Superantigens, endothelial injury and vasculitis in the young.

Paul Anthony Brogan BSc (Hon) MBChB (Hon), MRCP, MSc.

Submitted for the degree of Doctor of Philosophy.

Department of Nephro-Urology, Institute of Child Health, University College London.
Acknowledgements

I would like to acknowledge my supervisors, Professor Michael Dillon and Dr Nigel Klein for constant support, enthusiasm, ideas and discussion. It was truly a great privilege to be Professor Dillon’s last research fellow before his retirement- Mike I hope this thesis honours the fantastic contribution you have made to the study of the paediatric vasculitides over the years. A special thanks must go to Mrs Vanita Shah for help and guidance in the laboratory, and for much support and nurturing throughout the whole duration of my PhD. Thanks also to Alka Patel for showing me how to turn on a flow cytometer, and to Professor Nancy Hogg for the kind gifts of the monoclonal antibodies, and to Ms Carol Hutchinson for collecting control samples. Acknowledgement must also go to the Charlotte Parkinson Research Fund and John Herring and Friends Trust, who funded this research. Thanks also to the children and parents who participated in the studies described in this thesis.

Lastly, I would like to say thankyou to my wife Gabi, for putting up with me throughout all of this.

Paul Brogan, October 2002.
Thesis Abstract

Superantigens, endothelial injury, and vasculitis of the young.

Limited data exist suggesting a role for superantigens (SAgs) in the aetiopathogenesis of vasculitis syndromes in children and adults. The best evidence relates to Kawasaki Disease (the second commonest vasculitic syndrome of childhood), although this remains a controversial issue. Even fewer data exist examining how SAgs could cause endothelial and/or vascular injury. This thesis addressed these issues by examining peripheral blood T cell activation and T cell Vβ skewing in children with vasculitis. Secondly, an in vitro model was established to examine the hypothesis that the endothelial cell may operate as a competent SAg-presenting cell for T cells. Further evidence of endothelial injury in children with vasculitis was derived by examining circulating endothelial microparticles (EMP) in children with vasculitis at various stages of disease activity. Children with active vasculitis had higher CD4 and CD8 peripheral blood T cell CD69 expression than those with inactive vasculitis and healthy controls, but not disease control children. No difference in CD25 expression in either the CD4 or CD8 T cell populations was observed between active and inactive vasculitis groups, however. There was a significantly increased variance of CD4 Vβ12, and Vβ17 and CD8 Vβ1 in the primary systemic vasculitis group as compared to control and disease controls. Moreover, 80% of the primary systemic vasculitis children had one or more CD4 Vβ expansions or deletions, as compared with 30% of controls and 37% of the disease controls (p<0.002). In the KD group, the mean % of CD4 Vβ2 T cells was higher than in controls or disease controls. Co-culture of purified T Cells and MHC class II⁺ HUVEC with SAg resulted in Vβ-restricted CD4 and CD8 CD69 upregulation. Additionally, there was CD4 and CD8 T Cell Vβ-restricted adherence to the HUVEC monolayer at 4 hours, which was partially abrogated by blockade of the integrin VLA-4, but not LFA-1. ICAM-1, E-selectin, and VCAM-1 expression were upregulated on the MHC class 1⁺ HUVEC following exposure to SAg in the presence of T cells, and there was increased EMP release from the activated HUVEC. Plasma from patients with active vasculitis contained increased numbers of E-selectin and CD105 positive EMP compared with patients in remission, controls and disease controls. EMP correlated with clinical and laboratory indices of vasculitic disease activity, but there was overall a poor correlation between EMP and acute phase reactants in the disease controls. In conclusion, these data provide indirect evidence of superantigenic involvement in vasculitis syndromes of the young, and suggest that one possible mechanism of endothelial injury mediated by SAg could involve dual signaling between the endothelial cell (EC) and T cell, with EC activation and injury. These data also underscore the endothelium as both a target for injury in vasculitis, and as a potential amplifier of aberrant inflammatory responses, and suggest that circulating EMP may provide a window to the activated endothelium in vasculitis.
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**CHAPTER 1: INTRODUCTION AND THESIS AIMS**

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<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>AECA</td>
<td>Anti endothelial cell antibody</td>
</tr>
<tr>
<td>AILD</td>
<td>Angioimmunoblastic T cell lymphoma disease</td>
</tr>
<tr>
<td>ALC</td>
<td>Angiotropic large cell lymphoma</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BPSU</td>
<td>British Paediatric Surveillance Unit</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BVAS</td>
<td>Birmingham Vasculitis Activity Score</td>
</tr>
<tr>
<td>CAA</td>
<td>Coronary artery abnormality</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>cANCA</td>
<td>Cytoplasmic antinuclear cytoplasmic antibody</td>
</tr>
<tr>
<td>CDR3</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CINCA</td>
<td>Chronic infantile neurological cutaneous articular syndrome</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II associated invariant chain peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRP</td>
<td>C reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebro-spinal fluid</td>
</tr>
<tr>
<td>CSS</td>
<td>Churg Strauss Syndrome</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>CD152</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>ECAF</td>
<td>Endothelial cell attachment factor</td>
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<tr>
<td>ECG</td>
<td>Electrocardiograph</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
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<tr>
<td>EMP</td>
<td>Endothelial microparticle</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow activated cell sorter</td>
</tr>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>Glomerular basement membrane</td>
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<tr>
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<td>Giant cell arteritis</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
</tr>
<tr>
<td>HMEC</td>
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<tr>
<td>HSP</td>
<td>Henoch Schönlein Purpura</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical endothelial cell</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IIF</td>
<td>Indirect immunofluorescence</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirit</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ISKDC</td>
<td>International Study of Kidney Diseases in Children</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>JDM</td>
<td>Juvenile dermatomyositis</td>
</tr>
<tr>
<td>JIA</td>
<td>Juvenile idiopathic arthritis</td>
</tr>
<tr>
<td>KD</td>
<td>Kawasaki disease</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte functional antigen-1</td>
</tr>
<tr>
<td>LR</td>
<td>Likelihood ratio</td>
</tr>
<tr>
<td>MAM</td>
<td>Mycoplasma arthritides mitogen</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence index</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>MPA</td>
<td>Microscopic polyangiitis</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthetase</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>PAN</td>
<td>Polyarteritis nodosa</td>
</tr>
<tr>
<td>pANCA</td>
<td>Perinuclear anti neutrophil cytoplasmic antibody</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PMP</td>
<td>Platelet microparticle</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PR3</td>
<td>Proteinase 3</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PSV</td>
<td>Primary systemic vasculitis</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinase activating gene</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>ROC curve</td>
<td>Receiver operator characteristic curve</td>
</tr>
<tr>
<td>SABE</td>
<td>Subacute bacterial endocarditis</td>
</tr>
<tr>
<td>SAg</td>
<td>Superantigen</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SV</td>
<td>Systemic vasculitis</td>
</tr>
<tr>
<td>SVV</td>
<td>Small vessel vasculitis</td>
</tr>
<tr>
<td>TC</td>
<td>T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>Takayasu disease</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TSS</td>
<td>Toxic shock syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Toxic shock syndrome toxin type 1</td>
</tr>
<tr>
<td>TTP</td>
<td>Thrombotic thrombocytopenic purpura</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
</tr>
<tr>
<td>WG</td>
<td>Wegener's granulomatosis</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction and thesis aims

1.1 Vasculitis of the young

1.2 The immuno-pathogenesis of vasculitis

1.3 Superantigens and vasculitis

1.4 Thesis aims
1.1 Vasculitis of the young

1.1.1 Introduction - definition of vasculitis

The vasculitides are a group of disorders characterised by the presence of inflammatory infiltrates (which may be predominantly neutrophilic, eosinophilic, or mononuclear) in the walls of blood vessels, with resultant tissue ischaemia and necrosis. The term vasculitis refers to the presence of inflammation in a blood vessel wall (Petty and Cassidy, 2001a). Perivasculitis describes inflammation around the blood-vessel wall but without involvement of the mural structure itself (Petty and Cassidy, 2001a). Vasculopathy is a broader term encompassing abnormalities of blood vessels that may be inflammatory, but may also be developmental or degenerative. More recently, the distinction between certain degenerative vasculopathies and inflammatory vasculopathy has become less clear with the emerging body of evidence that the pathogenesis of atherosclerosis (previously considered to be a degenerative vasculopathy) involves an important inflammatory component (Alexander and Dzau, 2000). Vasculitis may affect blood vessels of all sizes and may be the major manifestation of a disease (primary systemic vasculitis e.g. microscopic polyangiitis), or one aspect of a more widespread disease (secondary vasculitis e.g. systemic lupus erythematosus and the other connective tissue diseases) (Flores-Suarez and Alarcon-Segovia, 2000).
1.1.2 Classification of the paediatric vasculitides

Classification of the paediatric vasculitic disorders has proved difficult because of the absence of sensitive and specific diagnostic tests and an incomplete understanding of the aetiology of most vasculitis syndromes. Moreover, as in adult vasculitic syndromes, many children have constellations of symptoms and findings that overlap the clinical features of the various individual diseases that will be discussed below.

The most widely used classification system for the purposes of research is the Chapel Hill Consensus on the classification of the primary systemic vasculitides (Jennette, Falk and others, 1994) which is widely accepted in adult practice and is shown in Appendix 1. This classification system is based upon the smallest blood vessel involved in the disease process: Wegener’s granulomatosis, for instance, is classified as a small vessel disease but vasculitis is detected in small and medium-sized arteries and larger vessels, including the renal artery and aorta.

Importantly, the Chapel Hill criteria and the other main set of classification criteria for the vasculitides- the American College of Rheumatology (ACR) criteria- both of which are used for the description of patients in this thesis (see Chapter 2 and Appendix 1) have never been evaluated in children (Petty and Cassidy, 2001a). Indeed there is much debate regarding the most reliable classification system, with recent studies favouring the Chapel Hill consensus. In this thesis, when considering aetio-pathogenesis (Chapter 3) both the Chapel Hill and the ACR classification criteria were utilised because neither system on its own classifies the paediatric vasculitides absolutely. For example, since the Chapel
Hill consensus defines a vasculitis based on involvement of the smallest artery manifesting vasculitic changes and as such the diagnosis of classical polyarteritis nodosa (PAN) would be precluded using this system if rash (such as purpura or livedo reticularis—signs of small vessel vasculitis) was present. In contrast, the presence of livedo reticularis is one of the defining criteria for classical PAN using the ACR criteria and small vessel vasculitic skin rash such as livedo reticularis is frequently observed in children with classical PAN (Dillon, 1998; Petty and Cassidy, 2001b; Ozen, Besbas and others, 1992).

