact, ns not significant

<table>
<thead>
<tr>
<th>Proposed predictors</th>
<th>PPV (95% confidence interval)</th>
<th>LR +</th>
<th>PPV in present study</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melbourne 1991 [2]</td>
<td>VI &gt; 40 and OI &gt; 40</td>
<td>77 (55-92)%</td>
<td>2.5</td>
<td>6/15 = 40 (16-68)%</td>
</tr>
<tr>
<td></td>
<td>PIP &gt; 40 and A-aDO(_2) &gt; 580</td>
<td>81 (58-95)%</td>
<td>3.5*</td>
<td>4/7 = 57 (18-90)%</td>
</tr>
<tr>
<td>Memphis 1993 [3]</td>
<td>A-aDO(_2) &gt; 450 for 24 h</td>
<td>100 (69-100)%</td>
<td>very high*</td>
<td>9/22 = 41 (21-64)%</td>
</tr>
<tr>
<td>Salt Lake City 1991 [4]</td>
<td>A-aDO(_2) &gt; 470</td>
<td>81 (61-92)%</td>
<td>1.4</td>
<td>17/42 = 42 (26-57)%</td>
</tr>
<tr>
<td></td>
<td>MAP &gt; 23</td>
<td>90 (72-98)%</td>
<td>3.0*</td>
<td>8/22 = 32 (17-59)%</td>
</tr>
<tr>
<td>Philadelphia 1993 [5]</td>
<td>A-aDO(_2) &gt; 420</td>
<td>87 (72-97)%</td>
<td>6.2*</td>
<td>17/44 = 40 (26-57)%</td>
</tr>
</tbody>
</table>
patients. Finally, since death in a previously normal marker of ventilation-related outcome should be child is now an infrequent end point in AHRF, our data sought.
reiterates a previously discussed idea [18], that other

References

Circulating platelet–neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing

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Summary. Platelets play a prominent role in linking the processes of inflammation, haemostasis and thrombosis. Recent studies have shown that platelets form heterotypic aggregates with leucocytes via platelet CD62P and leucocyte \( \beta 2 \) integrins. These interactions have been observed in vitro in blood taken from healthy volunteers and in clinical conditions in which thrombosis and inflammation are prominent.

This study investigated the properties of platelet-neutrophil complexes (PNCs) in anticoagulated whole blood. At rest, neutrophils in PNCs exhibit a significantly more activated adhesion molecule profile than free neutrophils with increased CD11b expression and activation (increased binding of the CD11b/CD18 'activation reporter' monoclonal antibody 24) and decreased CD62L expression. In addition, neutrophils in PNCs phagocyted significantly more Neisseria meningitidis and produced more toxic oxygen metabolites than free neutrophils.

Stimulation with the platelet agonist adenosine diphosphate (ADP) led to further increases in CD11b expression and activation, loss of CD62L as well as increased phagocytosis and toxic oxygen metabolite production throughout the whole neutrophil population. When these experiments were repeated with the CD62P blocking antibody G1 the effects were inhibited to a variable extent, dependent upon the parameter under investigation. These results indicate that both soluble and contact-dependent factors contribute to platelet-mediated neutrophil activation.

Platelet neutrophil complexes represent a large subpopulation of neutrophils with a more activated adhesion molecule profile, and a greater capacity for phagocytosis and toxic oxygen metabolite production. This study provides further support for a role for PNCs in both health and disease.

Keywords: neutrophils, platelets, adhesion molecules, phagocytosis, flow cytometry.

There is increasing evidence that the complex multicellular processes of inflammation, haemostasis and thrombosis are closely linked. It has been established that inflammatory cytokines such as tumour necrosis factor (TNF)-\( \alpha \) and interleukin (IL)-1 can induce a prothrombotic state. This results from increased monocyte and endothelial cell tissue factor (TF) expression and pathway activation (Levi et al, 1997; Heyderman et al, 1995) in combination with reduced fibrinolytic activity secondary to raised levels of plasminogen activator inhibitor-1 (Gando et al, 1995). Conversely, key components of the coagulation pathways, such as thrombin, have now been shown to contribute to inflammation by inducing IL-6 and IL-8 release from monocytes and endothelial cells (Johnson et al, 1998).

Platelets are pivotal in regulating this haemostatic/inflammatory axis, expressing surface adhesion molecules responsible for primary haemostasis and accelerating the conversion of prothrombin to thrombin (Zwaal et al, 1977). In addition, platelets can secrete pro-inflammatory mediators such as IL-1 (Hawrylowicz et al, 1989) and express surface molecules capable of modulating inflammatory processes (e.g. CD40L) (Henn et al, 1998). The importance of platelets in the link between haemostasis and inflammation...
is apparent clinically in cases of Gram-negative sepsis. Thrombocytopenia is a very poor prognostic sign in these patients (Kornelisse et al., 1997).

The capacity for platelets to influence the haemostatic/inflammatory axis may rely upon direct contact with inflammatory cells. Heterotypic aggregation of platelets to neutrophils (and other leucocytes) has been observed in vitro (Larsen et al. 1989) and in blood taken from healthy volunteers (Li et al. 1997; Peters et al. 1997; Rinder et al., 1991; Anonymous 1882). These interactions are mediated by platelet CD62P expression and leucocyte β2 integrins (Ostrovsky et al., 1998; Evangelista et al., 1996). Indirect evidence that these complexes may themselves have a physiological function is provided by studies which have shown changes in the numbers of, or capacity to form, these heterotypic cell complexes in clinical conditions in which thrombosis and inflammation are prominent features (Gawaz et al., 1994, 1997; Ott et al., 1996). How platelet-leucocyte complexes contribute to these conditions has yet to be elucidated.

We showed that platelet-neutrophil complexes (PNCs) represent a large subpopulation of neutrophils with a more activated adhesion molecule profile, a greater capacity to phagocytose Neisseria meningitidis and to produce toxic oxygen metabolites. These functions were regulated by both contact dependent and independent pathways.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies were purchased as follows: fluorescein isothiocyanate (FITC) conjugated IgG1 irrelevant control antibody, phycoerythrin (PE) conjugated IgG2a anti-CD42b and FITC conjugated IgG2a anti-CD42b from Dako Ltd, U.K.; FITC labelled IgG1 anti-CD11b from Serotec, U.K.; IgG1:FITC/IgG2a: PE negative control, and IgG1 anti-CD61 (PerCP) from Becton Dickinson, U.K.; and IgG1 anti-CD62L (PE) from Pharmingen (U.K.). The CD62P blocking antibody G1 was a kind gift from Dr Rodger McEver from the University of Oklahoma. Antibody 24 (Mab24), which binds to the activation dependent binding site of CD11b/CD18, was a kind gift from Dr Nancy Hogg, ICRF, London, and was conjugated to FITC by standard techniques (Jack et al. 1998). Adenosine diphosphate (ADP) and f-met-leu-phe (FMLP) and phorbol myristate acetate (PMA) were purchased from Sigma Ltd, U.K., and FACSlyse solution from Becton Dickinson, U.K.