For the purposes of this thesis, therefore, both the ACR and Chapel Hill criteria were used, which classified the subjects adequately (but by no means absolutely), to allow comparison with previously published studies. The limitations of such classification systems must be borne in mind, however. Indeed, the sensitivity and specificity of the ACR criteria for the classification of PAN in adults are relatively low at 82.2% and 86.6% respectively (Lightfoot, Michel and others, 1990), emphasising the limitations of using such criteria for the classification vasculitis for research purposes.

In addition, an important concept worthy of emphasis is that classification criteria are not the same as diagnostic criteria although the former are often confused and misused as the latter (Hunder, 1998). Classification criteria work best in the study of groups of patients and work less well in the diagnostic evaluation of individual patients. As an example, the ACR criteria for PAN were designed to differentiate PAN from other types of vasculitis but not to diagnose vasculitis in the first instance (Hunder, 1998).
In clinical practice, one classification that relates specifically to the paediatric vasculitides that has proved useful is based on that of Fink (Fink, 1986), and is outlined in Table 1.1. This system, since it pertains to paediatric vasculitides, will be used as a template for the consideration of individual vasculitic syndromes in this chapter.
Table 1.1: The Classification of Paediatric Vasculitides based on Fink

<table>
<thead>
<tr>
<th>Classification</th>
<th>Disease</th>
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<tbody>
<tr>
<td>Polyarteritis</td>
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<tr>
<td>Macroscopic</td>
<td></td>
</tr>
<tr>
<td>Microscopic</td>
<td></td>
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<tr>
<td>Cutaneous</td>
<td></td>
</tr>
<tr>
<td>Kawasaki disease</td>
<td>Mucocutaneous Lymph Node Syndrome</td>
</tr>
<tr>
<td>Granulomatous vasculitis</td>
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<tr>
<td>Wegener's Granulomatosis</td>
<td></td>
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<tr>
<td>Churg-Strauss Syndrome</td>
<td></td>
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<tr>
<td>Primary angiitis of the central nervous system</td>
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<tr>
<td>Leukocytoclastic vasculitis</td>
<td></td>
</tr>
<tr>
<td>Henoch-Schonlein purpura</td>
<td></td>
</tr>
<tr>
<td>Hypersensitivity angiitis</td>
<td></td>
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<tr>
<td>Hypocomplementemic urticarial vasculitis</td>
<td></td>
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<tr>
<td>Vasculitis associated with connective tissue disease</td>
<td></td>
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<tr>
<td>Systemic lupus erythematosus</td>
<td></td>
</tr>
<tr>
<td>Juvenile chronic arthritis</td>
<td></td>
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<tr>
<td>Mixed connective tissue disease</td>
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<tr>
<td>Dermatomyositis</td>
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<tr>
<td>Scleroderma</td>
<td></td>
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<tr>
<td>Giant cell arteritis</td>
<td></td>
</tr>
<tr>
<td>Takayasu disease</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous vasculitides</td>
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</table>
1.1.3 Description of individual vasculitic syndromes

1.1.3.1 Polyarteritis nodosa (PAN) and cutaneous polyarteritis

The classic form of polyarteritis, polyarteritis nodosa (PAN), is a necrotizing vasculitis associated with aneurysmal nodules along the walls of medium-sized muscular arteries, and first described in detail in 1866 by Kussmaul and Maier (Kussmaul and Maier, 1866; Dillon, 1998; Petty and Cassidy, 2001b). Although there is an overlap with smaller vessel disease, it is distinct from microscopic polyangiopathy, and occurs more commonly in childhood than this latter disorder (Brogan and Dillon, 2000a). The main clinical features are malaise, fever, skin rash (figure 1.1), abdominal pain and arthropathy (figure 1.2) (Besbas, Ozen and others, 2000; Brogan and Dillon, 2000a). Other features include testicular pain, myalgia, hypertension, neuropathy, renal failure, organic psychosis, and myocardial ischaemia (Besbas, Ozen and others, 2000; Brogan and Dillon, 2000a; Lightfoot, Jr., Michel and others, 1990). Visceral angiography plays a key role in the diagnosis (figure 1.3) (Bron, Strott, and Shapiro, 1965; McLain, Kelsch, and Bookstein, 1972; Brogan, Davies and others, 2002).

Cutaneous polyarteritis is a syndrome associated with crops of painful skin nodules, livedo reticularis, and often with a story of preceding upper respiratory tract infection (David, Ansell, and Woo, 1993; Diaz-Perez and Winkelmann, 1974; Fink, 1991). The condition responds to non-steroidal anti inflammatory drugs but can require steroids. If streptococcus induced, prophylactic penicillin has a role. Cutaneous vasculitis usually
runs a benign course although in some patients the cutaneous features evolve into systemic polyarteritis nodosa. Relapses, particularly in association with recurrent
Figure 1.1 Spectrum of vasculitic skin rash in PAN

1A: Severe skin necrosis and gangrene; 1B: Ecchymotic and purpuric skin lesions; 1C: Livedo reticularis; 1D: Patchy necrotic skin ulceration ("punched-out" vasculitic rash)
Figure 1.2: Symmetrical polyarthritis in a 12 year old boy with PAN affecting knees and ankles.
Figure 1.3: Renal angiography in PAN

Figure 1.3A: Renal angiogram from a 6 year old girl with PAN demonstrating florid aneurysmal and non-aneurysmal changes. Large aneurysms (LA), small aneurysms (SA), perfusion defect (PD), arterial cut-off (CO), lack of crossing of peripheral renal arteries (X), collateral artery (Col).

Figure 1.3B: Renal angiogram from an 8 year old boy with PAN demonstrating less florid aneurysmal and non-aneurysmal changes. Perfusion defect (PD), small aneurysm (SA) in association with arterial cut-off (CO).
streptococcal infection are seen in up to 25% of cases. Clinicians seeing this condition usually advise continuing penicillin prophylaxis throughout childhood to prevent relapses since it is amongst the relapsing group that systemic vasculitis tends to occur.

1.1.3.2 Microscopic polyangiitis

Microscopic polyangiitis (MPA, formerly microscopic polyarteritis) differs from classic PAN by the presence of extensive glomerular involvement, and may be defined as small vessel vasculitis with focal segmental glomerulonephritis but without granulomatous disease of the respiratory tract (Savage, Winearls and others, 1985). Clinically, it can be difficult to distinguish from Wegener’s granulomatosis, and often presents with rapidly progressive pauci-immune glomerulonephritis (Jardim, Leake and others, 1992), in association with perinuclear anti neutrophil cytoplasmic antibody (pANCA) positivity (Jennette, Falk and others, 1994).

Treatment of PAN and MPA

Treatment for both PAN and MPA consists of steroids, anti-platelet agents, and an additional cytotoxic agent, ordinarily cyclophosphamide (Adu, Pall and others, 1997; Fauci, Katz and others, 1979). Cyclophosphamide is usually administered orally for 2-3 months at 2 mg/kg/day to induce remission (Brogan and Dillon, 2000b). Pulsed intravenous cyclophosphamide may have advantages over the oral route in reducing the total cumulative dose and hence side effects, but it may not be as effective as the daily oral regimen in aggressive disease for the prevention of relapses (Gaskin G and Pusey, 1998). Maintenance therapy is usually with oral azathioprine at a dose of 2 mg/kg/day, with low dose alternate day prednisolone (0.2-0.5 mg/kg), and anti-platelet agents
(Brogan and Dillon, 2000b). If remission with this regimen is not maintained, then
cyclosporin or mycophenolate mofetil may prove useful, although the published evidence
for the use of these agents in this context is lacking. The guideline that is currently used
at Great Ormond St Hospital for Children, London (and hence the treatment that the
majority of the patients described in this thesis received) is outlined in figures 1.4 and
1.5. Currently, the mortality for PAN Great Ormond Street Hospital, London is about
10%, which compares favourably with many adult series, and other paediatric series of
PAN (Besbas, Ozen and others, 2000; Dillon, 1998).
Figure 1.4: Guideline for the treatment of childhood PAN and related vasculitides

**INDUCTION THERAPY (2/12)**
- Prednisolone 60 mg/m² OD for 4/52, weaning over next 6-8 weeks (depending on response to Rx) to 0.5 mg/kg on alternate days, OR IV methyl prednisolone 600 mg/m² (max 1g) for 3 consecutive days followed by oral prednisolone as above
- Cyclophosphamide 2 mg/kg PO OD for 2/12 OR 750 mg/m² IV once a month for 6 months (reduce dose if renal failure)
- Aspirin 2 mg/kg OD (OR Dipyridamole 2.5 mg/kg BD if aspirin contraindicated)

**MAINTENANCE THERAPY for 18/12 to 3 years for PAN; lifelong therapy for Wegeners**
- Azathioprine 2-2.5 mg/kg PO OD (start 3-5 days after stopping CYC)
- Prednisolone 0.2-0.5 mg/kg alternate days
- Aspirin 2 mg/kg OD OR Dipyridamole 2.5 mg/kg BD
- Consider ranitidine if abdo pain
- If stopping Rx, stop azathioprine first, then wean prednisolone over next 12 months

**CONSIDER**
- Single dose of IV Cyclophosphamide 750 mg/m² if previously given oral CYC for induction of remission
- Methylprednisolone 600 mg/m² (max 1g) IV X 3 if not previously given as first line
- 5 or 10 day course of daily 2 volume plasma exchange with 4.5% human albumin solution
- Second course of oral cyclophosphamide 2mg/kg OD for 2/12

**NOTES:**
1. Second line maintenance agents
   1. MMF
   2. Cyclosporine A
   3. MTX
2. Consider sperm cryopreservation for all post-pubertal males receiving CYC, and oocyte preservation for females
3. For monitoring of complications of therapy refer to table 2 [4]
4. Beware neutropaenia as prednisolone dose is weaned during maintenance phase of therapy
5. Miscellaneous vasculitides such as Behçet’s may require colchicine or thalidomide

**FAILED REMISSION**

- Minor relapse: increase oral prednisolone if not steroid toxic
- Recurrent minor relapses or "grumbling vasculitis": consider IV pulsed Methylprednisolone and/or switch to second line maintenance therapy
Figure 1.5: The treatment of paediatric microscopic polyangiitis and related crescentic nephritides

Crescentic GN on renal biopsy

- Linear staining on immunofluorescence
  - Anti GBM +

- Pauci-immune on immunofluorescence—consider microscopic polyarteritis or 'renal-limited vasculitis'—may be ANCA +
  - Treatment as per vasculitis algorithm above, but use plasma exchange as first line induction therapy in conjunction with steroid and cyclophosphamide

- Granular deposits on immunofluorescence—indicative of immune complex disease
  - Electron microscopy
  - Treat specific disorders (refer to relevant protocols):
    - HSP
    - IgA nephropathy
    - Post streptococcal
    - SLE
    - Membranoproliferative GN
    - Membranous GN

Consider prolonging therapy if Anti GBM still detectable

- Plasma exchange for 10-14 days (2 volume, 4.5% HAS)—or until anti GBM Ab disappears
- Pulsed IV Methyl prednisolone 600 mg/m2 (max 1 g) X3, then oral prednisolone 60 mg/m2 OD (weaned over 2 months then stop)
- Cyclophosphamide 2 mg/kg PO OD for 2/12, OR IV at 500 mg/M2 monthly (reduced dose because of renal failure), possibly increasing by 250 mg/m2 per month (response dependent) to maximum of 1000mg/m2 for 6 months

Treat specific disorders (refer to relevant protocols):
- HSP
- IgA nephropathy
- Post streptococcal
- SLE
- Membranoproliferative GN
- Membranous GN

29
1.1.3.3 Kawasaki disease (KD)

In 1967 Tomisaku Kawasaki described 50 Japanese children with an illness characterised by fever, rash, conjunctival injection, erythema and swelling of hands and feet, and cervical lymphadenopathy (Kawasaki, 1967). The mucocutaneous lymph node syndrome that he described is now recognised as Kawasaki disease (KD), and is the second commonest vasculitic illness of childhood (Henoch-Schonlein Purpura being the commonest). KD is associated with the development of systemic vasculitis complicated by coronary and peripheral arterial aneurysms, and myocardial infarction in some patients (Shulman, De Inocencio, and Hirsch, 1995). It is the commonest cause of acquired heart disease in children in the United Kingdom and the USA (Shulman, De Inocencio, and Hirsch, 1995).