Whole blood stimulation. The protocol was approved by the local research ethics committee. Blood was drawn via a 21G butterfly needle without the use of a tourniquet from non-smoking healthy volunteers who had not been on any medication for at least 2 weeks. The first 2 ml of blood were discarded, and the required volume collected into sodium citrate to a final concentration of 0.38%. Whole blood was added to each FACS tube to which 4 ml of FACSlyse solution from Becton Dickinson, U.K. was easily distinguished on forward and side scatter characteristics. Results were compared to isotype-matched antibody staining and considered positive if the fluorescence intensity exceeded that of 98% of the control antibodies. Cross talk of the respective fluorophores was controlled by examination of samples stained with a single fluorophore and subtraction of observed fluorescence in the secondary channel. Events staining positive for both neutrophil and platelet antigens (e.g. CD11b and CD42b) were considered to represent PNCs and were distinguishable from events staining positive for CD11b alone. Samples were analysed at the highest flow rate which allowed a total event rate of <4500 events/s in order to minimize the possibility of mistakenly identifying separate but coincident platelets and neutrophils as PNCs.

Phagocytosis study Neisseria meningitidis type B1940 (0·5 ml in RPMI w/o phenol red at an A540 of 1) were mixed with an equal volume of FITC (1 mg/ml in RPMI w/o phenol red) and incubated at 37°C for 1 h as previously described (Heyderman et al. 1999). The organisms were then spun at 9780 g for 1 min, and washed twice with 0·5 ml RPMI before resuspension in RPMI w/o phenol red at an A540 of 1. 200 μl of this suspension was then added to 4 ml of citrate anticoagulated whole blood which was incubated at 37°C with gentle mechanical agitation for 60 min. 50 μl samples were added to FACS tubes containing CD42b-PE and prepared as above. Compensation for two-colour fluorescence was undertaken as for PNC estimation above.

Oxidative burst. Oxidative burst was assessed by the capacity of neutrophils to oxidize dihydrorhodamine 123 (DHR) to the fluorescent dye rhodamine 123 as previously described (Vowells et al. 1995). Briefly, 300 μl of heparinized whole blood was added to each FACS tube to which 4 ml of warmed (37°C) red cell lysis buffer was added (150 mm NH4Cl, 20 mm NaHCO3, 1 mm EDTA, pH 7.2). After gentle mixing, and 5 min incubation all erythrocytes were completely lysed. The tubes were centrifuged at 900 g for 5 min. the supernatants removed and the pellets resuspended in HBSS + 5% FCS. A further washing step was performed before resuspension of the pellet in 400 μl of HBSS + 5% FCS. To this 1·8 μl of DHR (25 μm solution in DMSO) was added.
and 20 µl of PerCP conjugated monoclonal antibody against CD61 (or isotype-matched control) and the samples incubated for 10 min at room temperature. PMA stimulation (final concentration 1 µg/ml) was used as a positive control. Flow cytometric analysis was carried out as described above, following compensation for cross-talk between the PerCP and rhodamine.

Statistics. Each experiment was performed on a minimum of six occasions. The median fluorescence intensity of a minimum of 5000 neutrophil events was recorded and compared to unstimulated samples by means of paired sample t-tests (SPSS version 8.0) or to unity in the investigation of PNC MFI to free neutrophil MFI ratio by one-sample t-tests. In all cases P<0.05 was considered significant.

RESULTS

Adhesion molecule expression

At rest, all neutrophils expressed CD11b and CD62L. Neutrophil activation with FMLP induced a significant increase in CD11b expression, and decrease in CD62L expression. Following incubation of whole blood with the platelet agonist ADP there was a significant increase in neutrophil CD11b expression detected at both 1 min (P = 0.02) and 30 min (P = 0.04). Similarly, ADP stimulation led to a significant fall in neutrophil CD62L at 30 min (P < 0.001) (Fig 1A).

As described above, examination of the neutrophils from whole blood stained with CD42b:PE and CD11b:FITC enabled the detection of two populations of cells: free

Fig 1. The influence of platelets on neutrophil adhesion molecule expression. (A) The neutrophil agonist FMLP caused a significant increase in CD11b expression and a significant fall in CD62L expression throughout the whole neutrophil population. The specific platelet agonist ADP also caused significant increases in CD11b and falls in CD62L although the changes were much smaller. (B) PNCs expressed more CD11b and less CD62L than unbound neutrophils. Means of at least three experiments and 95% CI are shown. (*P < 0.05, **P < 0.01 compared to unstimulated samples in A, or compared to unity, i.e. no difference between N and PNC, in B). ●, CD62L MFI after 30 min stimulation with FMLP was only just detectable.

neutrophils and platelet–neutrophil complexes (PNCs). FMLP stimulation increased the intensity of CD11b staining in both PNCs and free neutrophils (ratio MFI (CD11b PNC/CD11b free neutrophil) = 1-0). However, CD11b was expressed at higher levels in PNCs than free neutrophils in unstimulated samples (1 min ratio 1-1, P < 0-05), and following ADP stimulation (1 min ratio 1-1, P < 0-05) (Figs 1B and 4A).

Following whole blood stimulation with FMLP for 30 min, CD62L (L-selectin) was only just detectable, and therefore no comparison could be made between PNCs and free neutrophils. Neutrophil activation with FMLP resulted in a mean 2-6-fold increase in MFI compared to unstimulated samples (P < 0-001) after 1 min and further by 30 min (3-6-fold, P < 0-001) (Fig 2A). Incubation with ADP also increased Mab24 binding (1 min, 1-4-fold, P < 0-05) (Fig 2A). As observed with the levels of CD11b, Mab24 binding was greater on PNCs than on free neutrophils (Figs 2B and 4C) in both unstimulated (ratio PNC/N Mab24 MFI 1-1, P < 0-05) and ADP stimulated (1 min ratio 1-1, P < 0-05) whole blood. There was no difference between Mab24 binding to PNCs or free neutrophils following FMLP stimulation (1 min ratio 1-0, P = 0-86).

Influence of platelet–neutrophil complex size
Each PNC, as detected by the flow cytometer, must include at least one neutrophil and one platelet. We and others believe that many platelets can bind to a single neutrophil. However, it is also possible that some PNCs could contain more than one neutrophil, and these would still be counted by the cytometer as a single event. Such complexes would be much more likely to demonstrate higher levels of CD11b expression, Mab24 binding, superoxide production, and phagocytosis. We addressed this possibility by reviewing the forward scatter profile (related to particle size) plotted against the parameters under investigation and we were unable to demonstrate an association between event size and any of the parameters. Furthermore, the observation of lower levels of CD62L amongst PNCs also suggests that any contribution made by larger aggregates must be small.

Influence of CD62P blockade
The changes in adhesion molecules of the whole neutrophil population observed with ADP stimulation were assessed in the presence of the blocking monoclonal antibody CD62P (G1) (Fig 3) or isotype matched control. G1 prevents the formation of PNCs as previously described (Peters et al, 1997). A significant reduction but not abolition of the ADP-induced changes in CD11b expression was observed (Fig 3A). The ADP-induced changes in Mab24 binding were completely inhibited by the presence of G1 antibody (Fig 3B), but the changes in CD62L expression were not altered significantly (Fig 3C).

Phagocytosis
In order to investigate the phagocytic capacity of PNCs, a previously reported model of whole blood phagocytosis utilizing Neisseria meningitidis was employed (Heyderman et al, 1999). After 60 min incubation of freshly sampled whole blood with FITC-labelled live Neisseria meningitidis group B, neutrophil phagocytosis could be clearly detected (Fig 5A). We observed greater phagocytosis of the micro-organism.
Functions of Platelet–Neutrophil Complexes

Unstimulated
Unstimulated + G1
ADP
ADP + G1
A
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6
CD11b MFI at 30 min

Unstimulated
Unstimulated + G1
ADP
ADP + G1
B
0 0.2 0.4 0.6 0.8 1 1.2 1.4
Mab24 MFI at 30 min

Unstimulated
Unstimulated + G1
ADP
ADP + G1
C
0 0.2 0.4 0.6 0.8 1 1.2
CD62L MFI at 30 min

Fig 3. Influence of platelet–neutrophil contact on platelet-induced changes in neutrophil adhesion molecules. The changes in adhesion molecules of the whole neutrophil population with ADP stimulation in the presence of the CD62P blocking antibody (G1) were assessed. The ADP-induced increase in CD11b expression was significantly attenuated but remained greater than the control (A). The ADP-induced changes in Mab24 binding were completely inhibited by the presence of G1 antibody (B) but the changes in CD62L expression were not altered significantly (C). Means and 95% confidence intervals of four or more experiments are shown. (*P<0.05, **P<0.01 by paired t-tests).

amongst PNCs than in the free neutrophil population (ratio at 60 min, 1.5x, 95% CI 1.38–1.62, P<0.001, n = 34) (Fig 3). As with the changes seen in adhesion molecules, ADP led to increased phagocytosis (mean % phagocytosis after ADP = 1.5x control 95% CI 1.2–1.8, P<0.01). In order to investigate the contribution of platelet–neutrophil contact via CD62P to these effects, experiments were repeated in the presence of the G1 blocking monoclonal antibody against CD62P (Fig 5C). The increase in phagocytosis observed with ADP was not inhibited by co-incubation with G1.

Neutrophil superoxide activity
PMA caused a log increase in fluorescence intensity reflecting the oxidation of DHR to fluorescent rhodamine 123 by reactive oxygen species. In unstimulated samples greater fluorescence was detected in PNCs than in free neutrophils (ratio DHR MFI PNC/N 1.69, P<0.001). This effect was further enhanced by platelet stimulation with ADP (ratio 1.74, P<0.001) (Figs 6A and 6C). Indeed, the fluorescence intensity of the whole neutrophil population was significantly increased by co-incubation with ADP (Fig 6B). G1 monoclonal blocking antibody against CD62P reduced PNC formation and also reduced rhodamine 123 fluorescence in both unstimulated and ADP stimulated samples (Figs 6B and 6C).

In order to investigate the possibility that these observations may be the result of toxic oxygen metabolite
Fig 4. Example of fluorescence profiles for adhesion molecules in free and PNC neutrophils. Events displaying positive CD42b staining in the neutrophil population on forward and side scatter are considered to represent PNCs. The profiles shown are following ADP stimulation of whole blood and the figures are the MFI for the respective antibodies and populations. There is more CD11b (A) and Mab24 (C) and less CD62L (B) binding in PNCs than free neutrophils.

Fig 5. PNC and free neutrophil phagocytosis. The figures represent the percentage of neutrophils in each quadrant. Phagocytosis of *Neisseria meningitidis* in an unstimulated sample (A). Neutrophils which have phagocytosed FITC-labelled micro-organisms are clearly visible as separate populations with FITC fluorescence $>10^3$. A higher proportion of PNCs have phagocytosed bacteria than unbound neutrophils. (B) Following ADP stimulation there was an increase in PNCs and phagocytosis, but, as shown in (C), the ADP-induced increase in phagocytosis was not affected by blockade of CD62P with the monoclonal antibody G1 although PNC formation was greatly attenuated.

Fig 6. Oxidative burst activity. (A) The DHR median fluorescence for the whole neutrophil population was significantly increased by ADP stimulation (*P<0.01 versus unstimulated control). This effect is completely abolished by the presence of the G1-blocking monoclonal antibody against CD62P (**P<0.01 versus no G1). G1 inhibition of control levels of DHR MFI suggests that background platelet activation was present and contributing to oxidative burst activity before ADP was added. (B) There was significantly greater oxidative burst activity in PNCs than unbound neutrophils. (Ratio PNC/N DHR MFI >1.0 at rest and following ADP stimulation, P<0.01.) The addition of ADP increased PNC oxidative burst further. (Means and 95% CI of three experiments are shown.) (C) Example of neutrophil DHR fluorescence. The DHR fluorescence was greater amongst those neutrophils which also stained positive for the platelet marker CD61;PerCP. The second panel displays the intensity of DHR fluorescence in a sample incubated with the G1 blocking monoclonal antibody against CD62P. There were fewer PNCs and less total DHR fluorescence.

production by platelets (Yeaman, 1997), purified platelets were studied in this assay. PMA induced changes in autofluorescence but did not induce a detectable oxidative burst.