Since the early descriptions of KD there have been 21 randomised controlled trials (RCTs), and at least 2 meta-analyses examining therapeutic interventions in KD. Despite intensive research into the illness the cause remains unknown, and although there have been significant improvements in diagnosis and treatment of children with the disease there are still a number of important unanswered questions regarding therapy. Furthermore, there is still no diagnostic test available for KD.

Epidemiology of KD

KD is commonest in Japan where more than 125 000 cases have been reported (Tizard, 1999a). The disease is also commoner in Japanese and other Oriental children living abroad (Shulman, De Inocencio, and Hirsch, 1995). Children aged 6 months to 5 years
are most susceptible, with peak incidence in children aged 9-11 months (Levin, Tizard, and Dillon, 1991). Seasonal variation in the disease incidence has been reported, with peak occurrence in the winter and spring months (Shulman, De Inocencio, and Hirsch, 1995). Outbreaks of KD have been linked to weather patterns with clusters of KD cases occurring in association with precipitation (Bronstein, Dille and others, 2000). Direct person to person spread is not observed, although in Japan the disease occurs more commonly in siblings of index cases with an estimated peak incidence of 8-9% in siblings under the age of 2 years (Fujita, Nakamura and others, 1989). Currently, the estimated incidence of KD in the United Kingdom is 8.1 per 100 000 children under the age of five—an incidence which has doubled since 1991 (Harnden, Alves, and Sheikh, 2002).

Although KD is believed to be caused by an infectious agent in an immunologically susceptible individual, the causative agent remains elusive.

**Diagnosis of KD**

There is no diagnostic test for KD, therefore diagnosis is based on clinical criteria (table 1.2, and figure 1.6) (Dajani, Taubert and others, 1993) and the exclusion of other diseases, particularly sepsis. The differential diagnosis includes toxic shock syndrome (streptococcal and staphylococcal), staphylococcal scalded skin syndrome, scarlet fever, and infection with enterovirus, adenovirus, measles, parvovirus, Epstein-Barr virus, cytomegalovirus, mycoplasma pneumoniae, rickettsiae, and leptospirosis (Brogan, Bose and others, 2002). The differential diagnosis of IVIG (intravenous immunoglobulin)-
Fever of ≥ 5 days duration + four of five criteria:

1. Oropharyngeal Changes
   (90%+ of cases)

2. Changes in peripheral extremities
   (90%+ of cases)

3. Bilateral non-purulent conjunctival injection
   (90%+ of cases)

4. Polymorphous rash
   (95%+ of cases)

5. Cervical lymphadenopathy
   (75% of cases)
resistant KD includes PAN, systemic-onset juvenile idiopathic arthritis, and malignancy (particularly lymphoma).

**Table 1.2: Kawasaki Disease: diagnostic criteria (Dajani, Taubert and others, 1993)**

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>Duration of 5 days or more PLUS 4 of the following:</td>
</tr>
<tr>
<td>1. Conjunctivitis</td>
<td>Bilateral, bulbar, non-suppurative</td>
</tr>
<tr>
<td>2. Lymphadenopathy</td>
<td>Cervical, &gt;1.5 cm</td>
</tr>
<tr>
<td>3. Rash</td>
<td>Polymorphous, no vesicles or crusts</td>
</tr>
<tr>
<td>4. Changes of lips or oral mucosa</td>
<td>Red cracked lips; &quot;strawberry&quot; tongue; or diffuse erythema of oropharynx</td>
</tr>
<tr>
<td>5. Changes of extremities</td>
<td>Initial stage: erythema and oedema of palms and soles Convalescent stage: peeling of skin from fingertips</td>
</tr>
</tbody>
</table>

KD may be diagnosed with fewer than 4 of these features if coronary artery aneurysms are detected.

Fever of 5 days duration plus 4 of the 5 remaining criteria or the presence of fever and coronary arterial abnormalities (CAA) with 3 additional criteria are required for the diagnosis of "complete" cases (Tizard, 1999a). "Incomplete" cases comprise those with less than the prerequisite number of criteria. Irritability is an important sign, which is nearly always present, although not included as one of the diagnostic criteria (Brogan, Bose and others, 2002). The exact mechanism of the irritability is unclear, but it may be related to the presence of aseptic meningitis. Another clinical sign not incorporated into the diagnostic criteria but which is relatively specific to KD is the development of erythema and induration at sites of BCG immunisations (Brogan, Bose and others, 2002). The mechanism of this clinical sign is cross-reactivity of T cells in KD patients between specific epitopes of mycobacterial and human heat shock proteins (Sireci, Dieli, and Salerno, 2000). With an increasing number of infants receiving the BCG in the UK, it is
likely that this sign will become more common, and awareness of it could result in earlier diagnosis and treatment.

An important point worthy of emphasis is that the criteria may present sequentially such that a so-called "incomplete" case can evolve with time into a "complete" case. Thus the diagnosis of KD must be considered in any child with a febrile exanthematous illness, particularly if it persists longer than four to five days.

Other relatively common clinical findings in KD include arthritis, aseptic meningitis, pneumonitis, uveitis, gastroenteritis, meatitis and dysuria, and otitis (Petty and Cassidy, 2001c). Relatively uncommon abnormalities include hydrops of the gallbladder, gastrointestinal ischaemia, jaundice, petechial rash, febrile convulsions, nerve deafness, and encephalopathy or ataxia (Petty and Cassidy, 2001c). Cardiac complications other than coronary arterial abnormalities include cardiac tamponade, cardiac failure, myocarditis, and pericarditis (Petty and Cassidy, 2001c).

**Laboratory findings in KD**

KD is associated with many non-specific laboratory findings. Acute phase proteins, neutrophils, and erythrocyte sedimentation rate are usually elevated. Thrombocytosis occurs towards the end of the second week of the illness and therefore may not be helpful diagnostically (Petty and Cassidy, 2001c). Liver function may be deranged. Sterile pyuria is occasionally observed, and also CSF pleocytosis (predominantly lymphocytes) representing aseptic meningitis (Shulman, De Inocencio, and Hirsch, 1995).
Treatment of KD

Treatment of KD is aimed at reducing inflammation, and preventing the occurrence of coronary artery aneurysms (CAA) and arterial thrombosis. The optimal doses of aspirin and IVIG are discussed, and the treatment of refractory KD is considered.

Aspirin

It is worthy of note that treatment of KD with aspirin alone has never been subject to a randomised controlled trial, although aspirin versus aspirin plus IVIG has been studied. Aspirin is given in the acute phase of the illness at relatively high "anti-inflammatory" doses (30-100 mg/Kg/day). Following defervescence of the disease aspirin is given as an anti-platelet agent in a dose of 2-5 mg/Kg/OD, the duration being dependent on findings on echocardiography but usually for a minimum of 6-8 weeks.

Recent reviews have recommended the higher anti-inflammatory dose of 100 mg/Kg/day in the acute phase of the illness (Tizard, 1999a) on the basis of reducing duration of fever and length of hospitalisation compared with low dose aspirin (3-8 mg/kg/day) (Melish, Takahashi, and Shulman, 1992). No such comparative data on fever and hospitalisation exists regarding high dose aspirin versus moderate doses (30-50 mg/Kg/day).

Meta-analysis comparing moderate anti-inflammatory doses of aspirin (30-50 mg/kg/day) with IVIG versus high dose aspirin (80-120 mg/Kg/day) with IVIG found no significant difference in the incidence of CAA between the groups (Terai and Shulman, 1997).
Currently, it is our practice to administer aspirin at a dose of 30 mg/Kg/day during the acute phase of the illness, since this may be better tolerated in terms of gastrointestinal and other side effects (Matsubara, Mason and others, 1996). Some have advocated the use of dipyridamole in addition to moderate dose aspirin as a synergistic anti-platelet agent. The evidence for the effectiveness of this in this situation is, however, lacking.

**Intravenous Immunoglobulin (IVIG)**

Early recognition and treatment of KD with aspirin and IVIG has been shown unequivocally by meta-analysis to reduce the occurrence of CAA (Durongpisitkul, Gururaj and others, 1995; Terai and Shulman, 1997). Moreover, the prevalence of coronary artery abnormalities in KD is highly dependent on total IVIG dose, but independent of aspirin dose (Terai and Shulman, 1997). Treated with aspirin alone, 20-40% of children develop CAA (Durongpisitkul, Gururaj and others, 1995; Terai and Shulman, 1997). Combined therapy with aspirin and high dose IVIG given as a single infusion reduces the occurrence of CAA to 9% at 30 days, and 4% at 60 days after the onset of the illness (Durongpisitkul, Gururaj and others, 1995). The prevalence of CAA is inversely related to the total dose of IVIG, 2 g/Kg of IVIG being the optimal dose, usually given as a single infusion (Durongpisitkul, Gururaj and others, 1995; Terai and Shulman, 1997). Meta-analysis of randomised controlled trials comparing divided lower doses of IVIG (400 mg/Kg/day for 4 consecutive days) versus a single infusion of high dose IVIG (2g/Kg over 10 hours) has clearly demonstrated the therapeutic benefits in the prevention of CAA with the latter regimen (Durongpisitkul, Gururaj and others, 1995). One important practical point, however, is that infants who have cardiac compromise
may not be able to tolerate the fluid challenge associated with the high dose single infusion, and consideration of divided doses given over several days may be appropriate for this patient group.

IVIG treatment should be started early in the disease, preferably within the first 10 days of the illness (Durongpisitkul, Gururaj and others, 1995; Terai and Shulman, 1997). Importantly, however, clinicians should not hesitate to give IVIG to patients who present after 10 days if there are signs of persisting inflammation.

Not all patients respond to a single dose of IVIG, and some require a second dose. It has been observed that those children who received IVIG very early in the illness may require a second infusion of IVIG for primary treatment failure or disease recrudescence (Han, Silverman and others, 2000). Thus, the timing of IVIG administration appears to be important, although this latter point should not dissuade clinicians from giving IVIG before day 5 of fever if the diagnosis of KD is suspected.

Lastly, one question that remains unanswered is whether the type of IVIG administered is important, perhaps as a result of the presence of antibodies to epitopes derived from different donor pools. There are currently no data to suggest that particular preparations derived from populations of different ethnicity or geography are superior.

The current UK guideline for the treatment of KD is given in figure 1.7 (Brogan, Bose and others, 2002).
Establish diagnosis- 1. *Complete KD (any age), or 2. **Incomplete KD < 1 year old

- IVIG 2g/kg as a single infusion over 12 hours (consider splitting the dose over 2-4 days in infants with cardiac failure)
- Aspirin 30-50 mg/kg/day in four divided doses
- Perform echocardiography, and ECG
- Aspirin 2-5 mg/kg/day when fever settled (disease defervescence) continuing for a minimum of 6 weeks.

Disease defervescence

- Repeat echocardiography at 2 weeks and 6 weeks

No Disease defervescence within 48 hours, or disease recrudescence within 2 weeks

Seek expert advice to consider:
- Second dose of IVIG at 2g/kg over 12 hours
- Consider pulsed methyl prednisolone at 600 mg/m^2 daily for 3 days, or Prednisolone 2 mg/kg/day OD weaning over 6 weeks

No CAA
- Stop aspirin at 6 weeks
- Lifelong follow up at least every 2 years

CAA < 8mm, no stenoses
- Continue aspirin
- Repeat echocardiography & ECG at 6 monthly intervals
- Discontinue aspirin if aneurysms resolve
- Consider exercise stress test if multiple aneurysms
- Specific advice RE minimizing atheroma risk factors
- Lifelong follow up

CAA > 8mm, and/or stenoses
- Lifelong aspirin 2-5 mg/kg/day
- Consider warfarin
- Consider coronary angiography and exercise stress testing
- Repeat echocardiography & ECG at 6 monthly intervals
- Specific advice RE minimizing atheroma risk factors
- Lifelong follow up

CAA > 8mm, and/or stenoses

Notes:
* Treatment can be commenced before full 5 days of fever if sepsis excluded; treatment should also be given if the presentation is > 10 days from fever onset
** Incomplete cases > 1 year old treated at discretion of clinician- seek expert advice.
* Refer to paediatric cardiologist
* Other specific interventions such as PET scanning, addition of calcium channel blocker therapy, and coronary angioplasty at discretion of paediatric cardiologist.
Cardiac complications of KD

Echocardiographic and cardiac angiographic data indicate that 20-40% of untreated KD patients develop coronary artery abnormalities. Approximately 50% of these lesions regress within 5 years, and in most with mild CAA (3-4 mm) regression occurs within 2 years (Shulman, De Inocencio, and Hirsch, 1995). Giant aneurysms (>8 mm) are unlikely to resolve, and some may develop stenosis with risk of coronary thrombosis, myocardial infarction, and death. In 1993, a report from the British Paediatric Surveillance Unit (BPSU) indicated a mortality rate of 3.7% in the UK for KD (Dhillon, Newton and others, 1993). Current mortality rates reported from Japan are much lower at 0.14% (Tizard, 1999a). The reasons for this difference include improved therapy, and better case recognition. Long-term sequelae may include the early development of coronary atherosclerosis (Burns, Shike and others, 1996).

All patients with KD should undergo echocardiography on diagnosis and six to eight weeks after the onset of the disease (Brogan, Bose and others, 2002). Some also advocate an intermediate echocardiograph at 10-14 days of disease onset to pick up any missed pathology (Brogan, Bose and others, 2002). Additionally, it is good practice to perform echocardiography at least weekly (and occasionally 48 hourly in the acute stages with ongoing active inflammation) for those who develop CAA to monitor aneurysm size progression, or the development of thrombus formation. Long term aspirin at 2-5 mg/Kg/day is recommended for those with persisting aneurysms on echocardiography (Tizard, 1999a). This can be discontinued if the aneurysms resolve. Depending on the
size of the aneurysms, ECG and echocardiography performed 6-12 monthly is recommended.

Patients with aneurysms greater than 8 mm may require stress testing and possibly coronary angiography to identify stenotic lesions. Most experts recommend the addition of warfarin to aspirin therapy for those with giant (greater than 8 mm) coronary aneurysms (Newburger, 2000), although randomised controlled trials supporting this practice are lacking. If warfarin is to be commenced, it is imperative to cover the initial warfarinization period with intravenous heparin to counteract the paradoxical prothrombotic state which can occur soon after starting warfarin in some patients (Weiss, Soff and others, 1987). More recently, it has been suggested that platelet glycoprotein IIb/IIIa receptor blockade therapy may be a useful addition to the therapeutic armamentarium for those with giant aneurysms (Etheridge, Tani and others, 1998).

Limitation of strenuous activity is recommended in all patients with giant coronary aneurysms and/or stenoses. Some patients with coronary artery stenoses may require surgical revascularisation. In those patients who develop myocardial infarction, treatment with streptokinase or tissue plasminogen activator (TPA) is indicated (Shulman, De Inocencio, and Hirsch, 1995).

A rare but serious complication of KD is the development of peripheral ischaemia and gangrene. This particular complication is associated with peripheral arterial aneurysm formation, particularly axillary aneurysms. Treatment with thrombolytic agents,
anticoagulants, and intravenous prostacyclin may be indicated in such patients (Shulman, De Inocencio, and Hirsch, 1995; Brogan, Bose and others, 2002).

**Prognosis of KD**

The overall outlook for children with KD is good, although CAA are present in 4% of treated cases 60 days after the acute illness (Durongpisitkul, Gururaj and others, 1995). A 1-2% acute mortality rate due to myocardial infarction has been reduced further in many countries by alertness of clinicians to the diagnosis and early use of gamma globulin with anti-platelet therapy- the mortality for the disease in Japan is currently 0.14% (Tizard, 1999a). Nonetheless, it has been postulated that adult atheromatous coronary disease in some cases may have its origins in childhood due to covert or overt KD (Brecker, Gray, and Oldershaw, 1988).

**1.1.3.4 Wegener’s granulomatosis (WG)**

WG is a necrotizing granulomatous vasculitis of the upper and lower respiratory tract, associated with glomerulonephritis and variable small vessel vasculitis and first described by Wegener in the 1930s (Stegmayr, Gothefors and others, 2000; Wegener, 1936). Particular features that might lead clinicians to the diagnosis include subglottic stenosis due to granulomatous involvement of the trachea, and other upper airway findings such as sinus opacity, and nasal septum disease. Lower respiratory tract features frequently masquerade as infection. Retro-orbital masses presenting as orbital pseudotumour are also well-recognized (Moorthy, Chesney and others, 1977; Valmaggia and Neuweiler, 2001). The association with ANCA showing a cytoplasmic immunofluorescence
appearance has created another means of diagnosis as well as a method of monitoring disease activity, although this latter point is controversial (Moorthy, Chesney and others, 1977; van der Woude, Rasmussen and others, 1985; Moorthy, Chesney and others, 1977; Rus and Handwerger, 2000).

Treatment is similar to polyarteritis and includes steroid, cyclophosphamide, anti-platelet agents, and prophylactic antibiotics such as cotrimoxazole, with plasma exchange in life-threatening situations (Besbas, Ozen and others, 2000; Brogan and Dillon, 2000b; Dillon, 1998; Rottem, Fauci and others, 1993; Stegmayr, Gotheors and others, 2000). Following induction of remission with oral or pulsed intravenous cyclophosphamide, remission is maintained with long term azathioprine or cyclosporin, with low-dose alternate day prednisolone (Brogan and Dillon, 2000b; Dillon, 1998; Besbas, Ozen and others, 2000), and further courses of cyclophosphamide (if necessary) to treat relapses. The mortality for WG at Great Ormond Street Hospital is currently around 15% (Dillon, 1998), although permanent morbidity is substantial.

1.1.3.5 Churg-Strauss syndrome

Churg-Strauss syndrome or allergic granulomatosis is extremely rare in childhood (Fink, 1977). The clinical picture consists of variable vasculitic features with asthma, eosinophilia, infiltrates on chest X-ray and extra-vascular granulomata on biopsy (Guillemin, Le Thi and others, 1988). Steroids are the mainstay of therapy but cytotoxic agents such as cyclophosphamide may need to be introduced to control disease activity (Guillemin, Le Thi and others, 1988; Roberti, Reisman, and Churg, 1993).
1.1.3.6 Primary angiitis of the central nervous system

A primary angiitis of the central nervous system (CNS), sometimes referred to as primary granulomatous angiitis, is predominantly seen in adults but can occur in childhood. The clinical presentation is usually of unexplained cerebral ischaemic episodes without systemic features. Multifocal segmental arterial lesions of small or medium-sized arteries are usually present at angiography, and histologically there is evidence of a lymphocytic necrotizing or granulomatous vasculitis. Aetiology is unknown and in spite of treatment with steroids and cyclophosphamide, prognosis is poor (Dillon, 1998; Moore, 2000).

1.1.3.7 Henoch Schönlein purpura (HSP)

Introduction

Henoch-Schönlein purpura (anaphylactoid purpura) is a multi-system small vessel systemic vasculitis with a prominent cutaneous component. It is the commonest vasculitis in the paediatric population, occurring with an incidence of around 14 per 100 000-child population (Stewart, Savage and others, 1988), and has the most favourable outcome, with the large majority of children requiring no treatment. Younger children are most frequently affected, the peak incidence occurring at around 4 to 5 years of age and the disease is more prevalent in boys. North American series report a higher incidence of Henoch-Schönlein purpura in White compared with Black children (Allen, Diamond, and Howell, 1960). The disease appears to follow a seasonal pattern, with a higher incidence during winter and the early spring.
Presentation often follows an upper respiratory tract infection. A number of early reports implicated \( \beta \)-haemolytic Streptococcus as a significant specific cause, though subsequent publications have failed to confirm this. Allergic reactions to both foods and drugs have also previously been implicated, although again, definitive evidence to support these theories is lacking.

**Clinical features of HSP**

Henoch-Schönlein purpura is a multi-system disease most frequently affecting the skin, joints, gastrointestinal tract and the kidney (Tizard, 1999b). Other organs less frequently involved include the central nervous system, gonads and the lungs. Many cases follow an upper respiratory tract infection and the onset of the disorder may be accompanied by systemic symptoms including malaise and mild pyrexia. Multiple organ involvement may be present from the outset of the disease, or alternatively an evolving pattern may develop, with different organs becoming involved at different time points over the course of several days to several weeks (Tizard, 1999b).