DISCUSSION

The data presented in this study indicate that neutrophils within platelet–neutrophil complexes are phenotypically and functionally distinct from uncomplexed neutrophils. In unstimulated whole blood, neutrophils with platelets attached displayed a more ‘activated’ pattern of adhesion molecule expression, produced more superoxide, and ingested more bacteria than neutrophils which were not associated with platelets. These findings are indicative that neutrophils within platelet–neutrophil complexes represent an activated subset of cells. In addition, it can be inferred that the platelet component of PNCs must also represent an activated subpopulation of platelets, as CD62P expression is an essential prerequisite for PNC formation (de Bruijne Admiral et al, 1992; Evangelista et al, 1993).

To investigate the effect of platelet stimulation on neutrophil phenotype and function, we performed experiments in which whole blood was incubated with ADP. We found that for all of the parameters studied, platelet stimulation led to an exaggeration of the profile observed in...
unstimulated blood. Therefore, following ADP stimulation, the entire neutrophil population was found to express more CD11b/CD18, less CD62L, more antibody 24 binding sites, produce more superoxide and phagocytose more N. meningitidis. These results extend the findings from previous studies which showed that platelet activation is a potent stimulus for both PNC formation and neutrophil function (Peters et al., 1997; de Bruijne Admiraal et al., 1992; Chignard & Renesto, 1994; Nagata et al., 1993).

In both resting and ADP-stimulated blood the observed phenotypic and functional changes were associated with an increased number of PNCs. It is tempting to postulate that heterotypic adhesion between platelets and neutrophils is a prerequisite for the neutrophil changes seen in this study. However, experiments using the CD62P blocking antibody, G1, indicate that the relationship between PNC formation and neutrophil activation is more complex.

We found that blockade of CD62P not only markedly diminished the number of PNCs formed in response to stimulation, but completely inhibited the platelet induced changes in Mab24 binding and superoxide production. For these parameters it would appear that CD62P was absolutely necessary. This was consistent with previous studies in which platelet agonists such as thrombin were shown to increase the capacity of platelets to induce neutrophil and superoxide anions (Nagata et al., 1993) via a CD62P-dependent mechanism. Furthermore, soluble CD62P has also been reported to increase superoxide production (Nagata et al., 1993).

Platelet-induced changes in CD11b and CD62L expression were only partially inhibited by CD62P blockade. These findings indicated that, in addition to CD62P other factors also contributed to platelet-mediated neutrophil adhesion molecule expression in whole blood. Platelet-derived platelet activating factor (PAF) (Chignard & Renesto, 1994), β-thromboglobulin (Walz & Baggilini, 1989) and IL-1 have all been shown to induce inflammatory cell activation. These and/or other mediators are likely to be involved in the changes observed in neutrophil adhesion molecule expression and would explain how platelet activation could effect the entire neutrophil population and not just those complexed to platelets.

ADP stimulation of blood increased the capacity of neutrophils to phagocytize N. meningitidis by 50%. This was associated with a similar increase in the ratio of PNCs containing bacteria compared to uncomplexed neutrophils. Purified P-selectin has been shown to promote neutrophil phagocytosis of labelled zymosan particles (Cooper et al., 1994), and platelets have been reported to increase mouse macrophage phagocytosis of Salmonella (Mandell & Hook, 1969). However, we could not detect any effect of CD62P blockade on the percentage of neutrophils which had phagocytosed the bacteria, indicating that platelet–neutrophil heterotypic adhesion is not required for platelet enhancement of phagocytosis. This raises the question as to why there was such a dramatic over-representation of phagocytosis within the PNCs.

This may be explained by considering the role of neutrophil integrins in complex formation. A number of groups have observed the capacity of immobilized platelets to bind leucocytes (including neutrophils) via a multistep selectin and β2 integrin dependent mechanism (Kuijper et al., 1996, 1997; Buttrum et al., 1993; Diacovo et al., 1996), analogous to that which occurs between activated endothelium and leucocytes. Platelet CD62P, platelet-derived PAF and neutrophil β2-integrin activation have recently been described as requirements for maximal adhesion of neutrophils on immobilized platelets (Evangelista et al., 1996). This requirement for selectin and integrin involvement in PNC formation has also been demonstrated in mixed cell conjugates under dynamic conditions (Ostrovsky et al., 1998).

We have previously shown that N. meningitidis is a potent stimulus for β2 integrin expression (Heyderman et al., 1999; Klein et al., 1996). Furthermore, exogenous stimuli, including FMLP and endotoxin, further enhance neutrophil integrin expression and phagocytosis in whole blood (Heyderman et al., 1999). Therefore it seems probable that soluble platelet release products may act in a similar fashion and are responsible for the ADP-induced increase in phagocytosis seen in this study. In this situation, the over-representation of phagocytosis in PNCs may simply reflect the stabilization of platelet–neutrophil complexes which occur in the presence of increased β2 integrin activation as a result of neutrophil/bacterial contact. Such a finding is supportive of the importance of neutrophil adhesion molecules in the formation of PNCs (Ostrovsky et al., 1998; Evangelista et al., 1996).

These observations in whole blood are consistent with previous studies using separated cells and imply a functional role for PNCs in vivo. Irrespective of the requirement of heterotypic adhesion for enhancing neutrophil function, the fact that PNCs contain the most activated neutrophils and platelets within the blood may facilitate both cell types to perform their respective functions in response to inflammatory stress or changes in local blood flow. Our results indicate that PNCs would be amongst the most adhesive cells in the circulation. As such they may also have the greatest capacity to bind to activated vascular endothelium. This may explain the recent data from adults with severe endothelial dysfunction associated with multi-organ failure in whom low circulating PNCs were found to be correlated with a poor outcome (Gawaz et al., 1997). This study provides further support for a role for PNCs in both health and disease.

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REFERENCES


*Functions of Platelet–Neutrophil Complexes* 399
EARLY SEVERE NEUTROPENIA AND THROMBOCYTOPIENIA IDENTIFIES THE HIGHEST RISK CASES OF SEVERE MENINGOCOCCAL DISEASE.

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Abstract

Objective
To determine the performance of established predictors of mortality in paediatric acute meningococcal disease (MD) in a contemporary population and to develop a simple predictive score that will not vary with observer.

Design
Prospective study for development set and mixed retrospective and prospective study for validation set.

Setting & Patients
227 cases of clinical MD referred to three multidisciplinary paediatric intensive care units 1993 -1999. Early deaths prior to transfer to PICU and deaths from cerebral herniation were included in the analysis.