Around one third of children have symptoms for less that 14 days, one third 2-4 weeks and one third greater than 4 weeks (Allen, Diamond, and Howell, 1960). Recurrence of symptoms occurs in around one third of cases, generally within four months of resolution of the original symptoms. Recurrences, which tend to be less severe than the initial presenting episode, are more frequent in those with renal involvement.
Skin changes in Henoch-Schönlein purpura are a uniform finding. The skin lesion is typically that of a purpuric rash which is generally symmetrical, affecting the lower limbs and buttocks in the majority of cases, the upper extremities being involved less frequently. The abdomen, chest and face are generally unaffected. The rash has a predilection for the extensor surfaces. The earliest skin changes may be those of urticaria with associated oedema, which evolve into non-blanching purpuric lesions, with lesions at different stages of evolution often being present at the same time. New crops of purpura may develop for several months after the disease onset, though generally fade with time. Lesions can be induced by mild trauma.

Around two thirds of children have joint manifestations at presentation. The knees and ankles are most frequently involved. Symptoms, which take the form of pain, swelling and decreased range of movement, tend to be fleeting and resolve without the development of permanent damage.

Around three-quarters of children develop abdominal symptoms. The extent of gastrointestinal involvement is highly variable, ranging from mild colicky abdominal pain to severe pain with associated ileus and vomiting and gastrointestinal bleeding manifest by haematemesis and melaena. Other complications include intestinal perforation and intussusception. The latter may be difficult to distinguish from abdominal colic, though the incidence of intussusception is significant enough to warrant exclusion by ultrasound where suspected.
Central nervous system involvement may present as headache, behavioural change, seizure, hemiparesis or coma. Testicular swelling is well-recognised in males. Lung involvement presents as pulmonary haemorrhage (Tizard, 1999b).

The reported incidence of renal involvement in Henoch-Schönlein purpura (Henoch-Schönlein nephritis) varies according to the intensity with which evidence of renal involvement is sought. Overall, studies report an incidence of between 20 and 100%, though in studies where careful routine in-patient urinalysis was performed, the reported incidence of renal involvement was 20-61% (Kobayashi, Wada and others, 1975; Koskimies, Rapola and others, 1974; Stewart, Savage and others, 1988). Renal involvement is normally manifest between a few days and a few weeks after clinical presentation, but can occur up to 2 or months or (rarely) more from presentation. There appears to be an increased risk of renal disease in those with bloody stools.

Renal involvement may present with isolated microscopic haematuria, proteinuria with microscopic or macroscopic haematuria, acute nephritic syndrome (haematuria with at least two of hypertension, raised plasma creatinine and oliguria), nephrotic syndrome (usually with microscopic haematuria) or a mixed nephritic-nephrotic picture.

Investigation and Diagnosis of HSP

The diagnosis and classification of Henoch-Schönlein purpura is usually straightforward and made clinically (see Appendix 1)- no single laboratory test has been shown to be helpful. Immunological investigations including complement levels and anti-nuclear
antibodies are normal, though IgA is elevated in around one half of children and a small number exhibit ANCA positivity (Tizard, 1999b). Coagulation studies are normal and platelet numbers are normal or occasionally increased. Where significant nephritis is present at presentation, renal function and electrolytes may be correspondingly abnormal.

The differential diagnosis includes sepsis and other systemic vasculitides (SLE, PAN, WG, and hypersensitivity vasculitis), all of which can present with similar clinical features. Familial mediteranean fever can also mimick HSP.

Indications for renal biopsy in HSP are: presentation with nephritic and/or nephrotic syndrome; impaired renal function (glomerular filtration rate (GFR) <80ml/min/1.73m2); nephrotic proportion proteinuria (protein: creatinine ratio >250mg/mmol); significant hypertension; plasma albumin <25 g/1; and persistently abnormal urinalysis after 1 year.

Pathological findings in HSP

The skin lesion of Henoch-Schönlein purpura is that of a leucocytoclastic vasculitis with perivascular accumulation of neutrophils and mononuclear cells (Bagga and Dillon, 2001). Immunofluorescence studies reveal vascular deposition of IgA and C3 in affected skin, though similar changes may be observed in skin unaffected by the rash (Bagga and Dillon, 2001).

The renal lesion of Henoch-Schönlein nephritis is characteristically a focal and segmental proliferative glomerulonephritis. The International Study of Kidney Diseases in Children
Classification of Henoch Schonlein nephritis was originally devised by Meadow (Meadow, Glasgow and others, 1972) and adopted in a modified form by the ISKDC (Counahan, Winterborn and others, 1977).

A broad correlation exists between the clinical presentation and the histological changes on renal biopsy. Those with haematuria alone without significant proteinuria have generally less severe histological changes which are highly likely to undergo spontaneous resolution, whilst those with heavy proteinuria, a persisting nephritic syndrome or nephrotic syndrome are likely to have more severe changes which are less likely to resolve.

The renal lesion bears many similarities to that observed in IgA nephropathy, and the two diseases are considered by many to share a common pathogenesis and perhaps represent different spectra of one disease. Patients with IgA nephropathy do not, however, have clinical evidence of extra-renal disease.

Treatment of HSP
The large majority of cases of Henoch-Schönlein purpura are mild and are associated with a good prognosis, and immunosuppressive treatment is usually not justified—children should receive symptomatic treatment only. The skin lesion usually requires no treatment (except where there is severe haemorrhagic oedem affecting the face or scrotum, where systemic corticosteroid therapy may be indicated) and the arthropathy
should be treated with rest and simple analgesia. Whilst never subjected to a controlled clinical trial, there is some evidence to suggest that the more severe gastrointestinal symptoms, particularly abdominal pain and gastrointestinal bleeding respond well to corticosteroid therapy (Rosenblum and Winter, 1987), which has also been used for the treatment of testicular involvement and pulmonary haemorrhage.

Few prospective randomised controlled studies have been completed relating to the treatment of renal disease associated with Henoch-Schönlein purpura. Mollica et al have reported the results of a prospective study where 168 children with no evidence of renal involvement at disease presentation were alternatively assigned to two weeks of daily prednisolone (1mg/kg) or no therapy (Mollica, Li and others, 1992). No children treated with steroids developed renal involvement, compared with 10 of the 84 control patients. To investigate this further, a randomised prospective study has recently commenced in the UK to determine whether the administration of corticosteroids in all patients at disease presentation results in an overall reduction in the subsequent incidence of severe renal involvement. Studies investigating anti-platelet therapy have failed to show any benefit in terms of modifying the course of the disease or preventing the development of nephropathy (Peratoner, Longo and others, 1990).

There is no evidence based on randomised controlled trials to support the use of any of the more potent immunosuppressive therapies in the treatment of more severe grades of Henoch-Schönlein nephritis. A number of uncontrolled studies have reported improvement following therapy with azathioprine, cyclophosphamide, and chlorambucil,
anticoagulants and anti-platelet drugs, often in association with oral corticosteroid therapy. Others, however, have failed to show any benefit. The presence of a large number of crescents may warrant more aggressive additional therapy with intravenous methylprednisolone and plasma exchange, and there are a number of uncontrolled studies reporting good outcomes with these therapies (Gianviti, Trompeter and others, 1996; Niaudet and Habib, 1998; Oner, Tinaztepe, and Erdogan, 1995). Where crescentic changes are present, there are reports of good outcomes following 5-10 days of plasma exchange in addition to steroid and cyclophosphamide, as in other forms of crescentic nephritis (figure 1.4). It is important to stress that there are no controlled trial data to support these therapeutic interventions.

Long-term outcome of HSP

The overwhelming majority of the long-term morbidity associated with Henoch-Schönlein purpura relates to the associated nephritis. All published data indicate that the great majority of children with the disease make a full and uneventful recovery with no evidence of ongoing significant renal disease. However, Henoch-Schönlein nephritis is reported to be the cause of end stage renal failure in 1.6-3% of children in the UK (Lewis MA, 1999) and Europe (Niaudet and Habib, 1998).

There is significant reported variability in the relative incidence of the various clinical presentations of Henoch-Schönlein nephritis and the long-term outlook associated with these. Series from tertiary referral institutions report a significantly higher incidence of long-term renal morbidity than unselected series.
The two largest unselected series of children with Henoch-Schönlein purpura are from Finland and Northern Ireland (Koskimies, Mir and others, 1981; Stewart, Savage and others, 1988). Koskimies et al found 39 of 141 (28%) children to have an abnormal urinary sediment for more than one month after presentation. 29 of these children were followed up, and at an average of 7.2 years post diagnosis, only 1 child had developed end stage renal failure and died, and two had developed chronic glomerular disease, the overall incidence of chronic renal disease being 2.1%. Stewart et al reported 270 patients presenting over a 13-year period. 55 (20%) were found to have initial evidence of renal involvement. One child died during the acute phase of the illness. At an average of 8.3 years follow-up, only 3 (1.1%) had evidence of persistent urinary abnormality with normal renal function. In both of these studies, the large majority of children had relatively minor changes in urinary sediment.

In contrast, series of highly selected patients from tertiary renal centres report significantly higher rates of long-term renal impairment. A 23-year follow-up study from Guys Hospital, London UK and Birmingham Children’s Hospitals, UK found the incidence of long-term renal failure to be 19.2% in a cohort of 78 patients who underwent renal biopsy early in the course of their illness (Goldstein, White and others, 1992). It must be stressed that the fact that these children were referred into a tertiary centre and underwent renal biopsy is indicative of the relative severity of their renal disease at presentation. A broad correlation was detected between clinical presentation and outcome: 44% of children who presented with a nephritic, nephrotic or mixed nephritic/nephrotic developed hypertension or renal failure on follow-up, whereas of
those with microscopic haematuria with or without proteinuria, 82% were entirely normal at follow-up, with 7.7% developing hypertension or renal failure. A small number of patients showed clinical improvement at 5-year follow-up, only to deteriorate in the longer term, and 16 of 44 successful subsequent pregnancies were complicated by hypertension or proteinuria, including 12 where the mother had made an apparent complete clinical recovery.

The severity of the histology on the initial biopsy correlated well with long-term outcome: 42% of those with WHO grade IV or V changes developed renal failure or died, compared with 7.4% of those with grade I or II changes.

From these studies and others, a number of conclusions can be drawn:

i] In unselected populations, the overall risk of significant long-term renal impairment is around 2%

ii] Children with isolated haematuria with no proteinuria have a negligible incidence of long-term renal morbidity

iii] Patients who present with isolated microscopic and or macroscopic haematuria may have microscopic haematuria that persists for many months and years. Recurrence of episodes of macroscopic haematuria may occur following upper respiratory tract infections. The prognosis generally remains good here unless there is evidence of significant proteinuria

iii] Where isolated haematuria is associated with proteinuria, the risk of long term renal dysfunction is around 5%
iv] Children presenting with the acute nephritic syndrome have a less favourable outcome, with a risk of long-term renal failure of 10-20%. Those with a mixed nephritic-nephrotic presentation have the worst long-term outlook, with up to 33% developing long-term renal failure.

v] Those with more aggressive renal biopsy changes are more likely to have a poorer long-term outlook.

vi] Children with significant renal impairment at presentation should remain under long-term follow-up.

vii] Some instances of hypertension have been reported many years after normalisation of renal function and urinalysis: hence the need for long-term follow-up. Most would advocate monitoring of blood pressure for 2 years after normalisation of urinary sediment.

1.1.3.8 Hypersensitivity vasculitis

Hypersensitivity vasculitis is frequently a drug-induced condition but can be associated with other antigens including infectious agents (Bagga and Dillon, 2001). The predominant clinical manifestations involve the skin with palpable nodules or purpura although other organs can be affected (Bagga and Dillon, 2001). Removal of the precipitating agent is usually followed by resolution, but sometimes non-steroidal anti-inflammatory drugs and steroids are indicated. The ACR classification criteria for this entity are given in Appendix 1 (Calabrese, Michel and others, 1990).
1.1.3.9 Hypocomplementaemic urticarial vasculitis

The clinical manifestations of this vasculitic disorder are recurrent urticarial lesions, angioedema, arthritis and arthralgia, vomiting and abdominal pain. Severe systemic disease with rapidly progressive glomerulonephritis and lung haemorrhage can occur (Martini, Ravelli and others, 1994). There is classic complement pathway activation. Therapy includes prednisolone and hydroxychloroquine but other immunosuppressive agents may be required (Worm, Muche and others, 1998; Worm, Sterry, and Kolde, 2000).

1.1.3.10 Vasculitis associated with connective tissue disorders

Vasculitis is seen at times in systemic lupus erythematosus (SLE), juvenile idiopathic arthritis (JIA), mixed connective tissue disease, juvenile dermatomyositis (JDM), and scleroderma.

Juvenile dermatomyositis

Vasculitis is a major component of JDM, and can pose a threat to life (Cassidy and Petty, 2001). The vasculitis affects striated muscle, skin, subcutaneous tissue and gastrointestinal tract (Cassidy and Petty, 2001). Gastrointestinal perforation, bleeding and acute pancreatitis can all result from mesenteric vasculitis (See, Martin and others, 1997). Treatment of severe disease typically includes steroid, oral or intravenous cyclophosphamide plus, in life threatening situations, plasma exchange (Dillon, 1998;
Brogan and Dillon, 2000b). IVIG and methotrexate also have a role in recalcitrant disease. Recently cyclosporin has been shown to be effective in this disease (Heckmatt, Hasson and others, 1989), and indeed many would now initially treat JDM with a combination of prednisolone and cyclosporin, reserving more aggressive treatment modalities for those in whom severe features emerge (Brogan and Dillon, 2000b).

For a recent comprehensive review of vasculitis in the connective tissue diseases, the reader is referred to the work of Flores-Suarez (Flores-Suarez and Alarcon-Segovia, 2000).

1.1.3.11 Takayasu disease

Takayasu disease (TD) is a giant cell arteritis causing stenosis and aneurysmal dilatation of large arteries, such as the aorta and its major branches (Lindsley CB, 2001). Worldwide, it is the third commonest vasculitis of childhood, and may be related to infection with tuberculosis (Lindsley CB, 2001; Wiggelinkhuizen and Cremin, 1978). Clinical features include fever, anorexia, weight loss, arthritis, and later the development of hypertension, heart failure and pulse defects (so-called “pulseless disease”) (Hall, Barr and others, 1985; Lindsley CB, 2001). Diagnosis involves doppler ultrasonography, magnetic resonance imaging, and conventional angiography (Lindsley CB, 2001; Southwood TR, 1988). Therapeutic regimens in the acute phase of the disease include steroids, cyclophosphamide and methotrexate (Shelhamer, Volkman and others, 1985). More recently, there have been case reports of the successful treatment of TD with mycophenolate mofetil (Daina, Schieppati, and Remuzzi, 1999).
1.1.3.12 Miscellaneous vasculitides of the young

These include Behçet’s syndrome, familial Mediterranean fever and Cogan’s syndrome. Behçet’s syndrome consists of the triad of aphthous stomatitis, genital ulceration and iritis (Petty and Cassidy, 2001d; Kari, Shah and others, 2001). Although relatively rare in childhood, it is well recognised that a vasculitic component is an important feature (Petty and Cassidy, 2001d).

Familial Mediterranean fever often manifests itself for the first time in childhood (Sohar, Gafni and others, 1967; Pras, Langevitz and others, 1996). Although not normally considered to be associated with vasculitis there are now a number of reports describing both polyarteritis nodosa and Henoch-Schönlein purpura in affected patients (Glikson, Galun and others, 1989). It is therefore important to consider the possibility of an associated vasculitis in patients with familial Mediterranean fever that may need treating in its own right.

Non-syphilitic interstitial keratitis with vestibuloauditory dysfunction was first described by Cogan (Cogan, 1945). Although rare and usually affecting young adults it has been reported in children (Cogan, 1945; Kundell and Ochs, 1980) and in addition to the main features there can be evidence of a widespread vasculitis that may require treatment with corticosteroids and/or cyclophosphamide.
1.1.4. Conclusion

There is a wide spectrum of vasculitic disease affecting children. The distinction from other conditions, especially those associated with infection, is often difficult, but it is usually possible to categorise the disorders into clinico-pathological entities even though there is a substantial degree of overlap. The current range of investigative tools usually allows the correct diagnosis to be established and modern therapeutic approaches have made a major impact on the treatment of these serious conditions. In spite of this, however, morbidity and mortality is not inconsequential.
1.2 The Immuno-pathogenesis of vasculitis

1.2.1 Introduction

The aetiology of vasculitis is unknown in most patients, although the immuno-pathogenetic mechanisms which culminate in vascular injury are beginning to be unravelled. Whilst an understanding of the immuno-pathogenesis is critical for the design of future “magic-bullet” therapies based on strategies targeted to specific immunological blockade (such as the success which has been achieved with monoclonal antibody therapy against tumour necrosis factor-alpha in rheumatoid arthritis), it must be borne in mind that many of the immunological processes that will be considered in this section represent downstream events that arise in individuals with as yet undefined predisposing factors, and triggered by some precipitating event. For example, whilst much is now known regarding the immuno-pathogenesis of the vascular injury associated with ANCA, comparatively little is known about the predisposing genetic or environmental factors which result in a loss of immunological tolerance to neutrophil azurophilic enzymes in the first instance.

This section will describe the current state of knowledge regarding the immuno-pathogenesis of vasculitis, and will consider aetiology where it is clearly defined in the context of particular vasculitic syndromes. The mechanisms involved in the pathogenetic process can be considered under the headings of infections, malignancy, autoantibodies, immune complexes, cryoglobulins, endothelial amplification of inflammation (cell adhesion molecules [CAMs] and cytokines), and T cells. It is proposed that a description
of these mechanisms will set the scene and place in context the detailed discussion of superantigens in vasculitis, which will be considered in section 1.3.

1.2.2 Infections and vasculitis

Many pathogens are known to cause vasculitis. For several of these pathogens, vasculitis may be the major manifestation of disease; others, however, typically present with vasculitis as an occasional manifestation. The viruses, bacteria, and fungi that cause vasculitis share a common target—blood vessels. Many infectious pathogens have tissue tropism that includes endothelium; other agents may bind to the vessel wall because the vascular endothelium expresses specific receptors for the pathogen (Naides, 2002). Even when the agent does not enter the endothelial cell, the immune response to the agent may be focused at the vessel wall because the pathogen is adherent to the endothelial cell surface, resulting in bystander injury to the vessel. The advent of sensitive molecular techniques for the detection of infectious agents has encouraged searches for various known pathogens in idiopathic vasculitis syndromes (Shingadia, Bose, and Booy, 2002).

1.2.2.1 Viruses causing vasculitis

Hepatitis C virus

Hepatitis C virus (HCV), discovered in 1989, has world-wide prevalence and is the cause of most cases of the vasculitic syndrome previously known as “essential mixed cryoglobulinaemic vasculitis”, and may also cause a vasculitic syndrome resembling PAN (Cacoub, Lunel-Fabiani, and Du, 1992; Cacoub, Costedoat-Chalumeau and others,
The 10 to 20-year latent period before hepatic or rheumatic manifestations of disease explains the increasing number cases of hepatitis C virus-mediated vasculitis currently being seen following the epidemic of new cases in the USA in the 1980s (Cacoub, Costedoat-Chalumeau and others, 2002).

Prior to the discovery of HCV in the late 1980s, the triad of arthritis, palpable purpura, and type II cryoglobulinemia was known as “essential mixed cryoglobulinaemia” and considered an idiopathic vasculitis (Cacoub, Lunel-Fabiani, and Du, 1992; Cacoub, Costedoat-Chalumeau and others, 2002). The advent of diagnostic testing for HCV demonstrated that almost all of these cases were associated with HCV infection. Clinical evidence of liver disease was found in 60 to 80% of patients with essential mixed cryoglobulinaemia, and liver biopsies showed chronic active hepatitis or cirrhosis (Cacoub, Maisonneuve and others, 2001; Levo, Gorevic and others, 1977a; Levo, Gorevic and others, 1977b).

In mixed cryoglobulinemia patients, the hepatotropic antigen triggering the production of antibodies that later can form immune complexes was sought for many years. Initially hepatitis B virus (HBV) was implicated as the causative agent of essential mixed cryoglobulinaemia (Levo, Gorevic and others, 1977b). Numerous studies using more recent tests for the detection of HBV surface markers and HBV DNA have failed to validate these observations: HBV surface antigen (or antibodies to surface antigen) was found in only 17% of 33 patients with essential mixed cryoglobulinemia, and no patients
had detectable HBV DNA in their sera or cryoprecipitates (Cacoub, Fabiani and others, 1994).

Many studies of patients with mixed cryoglobulinemia have investigated the prevalence of anti-HCV antibody and HCV RNA. Studies on large populations found anti-HCV antibodies in 43-85% of highly selected patients with mixed cryoglobulinemia (Ferri, Zignego and others, 2002). All patients were of Italian origin, and many also had evidence of HBV infection. In a prospective study (Cacoub, Fabiani and others, 1994), anti-HCV antibody was demonstrated in 52% of patients with essential mixed cryoglobulinemia. The anti-HCV antibody-positive patients had more cutaneous involvement (Raynaud phenomenon, purpura, livedo reticularis, distal ulcers, or gangrene), higher alanine aminotransferase, higher cryoglobulin levels, and lower serum complement (CH50 and C4) levels.