Measurements and Main Results.
The product of platelet and neutrophil counts at presentation (PN product) predicts mortality from MD better than either count alone and at least as well as established severity scores. The Glasgow meningococcal septicaemia prognostic score (GMSPS) and Malley scores performed poorly in these populations. The positive predictive value (PPV) for GMSPS score \( \geq 8/15 \) was 17.5% (16/91, 95% ci =9-25%) was significantly lower than published estimates of 30%-74%, (p<0.01). The PPV for death (or amputation) with a Malley score 3/3 was 50%, (12/24, 29-71%), significantly lower than the published value.
of 100%, \( p<0.001 \). The PN product appears to be a useful predictor: PN <40, PPV =82\% (9/11), Specificity =99\% (195/197) and sensitivity = 73\% (23/30). The performance of this score was greatest in younger children (<5 years), in whom clinical cerebral herniation was not seen as a cause of death (0/21 deaths <5 years, 4/9 deaths ≥5 years).

**Conclusion**

Established scores significantly overestimate the incidence of adverse outcomes in MD. This may reflect improved resuscitation and outcome, or variability in the application of these scores. The PN product achieves similar prediction to the scores currently in use and is independent of the observer. Factors that reflect the extent of the inflammatory response rather than the care prior to presentation are becoming of increasing importance.
Introduction

Factors that relate to a poor outcome from acute meningococcal disease (MD) have been identified for more than 30 years\(^1\). Detailed clinical assessments such as the Glasgow meningococcal septicaemia prognostic score (GMSPS) have been proposed\(^2\), validated\(^3\) and widely employed in clinical studies to date\(^4,5\). Others have described the use of simple laboratory values such as C-reactive protein\(^6\) or abnormal coagulation\(^7\) and neutrophil counts\(^8\) to develop predictive models.

In the last ten years there have been many advances in the understanding of the pathophysiology of systemic inflammation\(^8\) and particularly MD, including the mechanisms of endothelial dysfunction\(^10-13\), myocardial depression\(^14\), control of the micro-circulation\(^15\), and the interactions between abnormal thrombosis and haemostasis and inflammation\(^16\). These developments have led to many attempts to attenuate the systemic inflammatory response to infection by targeting the actions of single important pro-inflammatory mediators. The results of such interventions have been disappointing\(^17\).

One possible contribution to difficulty in the investigation of immunotherapies for sepsis is that heterogenous groups of patients have been studied\(^18\) including those who may have been anticipated to have a good outcome with standard therapy. Therefore small alterations in outcome may have been diluted by the inclusion of cases with a low risk of mortality (type II error).

The improved understanding of the pathophysiology of acute sepsis and MD should allow us to identify factors which are associated with a poor outcome in a modern PICU
population. Ideally these should relate to disease processes and be unaffected by observer variability or therapy prior to admission.

There is now a suggestion that outcome from MD may be improving\textsuperscript{3,20-22}. Decreased positive predictive values for death with the GMSPS have been observed in the same institution with time\textsuperscript{2,22}. Recently published multivariate predictive models of clinical observations and readily available laboratory values\textsuperscript{8,19} which employ data from 1985-90 in the development set may also require adjustment.

For clinicians, the ideal predictor must be easily identifiable on presentation, have a high positive predictive value for death from overwhelming shock (but not necessarily from other causes) in a contemporary population, and should not rely on clinical assessment which is prone to extreme variability\textsuperscript{23}. Such a score would allow improved targeting of investigations of new therapies to those patients most at risk. In addition, cases likely to require support to multiple organ systems would be identified early in the clinical course.

We undertook a prospective study of admissions with MD to our PICU 1997-1999 to investigate the performance of pathophysiologically relevant extreme neutropenia and thrombocytopenia on presentation as predictors of outcome, and to record the performance of established severity scores in the same population. The findings were then applied to two larger retrospective series from other PICUs.
Materials and Methods

Development Set

From November 1997 to December 1999 all cases of clinical meningococcal disease admitted to Great Ormond Street Hospital for Children PICU were assessed by one of three investigators for inclusion in a number of studies investigating neutrophil and platelet function. This assessment included the Glasgow Meningococcal Septicaemia Prognostic Score\(^2\) and the recently proposed 'Malley Score\(^{19}\) and the Paediatric Index of Mortality (PIM)\(^{24}\) which was recorded on first contact of the emergency transport team with the patient at the referring hospital. All three scores were performed by the investigator who accompanied the transport team. In addition early clinical and laboratory parameters were recorded. No child died before arrival of the transport team, but cases (n=2) in which it was not possible to achieve haemodynamic stability and in which the patient died before secondary transport were included in the study. One further case died with clinical evidence of cerebral herniation after haemodynamic stability was achieved.

The age of the child at presentation, the first neutrophil and platelet counts, PICU outcome, GMSPS, Malley score and PIM score were available in all cases.

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS) 8.0 for Windows. Mann-Whitney tests were used to compare values between survivors and non-survivors. Multiple logistic regression models were used to identify predictors of death after transformation of the predictor variables where necessary. Receiver operating characteristic plots were constructed\(^{25}\) to illustrate the differences in sensitivity and specificity between potential predictive models using a variety of cut-offs.
Established severity scoring systems were applied to the current datasets and compared with the most recent published positive predictive values, sensitivities and specificities. One way tests of proportions, incorporating corrections for small sample size where necessary, were used.

**Validation Set**

The predictors identified in the development set were further investigated in collaboration with two further multidisciplinary UK tertiary PICUs. Age, presentation neutrophil and platelet counts, haemodynamic data on presentation and PICU outcome were requested. A total of 195 cases were identified and outcome data were available in every case. The cases were made up as follows; 92 cases from Addenbrooke's Hospital, PICU, Cambridge (1993-1999) in which clinical and laboratory details but not GMSPS or Malley Scores had been recorded prospectively as part of an on-going audit programme of acute MD; 103 patients from the two Manchester PICUs (1996-1999) in which data on presentation cell counts, Malley score and GMSPS were obtained retrospectively. In 26 cases (2 deaths) the initial haematology was unavailable or incomplete or had not been recorded as the child died very shortly after presentation. Analysis was performed with missing data scored as normal values (i.e. lowest risk) and repeated with all cases with missing data excluded.

Cases that were too unstable to transfer (n=2) were included in the final analysis. Cases that had died of cerebral herniation rather than multiple organ failure were identified by the clinicians at each institution (n=3 Manchester, n=0 Cambridge).
The proposed scoring system was then applied to this validation set of Cambridge and Manchester data (n= 195) and the performance of established scores assessed.

**Results**

Between 1997 and 1999, 6/32 (19%) cases of severe meningococcal disease referred to GOS requiring intensive care died. One case died with clinical evidence of cerebral herniation but the remainder had severe irreversible cardio-respiratory failure unsupportable within 24 hours of diagnosis, 2 of whom died before arrival at GOS. No amputations were required in this population.

**Performance of established scores: development set**

The performance of the widely used GMSPS clinically based score and the recently described Malley score (table 1) were assessed prospectively in this population. Outcomes were better than predicted in the most severely ill cases. The observed positive predictive values of the 'high risk' cases identified by Malley (score 1-3) and GMSPS (≥12) were significantly less than published values (table 2). This difference was apparent despite the high overall mortality rate of 19%, indicating that this group has been selected as having particularly severe disease. This is likely to reflect local patterns of referral to our institution, which acts as a tertiary referral centre only. Although the sensitivity of the scores observed in this population was not altered, specificity was significantly reduced.

*Development of a predictive score.*
On inspection of physiological and laboratory data the wide difference between presentation neutrophil and platelet counts amongst survivors and non-survivors was apparent as it has been in several recent series\textsuperscript{8,19}. However, the platelet and neutrophil counts observed in non-survivors (table 3) were much lower than those previously suggested to be critical.

The relationship of platelet and neutrophil counts to outcome was investigated using a multiple logistic regression model. The product of the first platelet and neutrophil counts (PN product) was calculated and used as an additional predictor. Platelet and neutrophil counts as well as their product were approximately lognormally distributed, hence log transformed values were used in the models. PN product was the strongest predictor of mortality (table 4). None of the other variables were significantly associated with outcome after accounting for differences in PN score. Hence a simple univariate model consisting of the platelet neutrophil product was identified as the best predictive model and was used in subsequent validations. Figure 1 illustrates the superiority of this simple model using several arbitrarily chosen cut-offs when compared to the traditionally used GMSPS and Malley scores. In fact, the PN score model was a perfect fit for the data excepting the death from cerebral herniation and most importantly for clinical use, the positive predictive value of the highest score, (PN<40) was high at 100%.

\textit{Validation set}

In order to investigate if this new prediction model could be more widely applicable, data from two other Paediatric Intensive Care Departments from Addenbrooke's Hospital Cambridge UK and the Royal Manchester Children's Hospitals were investigated. A total
of 195 cases were identified. In this series the overall mortality rate was 12.3% (24 deaths). Similar comparisons with previous scores were made to establish whether this represented a change from previous survival. The observations made in the development are confirmed; the observed PPV for mortality (GMSPS) or adverse outcomes (death or amputation, Malley score) and the specificity of these scores were significantly reduced in comparison to published series (table 2).

The performance of the proposed platelet-neutrophil score is shown in table 5 and in figure 2. While the model does not perform as well as the development set, it is still superior to the other published scores. All predictors perform poorly in this population though the simple PN score has the greatest area under the ROC curve (figure 2A).

Cerebral herniation is a potential confounding factor that may lessen the performance of the PN product as a predictive score. This complication is rarely seen in younger children in whom cardio-respiratory failure is the predominant cause of death. When the PN score was investigated in the subgroup of cases of <5 years of age, prediction was improved (area under curve PN score =0.89 figure 2B).

These results are not significantly altered by the exclusion of all cases with missing values or inclusion of all cases with missing values as lowest risk.
Discussion

This study demonstrates that established scoring systems in MD significantly overestimate the risk of poor outcome in this recent large data set. In particular, cases at the higher end of the severity spectrum were not well discriminated into survivors and non-survivors. Prediction in this high-risk group can be improved by the identification of very low neutrophil and platelet counts in preference to clinical assessments such as those used to construct the GMSPS.

These differences may be the result of different interpretations of the scores or of a genuine improvement in outcome in comparison to these historical data. The GMSPS can be difficult to assess, especially in infants and smaller children when the Glasgow Coma Score and a clinical assessment of meningism can be observer dependent\(^2\). In addition, 'parents description of deterioration in an hour before scoring\(^2\) may be very variable. The Malley score limits these potential problems of observer dependence by the use of simple, well-defined laboratory and clinical parameters but does require a clinical assessment of perfusion (and an adjustment of BP for age)\(^9\). The collection of the Malley score in the development set here was the initial contact with the transport team rather than Emergency Room which is likely to contribute to altered outcomes.

However, data from all three institutions indicates that the GMSPS and Malley scores overestimate mortality. Therefore, a genuine improvement in outcome rather than observer variability alone is a possible explanation. This interpretation is also supported
by understanding that the data on which these scores were developed dates from 1985-1994 for the Malley score\textsuperscript{19} and 1977 to 1986 for the GMSPS\textsuperscript{2}.

It is interesting to speculate why the established scores perform poorly at the higher end of the severity scale. Simply updating scores to recognise new management which now includes high frequency oscillatory ventilation, continuous veno-venous haemofiltration and more informed use of coagulation factor replacement may be an explanation (In cases admitted to GOSH, HFOV was started on ICU admission in severe cases and in all cases requiring a mean airway pressure of $>16$ cmH\textsubscript{2}O and FiO\textsubscript{2} of $>0.6$, and CVVH was used in all cases requiring renal support and those who required large volume coagulation factor transfusions). Another related possibility must be considered. Improved recognition of early MD and widespread formal resuscitation training (e.g. APLS) may mean that early care is improved. Therefore, scores based on acute physiology which is amenable to resuscitation may have lost predictive value. As such the bias of the GMSPS and Malley scoring systems towards shock may explain their decreased performance as predictors.

Numerous studies have identified neutrophils and platelets as central to the pathophysiological events associated with bacterial sepsis. In MD there is now considerable evidence to show that both these cells are important in the induction of endothelial injury\textsuperscript{11,26-28}. It has been shown that meningococci are potent inducers of both neutrophil\textsuperscript{29} and endothelial cell adhesion molecule expression\textsuperscript{13} which leads to enhanced adherence of neutrophils to the vascular endothelial surface. Once activated neutrophils can cause endothelial injury\textsuperscript{11}. A profoundly low neutrophil count in a child with normal bone marrow function is likely to occur as a result of severe endothelial activation and
rapid neutrophil recruitment\textsuperscript{19}. Thrombocytopenia also reflects the extent of endothelial activation and damage as platelets adhere to exposed sub-endothelial matrices and activated coagulation factors to accelerate disseminated intravascular coagulopathy. In addition there is now evidence that platelets and neutrophils act in concert to aid adherence to inflamed vascular endothelium\textsuperscript{13,27,30}. Therefore, low platelet and neutrophil counts at presentation could provide an indication of the degree of vascular injury induced largely because of inflammation established before initiation of therapy.