Clinically, anti-HCV antibody-positive patients with mixed cryoglobulinemia have cryoglobulin-related manifestations (see section 1.2.5) more often than anti-HCV antibody-negative patients. There is a 2 to 3-fold increased frequency of mixed cryoglobulinaemia in HCV chronic hepatitis compared with HBV or non-A, non-B, non-C chronic liver disease (Ferri, Zignego and others, 2002).

A possible explanation for the HCV-mixed cryoglobulinaemia association could be an antigenic cross-reaction between HCV and the liver, particularly damaged liver (Cacoub, Costedoat-Chalumeau and others, 2002; Ferri, Zignego and others, 2002). The production
of antibody cross-reacting with liver and viral antigens is suggested by the finding that
HCV infection induces an antibody response to an endogenous antigen (Mishiro, Hoshi
and others, 1990). Hepatitis C virus RNA sequences have been detected in sera and the
cryoprecipitates from patients with mixed cryoglobulinemia and HCV antibody (Munoz-
Fernandez, Barbado and others, 1994). Among 27 HCV-positive patients with mixed
cryoglobulinemia, 66% were HCV RNA-positive in the supernatant and 85% in the
cryoprecipitate. In fact, the presence of entire viral particles has been proposed to be
present in the cryoprecipitate. Furthermore HCV RNA sequences have been detected in
vasculitic lesions of patients with HCV-mixed cryoglobulinemia (Agnello and Abel,
1997; Casato, Agnello and others, 1997).

Thus it is clear that HCV infection can produce vasculitis, and this discovery has led to
effective anti-viral therapeutic regimens with interferon alpha and ribavirin (Zuckerman,
Keren and others, 2000a; Zuckerman, Keren and others, 2000b).

**Hepatitis B virus**

Hepatitis B virus (HBV) infection is the classic example of virally mediated immune
complex disease (Dienstag, 1981a). Histologically there is lymphocytic venulitis and/or
neutrophilic vasculitis of small vessels with leukocytoclasia and fibrinoid changes
present. Clinical presentation is with an “urticaria-arthritis syndrome” (Berretty,
Neumann, and von Joost, 1981). Immune complexes of HBV surface antigen (HBsAg)
and antibodies to HBV surface antigen (HBsAb) circulate in the blood and are found
deposited in vessels in association with complement (Neumann, Berretty and others,
The long latency period of HBV allows time for an immune response to occur. Viral replication increases HBsAg load, and is temporally associated with jaundice (Hoofnagle and Di Bisceglie, 1991). The immune complexes eventually no longer form in antigen excess and the serum sickness-like illness resolves.

HBV has also been associated with a vasculitic syndrome resembling PAN (Anon, 1983; Avsar, Savas and others, 1998; Balkaran, Teelucksingh, and Singh, 2000; Chauveau and Christophe, 1995; Darras-Joly, Lortholary and others, 1995). Onset of this vasculitis is early in the course of chronic HBV hepatitis. Again, immune complexes containing HBsAg, HBsAb, and complement are found in the vessel wall (Dienstag, 1981b). The determinants of small vessel versus larger vessel vasculitis in response to HBV infection are unknown.

**Human Immunodeficiency virus**

Infection with Human Immunodeficiency virus (HIV) may present with a variety of vasculitic syndromes. However, it is difficult to specifically attribute the various vasculitides seen in HIV infection because of frequent co-infections with other pathogens that may cause vasculitis in their own right. That said, HIV infection has been associated with vasculitis affecting the central nervous system, skin, and retina (Berkefeld, Enzensberger, and Lanfermann, 2000; Font, Miro and others, 1996; Gisselbrecht, Cohen and others, 1998; Picard, Brunereau and others, 1997).
Herpes viruses

Herpes viruses are especially attractive as possible causes of vasculitis (Shingadia, Bose, and Booy, 2002). They are ubiquitous in the population, and many have been shown to infect cells of a vascular lineage (Dal Canto and Virgin, 2000). All classes of herpes virus (α, β, and γ herpes viruses) can cause panarteritis in animal models (Virgin, 2002). For example, murine cytomegalovirus is widely used as a model for studying many aspects of a human cytomegalovirus infection, particularly small vessel vasculitis (Dangler, Baker and others, 1995). Another interesting model for analysis of mechanisms of vascular injury and immune response in the large vessels is the rodent γ herpes virus γHV68. Analysis of the γHV68 genome demonstrates that this virus is closely related to primate herpes viruses, including EBV and human herpes virus (Shingadia, Bose, and Booy, 2002). γHV68 infection causes a severe chronic vasculitis of the great elastic arteries in mice (Weck, Dal Canto and others, 1997) and results in an expansion of splenic B cells. This expansion is associated with hypergammaglobulinaemia and much of the antibody is not apparently directed to γHV68 antigens. Another unusual aspect of the response of CD8+ T cells to γHV68 infection is the dramatic expansion of Vβ4 CD8+ T cells (Tripp, Hamilton-Easton and others, 1997; Weck, Dal Canto and others, 1997). The mechanism underlying this selective Vβ expansion and the physiologic importance of this phenomenon are currently unknown (Tripp, Hamilton-Easton and others, 1997; Weck, Dal Canto and others, 1997). Lack of either interferon-γ (INF-γ) or the INF-γ receptor and young age predisposes γHV68-infected mice to severe vasculitis of the great arteries. INF-γ receptor deficient mice died 1.5 to 14 weeks after infection with γHV68 and were found to have severe large-vessel arteritis. It is important to be cautious about making
extrapolations from animal models to human disease, however, as there are species
differences in the pathogenesis of many diseases.

Newer molecular methods such as degenerate PCR are increasingly used to identify new
infectious agents that cause human disease. However, molecular methods have been used
only to a limited extent in human vasculitis syndromes. Kikuta et al identified EBV DNA
in peripheral blood mononuclear cells and cardiac tissue of KD patients by selective
DNA amplification techniques (Kikuta, Sakiyama and others, 1993). Rowley et al failed
to detect highly conserved viral and bacterial nucleic acid sequences in patients with KD
(Rowley, Wolinsky and others, 1994).

Herpes viruses including cytomegalovirus, varicella-zoster, herpes simplex viruses 1 and
2, and herpes hominis may be associated with retinal vasculitis in immunocompromised
patients (Kuo, Yip, and Chen, 2001; Savir, Grosswasser, and Mendelson, 1980). Varicella-zoster may also cause a diffuse central nervous system small arterial
granulomatous vasculitis (Naides, 2002). Herpes simplex viruses 1 and 2 have been
associated with cutaneous vasculitis and necrotizing arteritis of small and medium vessels
(Schmitt, Dietzmann and others, 1992). Epstein-Barr virus has been proposed as a cause
of both small- and large-vessel disease in a number of cases and short series (Naides,
2002). However, the ability to convincingly infer causality in many studies is made
problematic by the latency of herpes virus infection.
Other viral agents

Parvovirus B19 has been implicated in the aetio-pathogensis of PAN (Corman and Dolson, 1992; Finkel, Torok and others, 1994; Martinelli, Azzi and others, 1997). However, the failure to eliminate B19 from pooled blood products is an important confounding factor to consider when inferring causality (Prowse, Ludlam, and Yap, 1997). Vasculitis has also been reported following infection with rubella virus, adenovirus, echovirus, coxsackie virus, parainfluenza virus, and hepatitis A virus (Naides, 2002).

1.2.2.2 Bacteria causing vasculitis

Mechanisms of bacterial vasculitic injury

Bacteria may cause vasculitis by direct microbial invasion of endothelial cells, by immune-complex-mediated mechanisms, or by stimulation of auto-reactive T or B-lymphocytes by bacterial superantigens. This latter hypothesis will be discussed in detail in section 1.3.

Bacterial seeding of vessels may lead to necrosis through direct bacterial infection. Vessels may be seeded intraluminally at sites of endothelial injury or flow turbulence, or seeding of vasa vasorum may cause destruction of vessels from the outside in ("mycotic aneurysm"). Spread from an infected site to a vessel may also occur. Immune responses to bacteria or to bacterial components may also lead to or contribute to vascular injury, usually by deposition of immune-complexes comprised of bacterial antigen, antibody to bacterial antigen, and complement components.
In subacute bacterial endocarditis (SABE), dissemination of septic emboli and immune complex injury occurs resulting in the classic vasculitic stigmata of SABE: myalgia, arthralgia, Osler’s nodes, Janeway lesions, and septic infarcts. Bacteraemia may also present as leukocytoclastic vasculitis.

**Specific bacteria causing vasculitis**

*Neisseria* species may be associated with small-vessel vasculitis. *Neisseria gonorrhoea* is associated with necrotic cutaneous papules. In *Neisseria meningitides* infection, vasculitis manifests in the skin and gastrointestinal tract, with the endothelial necrosis and widespread thrombosis (Seaton, Nathwani and others, 2000).

*Rickettsiae* (causing Rocky Mountain and Mediterranean spotted fever) are obligate intracellular bacteria with tropism for vascular endothelium (George, Brouqui and others, 1993). Infection results in widespread microvascular leak, focal thrombosis, and ultimately multisystem failure.

Another example of microbial invasion of endothelial cells is *Staphylococcus aureus* (*S. aureus*). It has been demonstrated that *S. aureus* binds more readily to endothelial cells than most other bacteria (Cohen Tervaert, 2002). Following binding, bacteria are internalised and can persist in phagosome-like vacuoles. The interaction between *S. aureus* and endothelial cells may result in activation of the endothelial cells resulting in enhanced expression of cell adhesion molecules and in the production of cytokines and
chemokines (Cohen Tervaert, 2002). Furthermore, endothelial cells may be damaged following internalisation of alpha-toxin producing strains of S. aureus (Cohen Tervaert, 2002).

Mycobacterial or fungal lung infections may mimic Wegener’s granulomatosis or Churg-Strauss syndrome by causing a granulomatous reaction in vessel walls (Naides, 2002). Spread of Mycobacterium tuberculosis to the aorta may causes tuberculous aortitis, coronary arteritis and mycotic aortic aneurysm. Pseudomonas aeruginosa and other gram-negative organisms can present as cutaneous vasculitis; vessel thrombosis results from direct bacterial invasion of the vessels (Naides, 2002).

Syphilis, the “great imitator” (Witkowski and Parish, 2002), can present as large or medium-sized vessel disease (aortitis or coronary arteritis), or as the small-vessel vasculitic rash of secondary syphilis. Syphilitic aortic aneurysms were insidious in their clinical presentation, and Treponema pallidum spirochaetes are rarely detected in the fibrosed and scarred aortas (Aizawa, Hasegawa and others, 1998).