We have suggested the use of the product of the initial platelet and neutrophil counts to identify the highest risk cases. The clear advantage of this approach is that it has no observer variability. This score has a high positive predictive value for death (PN product \textless{}40 = 82\% in the combined series of cases). There are several possible mechanisms of death in MD in addition to overwhelming cardio-respiratory failure. These include cerebral herniation from oedema or haemorrhage, adrenal failure, arrhythmias secondary to electrolyte disturbances, and cardiac tamponade and a significant delay in providing effective resuscitation. Mortality due to these causes may be less dependent upon endothelial injury as indicated by the PN product. This may explain why the PN product has a high PPV but fails to identify a significant proportion of deaths (sensitivity of PN product \textgreater{}100 = 42\%). 30\% of the deaths with PN product \textless{}100 died from cerebral herniation. Details of other mechanisms of death were not recorded.

In the patient group in which a prospective analysis was possible the only death not predicted by the PN score was due to cerebral herniation. Although there are many factors that overlap between severe systemic disease and predominant CNS pathology,
the two outcomes can be considered to represent separate therapeutic challenges.
Interestingly there were no cases of cerebral herniation in patients with a very low PN product and in children aged less than five.

Classification of cases by level of risk and regardless of pathology is admirably achieved by multi-variable scores such as the Paediatric Index of Mortality or the Pediatric Risk of Mortality Scores. This is demonstrated by the capacity of the PIM and PRISM II scores to correctly classify the risk of the entire population. However, as more targeted interventions become available, these scores will not be able to identify the populations most likely to benefit from such therapies.

In this study, which included a large proportion of retrospective cases, the PN product was the most accurate predictor of death in MD. It cannot be manipulated by the observer, can be very readily assessed on presentation, and focuses solely on the clinical problem of severe shock/MOSF. As such, it may maximise the chance of identifying those cases most likely to benefit from an anti-inflammatory intervention. Dilution of results with patients at little or no risk of death or whom outcome is unlikely to be influenced by the therapy under investigation is ethically questionable, increases the cost of studies and reduces their power. We propose that the PN product should be included in the patient entry criteria for future anti-inflammatory trials in MD.
References


### Table 1

#### A) Glasgow Meningococcal Septicaemia Prognostic Score

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;4 years: BP &lt;75 Systolic</td>
<td></td>
</tr>
<tr>
<td>Age &gt;4 years: BP &lt;85 Systolic</td>
<td>3</td>
</tr>
<tr>
<td>Skin/rectal temp difference &gt;3°C</td>
<td>3</td>
</tr>
<tr>
<td>Modified coma score &lt;8 OR deterioration of ≥ 3 points in 1 hour</td>
<td>3</td>
</tr>
<tr>
<td>Parents description of deterioration in hour before scoring</td>
<td>2</td>
</tr>
<tr>
<td>Absence of meningism</td>
<td>2</td>
</tr>
<tr>
<td>Extending purpuric or widespread ecchymoses</td>
<td>1</td>
</tr>
<tr>
<td>Base deficit (capillary or arterial) &gt;8</td>
<td>1</td>
</tr>
</tbody>
</table>

**maximum score** 15

#### B) Malley score

Presence of 1, 2 or 3 of following parameters on presentation as predictor of death or amputation (limb or all digits on a single limb).

- ANC: $<3000/mm^3$
- Platelet count: $<150,000/mm^3$
- Poor perfusion: (cool extremities, mottled skin or delayed CRT (>2 seconds) in association with low systolic BP.

**Low BP:**
- $<70$ mmHg up to 1 month of age
- $<80$ mmHg 1 month to 5 years of age
- $<90$ mmHg over 5 years of age

In original study, patients who did not survive long enough to be admitted to 'inpatient services' were excluded.
<table>
<thead>
<tr>
<th>Published Malley</th>
<th>Observed DS</th>
<th>n=32 deaths =6</th>
<th>Observed VS</th>
<th>n=195 AO =27</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3</strong> PPV</td>
<td>100%</td>
<td>83% 5/6 (35.9-99.6) p&lt;0.05</td>
<td>39% 7/18 (17-64) p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>30%</td>
<td>83% 5/6 (35.9-99.6) p&lt;0.05</td>
<td>26% 7/27 (11-46) ns</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
<td>96% 25/26 (80.3-99.9) p&lt;0.05</td>
<td>93% 157/168 (69-87) p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>2</strong> PPV</td>
<td>80%</td>
<td>30% 6/20 (12-54) p&lt;0.001</td>
<td>26% 20/76 (16-36) p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>60%</td>
<td>100% 6/6 (54-100) ns</td>
<td>74% 20/27 (64-69) ns</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>97%</td>
<td>46% 12/26 (27-66) p&lt;0.001</td>
<td>67% 112/168 (60-73) p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td><strong>1</strong> PPV</td>
<td>54%</td>
<td>22% 6/27 (9-42) p&lt;0.01</td>
<td>17% 23/133 (11-24) p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>93%</td>
<td>100% 6/6 (54-100) ns</td>
<td>85% 23/27 (66.3-95.8) ns</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>79%</td>
<td>19% 5/26 (6.6-39.3) p&lt;0.001</td>
<td>35% 58/168 (28-42) p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td><strong>0</strong> PPV</td>
<td>13%</td>
<td>19% 6/32 (7-38) ns</td>
<td>14 27/195 (9-15)</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>100% 6/6 (54-100) ns</td>
<td>100% 27/27 (37.2-100) ns</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>0%</td>
<td>0% 0/26 (0-13.2) ns</td>
<td>0% 0/168 ns</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GMSPS</th>
<th>n=32 deaths =6</th>
<th>n=102 deaths =13</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12-15</strong> PPV</td>
<td>100%</td>
<td>42% 6/14 (18-71) p&lt;0.001</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>36%</td>
<td>100% 6/6 (54-100) ns</td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
<td>69% 18/26 (48.2-85.7)p&lt;0.001</td>
</tr>
<tr>
<td><strong>≥10</strong> PPV</td>
<td>42-88%</td>
<td>35% 6/16 (15-64) ns</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>100% 6/6 (54-100) ns</td>
</tr>
<tr>
<td>Specificity</td>
<td>40-98%</td>
<td>62% 16/26 (40.6-79.8) ns</td>
</tr>
<tr>
<td><strong>≥8</strong> PPV</td>
<td>30-74%</td>
<td>26% 6/23 (10-48) ns</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>100% 6/6 (54-100) ns</td>
</tr>
<tr>
<td>Specificity</td>
<td>20-95%</td>
<td>31% 8/26 (18.1-61.6) ns</td>
</tr>
</tbody>
</table>

Table 2. Performance of established severity scores in development set (DS) and validation set (VS). The 26 cases (2 deaths) in the validation set with missing or incomplete haematological data were scored as having normal values for the Malley score.
<table>
<thead>
<tr>
<th></th>
<th>Survivors n=26 Median (IQR)</th>
<th>Non-survivors n=6 Median (IQR)</th>
<th>Significance Mann-Whitney</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>2.0 (1.0-5.0)</td>
<td>0 (0-2.5)</td>
<td>p=0.029</td>
</tr>
<tr>
<td><strong>GMSPS</strong></td>
<td>9 (3-11)</td>
<td>15 (15-15)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td><strong>Neutrophil count (x10⁹/l)</strong></td>
<td>10.9 (5-17)</td>
<td>0.5 (0.2-3)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td><strong>Platelet count (x10¹²/l)</strong></td>
<td>196 (84-236)</td>
<td>22 (11-44)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td><strong>PN product</strong></td>
<td>1295 (634-3689)</td>
<td>17.8 (2.6-84)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td><strong>Paediatric Index of Mortality</strong></td>
<td>0.06 (0.01-0.09)</td>
<td>0.28 (0.24-0.61)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
Table 4

Development set logistic regression models.

-2log likelihood indicates the performance of the predictor with lower values being better predictors. The exponential of the estimated coefficient is the relative change in odds of death with a unit change in the predictor. For example, a unit change in $\log_{10}$ PN product (1 to 2 = change in PN product 10 to 100) is associated with a reduction of odds of death by a factor of $0.02 = 1/50$ and an increase in Malley score by one is associated with a $66.7\times$ risk of death.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>-2log likelihood</th>
<th>Exponential of estimated coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\log_{10}$ neutrophil count</td>
<td>15.0</td>
<td>0.013</td>
</tr>
<tr>
<td>$\log_{10}$ platelet count</td>
<td>14.5</td>
<td>0.005</td>
</tr>
<tr>
<td>$\log_{10}$ platelet neutrophil product</td>
<td>9.1</td>
<td>0.02</td>
</tr>
<tr>
<td>$\log_{10}$ age</td>
<td>25</td>
<td>0.024</td>
</tr>
<tr>
<td>$\log_{10}$ PIM score</td>
<td>11.4</td>
<td>1439</td>
</tr>
<tr>
<td>GMSPS</td>
<td>19.1</td>
<td>6816</td>
</tr>
<tr>
<td>Malley Score</td>
<td>12.6</td>
<td>66.7</td>
</tr>
<tr>
<td>PN product</td>
<td>10.8</td>
<td>0.988</td>
</tr>
<tr>
<td>PN product</td>
<td>Observed DS</td>
<td>Observed VS</td>
</tr>
<tr>
<td>------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>$n=32$ deaths =6</td>
<td>$n=195$ deaths =24</td>
</tr>
<tr>
<td>&lt;40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>100% 5/5 (48-100)</td>
<td>66% 4/6 (22-94)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>83% 5/6 (35.9-99.6)</td>
<td>17% 4/24 (4.7-37.4)</td>
</tr>
<tr>
<td>Specificity</td>
<td>100% 26/26 (86.8-100)</td>
<td>99% 169/171 (97-100)</td>
</tr>
<tr>
<td>40-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>83% 5/6 (35.9-99.6)</td>
<td>57% 8/14 (29-82)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>83% 5/6 (35.9-99.6)</td>
<td>33 8/24 (15.5-55.3)</td>
</tr>
<tr>
<td>Specificity</td>
<td>96% 25/26 (80.4-99.9)</td>
<td>95 165/171 (93-99)</td>
</tr>
<tr>
<td>101-250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>63% 5/8 (24-92)</td>
<td>39% 14/36 (23-55)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>83% 5/6 (39-99.6)</td>
<td>58 14/24 (36.6-77.9)</td>
</tr>
<tr>
<td>Specificity</td>
<td>88% 23/26 (69.9-97.6)</td>
<td>93 159/171 (89-97)</td>
</tr>
<tr>
<td>250-1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>30% 6/18 (13-59)</td>
<td>20% 17/84 (11-29)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100% 6/6 (54-100)</td>
<td>71% 17/24 (36.6-77.9)</td>
</tr>
<tr>
<td>Specificity</td>
<td>54% 14/26 (33.4-73.4)</td>
<td>61% 104/171 (55-68)</td>
</tr>
<tr>
<td>&gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>19% 6/32 (7-38)</td>
<td>12% 24/195 (8-15)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100% 6/6 (54-100)</td>
<td>100 24/24 (85.8-100)</td>
</tr>
<tr>
<td>Specificity</td>
<td>0% 0/26 (0-13.2)</td>
<td>0 0/171</td>
</tr>
</tbody>
</table>

Table 5

The 26 cases (2 deaths) in the validation set with missing or incomplete haematological data were scored as having normal values i.e. PN product >1000.
Table 6

Performance of established multivariate predictive models in combined DS and VS.

<table>
<thead>
<tr>
<th>Risk of Mortality</th>
<th>Actual n</th>
<th>Risk of Mortality</th>
<th>Actual n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (95% ci)</td>
<td>% (95% ci)</td>
<td></td>
</tr>
<tr>
<td>&gt;30%</td>
<td>3/12</td>
<td>&gt;30%</td>
<td>6/12</td>
</tr>
<tr>
<td></td>
<td>23 (6-57)</td>
<td></td>
<td>50 (21-79)</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>15-29%</td>
<td>2/9</td>
<td>15-29%</td>
<td>2/9</td>
</tr>
<tr>
<td></td>
<td>22 (3-60)</td>
<td></td>
<td>22 (2-60)</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>&lt;15%</td>
<td>0/36</td>
<td>5-14%</td>
<td>4/27</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>15 (4-34)</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>&lt;5%</td>
<td>0/21</td>
<td></td>
<td>0 (0-16)</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td></td>
<td>ns</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1

Receiver operating characteristic curves for development set.

Area under curve for PN product = 0.97, GMSPS = 0.81 and Malley = 0.92.

If the single case of cerebral herniation is excluded then areas under the curve become
PN product 1, Malley 0.98 and GMSPS 0.81. The cut-offs used for plotting the curve for
PN product are those shown in table 5.

Figure 2

ROC curves for validation set. A) Data from each unit are presented separately to allow
comparison with GMSPS which was only available from the Manchester cases. Missing
values are scored as normal (i.e. lowest score). While no score predicts outcome ideally
in these populations, the objective PN product (plotted using the cut-offs as shown in table
5) is at least as good as established scores.

B) ROC curves of validation set, cases limited to those <5 years. All scores show
improved prediction compared to the whole population but PN product retains the greatest
area under the ROC curve (0.89).