Borrelia burgdorferi (the cause of Lyme disease) produces vasculitic changes in the central nervous system, retina, and temporal arteries (Naides, 2002).
1.2.2.3 Parasites causing vasculitis

Parasites rarely cause vasculitis. Toxocara canis can present as palpable purpura with features suggesting Henoch-Schönlein purpura (Hamidou, Gueglio and others, 1999; Magnaval and Morassin, 2000), or a more widespread vasculitis mimicking giant cell vasculitis (GCA) (Hamidou, Fradet and others, 2002). Cysticercus has reportedly caused vasculitis and arachnoiditis as it infects the central nervous system (Wolf, Allert and others, 1966). Loa loa (a filarial parasite) can present with cutaneous leukocytoclastic vasculitis and angioedema (Rakita, White, Jr., and Kielhofner, 1993). Angiostrongylus nematodes may cause pulmonary vasculitis mimicking WG (Pirisi, Gutierrez and others, 1995).

1.2.2.4 Conclusion

Many infectious agents can cause vasculitis, but few have been convincingly demonstrated as the cause of most idiopathic vasculitic syndromes. The advent of molecular techniques for the detection of infectious pathogens has renewed efforts to look for infectious causes for otherwise presumed autoimmune diseases, including the systemic vasculitides. The predisposing factors which cause one individual to develop vasculitis in response to infection, whereas another individual may escape this manifestation remain elusive.
1.2.3 Malignancy and vasculitis

Malignancy is a well-recognized association with systemic vasculitis, and is mainly associated with cutaneous vasculitis, although systemic features are also described. Vasculitis in this context can arise as a result of an infectious complication operating on the background of impaired immune function secondary to the presence of malignancy, as a paraneoplastic phenomenon (where the inflammatory cells infiltrating the blood vessel wall are non-malignant), or as a result of direct involvement of the blood vessels with the malignant process—usually associated with the so-called “angiocentric lymphomas”. These mechanisms will be considered in turn.

1.2.3.1 Infectious causes of vasculitis in malignancy

Varicella zoster virus (VZV) has been reported to cause vasculitis in patients with lymphoma (McKelvie, Collins and others, 2002). The presentation was that of a central nervous system meningoencephalitis with vasculitis presenting in a patient with multiple malignancies (non-Hodgkin’s lymphoma and cancers of breast and colon). Autopsy showed necrotizing vasculitis of the leptomeningeal vessels, and polymerase chain reaction detected VZV DNA in the cerebro-spinal fluid. Similarly, VZV vasculitis affecting the skin was described in a patient with B cell lymphoma (Uhoda, Pierard-Franchimont, and Pierard, 2000). Histology revealed a lymphocytic vasculitis, and immunohistochemistry demonstrated VZV in endothelial cells.
**1.2.3.4 Paraneoplastic vasculitis**

This entity is relatively common in adults, and usually takes the form of a cutaneous vasculitis (Pavlidis, Klouvas and others, 1995). It has been estimated that 1-7% of adults with cutaneous vasculitis will have an underlying malignancy (Gran, 1997). Paraneoplastic vasculitis has been described in association with several types of malignancy. Erythema nodosum has been described with Hodgkin’s lymphoma (Blanco, Neau and others, 1999). Henoch Schonlein purpura (HSP) is associated with non-Hodgkin’s lymphoma (Day, Savage and others, 2001), carcinomas (lung, breast, prostate, renal) (Pertuiset, Liote and others, 2000), adenocarcinoma of the gastro-intestinal tract (Pertuiset, Liote and others, 2000), and other haematological malignancies including Hodgkin’s lymphoma, IgA multiple myeloma (Pertuiset, Liote and others, 2000), splenic lymphoma (Farrell, Stern and others, 1999), and hairy cell leukaemia (Farrell, Stern and others, 1999). In addition, in a series of 14 adult patients with HSP, 4 cases were associated with underlying malignancy (Pertuiset, Liote and others, 2000).

Proposed pathogenetic mechanisms for paraneoplastic vasculitis include formation of immune complexes with tumour antigens (Pertuiset, Liote and others, 2000), dysregulated IgA formation leading to immune complex formation (Pertuiset, Liote and others, 2000), or the formation of cryoglobulins (Wooten and Jasin, 1996a; Wooten and Jasin, 1996b).
1.2.3.5 Angiocentric lymphomas

Angiocentric lymphomas are a heterogeneous spectrum of haematolymphoid malignancies that share a particular histological characteristic, namely, an angiocentric or perivascular growth pattern (Natkunam and Warnke, 2001). The consequence of this is that they present with lymphomatous vasculitis, which may mimic lymphocytic vasculitis unless specific tumour markers are examined on the infiltrating tumour cells. Angiocentric lymphoma should therefore be included in the differential diagnosis of systemic vasculitis (al Chalabi and Abbott, 1995). They include a variety of T-, B-, and natural killer-cell derived lymphomas that have many different clinico-pathological features, immunophenotype, and prognosis.

Vasculitic syndromes described in association with this group of malignancies include mononeuritis multiplex secondary to angiotropic large cell lymphoma (ALCL) (Roux, Grossin and others, 1995), CNS vasculitis with ALCL, and cutaneous vasculitis with angioimmunoblastic T cell lymphoma (Sugaya, Nakamura and others, 2001; Thomas, Vuitch, and Lakhanpal, 1994).
1.2.4 Autoantibodies and vasculitis

Autoantibodies associated with vasculitis include antineutrophil cytoplasmic antibodies (ANCA) and anti endothelial cell antibodies (AECA). Many other autoantibodies have been described with propensity for vascular injury but for the purposes of this thesis the above two autoantibodies will be discussed in detail.

1.2.4.1 Antineutrophil cytoplasmic antibodies (ANCA)

Studies relating to ANCA and associated small vessel vasculitides have dominated the vasculitis literature since these autoantibodies were first described by Davies in 1982 (Davies, Moran and others, 1982).

Vasculitic syndromes associated with ANCA

The small vessel vasculitides (SVV) associated with ANCA are Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS) (Jennette and Falk, 1997; Pirisi, Gutierrez and others, 1995; Pirisi, Gutierrez and others, 1995; Savage, Harper and others, 2000). ANCA target autoantigens are contained primarily within neutrophil azurophil granules, although it is becoming increasingly apparent that the tissue distribution of autoantigens targeted by ANCA is wider than first appreciated (Pirisi, Gutierrez and others, 1995; Savage, Harper, and Holland, 2002). In WG, the ANCA recognise proteinase-3 in more than 90% cases, whereas in MPA either proteinase-3 or myeloperoxidase may be recognized. In CSS myeloperoxidase is recognized most often (Pirisi, Gutierrez and others, 1995; Savage, Harper, and Holland, 2002).
The aetiological agents that initiate the autoimmune response resulting in loss of
tolerance to neutrophil enzymes and the development of ANCA are unknown, although
certain drugs (such as propylthiouracil (Casis and Perez, 2000; Chastain, Russo and
others, 1999; Colakovski and Lorber, 2001; Dolman, Gans and others, 1993),
minocycline (Schaffer, Davidson and others, 2001; Schrodt, Kulp-Shorten, and Callen,
1999; Schrodt and Callen, 1999)), as well as infections (superantigen-producing strains of
*S. aureus* (Stegeman, Tervaert and others, 1994; Popa ER, van der Meer B and others,
2002; Popa, Stegeman and others, 1998)), and silica (Hogan, Satterly and others, 2001;
Koeger, Rozenberg, and Bourgeois, 1994) have been implicated.

**Do ANCA cause small vessel vasculitis?**

There is strong circumstantial evidence that the vasculitis in ANCA-associated SVV is
the result of the presence of ANCA, but until very recently direct evidence was lacking.
Recently, however, 4 studies have demonstrated in animal models that ANCA are the
cause of the vascular injury. At the time of writing of this thesis, these have only been
published in abstract and were presented at the 10th International Vasculitis and ANCA
Workshop, Cleveland USA, 2002.

Jennette *et al* were able to induce pauci-immune necrotizing crescentic
glomerulonephritis by the intravenous administration of anti-MPO antibodies to a mouse
model deficient in recombinase activating gene-2 (RAG-2 /-) (Jennette JC, Xiao H and
others, 2002). These mice effectively lack B and T lymphocytes and therefore serve as a
pure model to study the passive transfer of potentially pathogenetic autoantibodies. Passive transfer of anti-MPO antibody resulted in the onset of haematuria and proteinuria. Mice sacrificed at day 6 that had received anti-MPO (n=5) all had focal necrotizing glomerulonephritis (mean 18% glomeruli with necrosis) and crescents (mean 11% crescents), whereas mice that received anti-BSA (bovine serum albumin) antibody (control, n=3) had no symptoms or histological lesions. The authors concluded that in mice with no T or B-lymphocytes, circulating anti-MPO (MPO-ANCA) causes pauci-immune necrotizing and crescentic glomerulonephritis.

Again using the RAG-2 -/- model, the same group were able to induce necrotizing and crescentic glomerulonephritis and SVV by transfer of anti-MPO producing splenocytes (Xiao H, Heeringa P and others, 2002). Mice that received anti-MPO splenocytes developed circulating anti-MPO within 3 days, and the titer rose until sacrifice at 13 days. Mice that received anti-BSA splenocytes developed circulating anti-BSA. Mice that received MPO-ANCA splenocytes developed renal failure, haematuria and proteinuria, whereas mice that received anti-BSA (control splenocytes) did not. All mice that received anti-MPO splenocytes (n=4) developed severe crescentic nephritis; 3 developed pulmonary capillaritis; 1 had necrotizing vasculitis in spleen and lymph nodes; and 1 had necrotizing granulomatous inflammation in spleen. Mice that received anti-BSA (n=14) splenocytes did not develop vasculitis.

Using another mouse model which spontaneously develops crescentic nephritis from early life (the SCG/Kj mouse) Ishida-Okawara et al correlated the development of
vasculitis with neutrophil MPO release, neutrophil superoxide generation, and MPO-ANCA titer in the serum of these mice (Ishida-Okawara, Ito-Ihara and others, 2002). They found positive correlation between neutrophil MPO release, MPO-ANCA, and histological scores of vasculitis severity, and suggested on the basis of these data that neutrophil activation, in association with MPO-ANCA contributed to the vascular injury observed in the kidneys of these mice.

Lastly, Smyth et al used a rat model to study MPO-ANCA associated pauci-immune focal segmental glomerulonephritis (Smyth CL, Smith J and others, 2002). In that study, Wistar Kyoto rats were immunised with purified human MPO (50 micrograms intramuscularly). Over 2 to 4 weeks all rats developed anti-myeloperoxidase antibodies as confirmed by ANCA indirect immunofluorescence (on rat and human neutrophils) and enzyme-linked immunosorbant assay (ELISA). Haematuria was detected in 95% by week 5, accompanied by mild proteinuria. Kidney sections taken from rats killed at 8 weeks showed glomeruli with segmental inflammation and occasional fibrinoid deposits, tubular red cell casts, and tubulo-interstitial inflammation. Lung sections showed evidence of fresh haemorrhage and haemosiderin deposition. Immunofluorescence microscopy revealed no deposits of IgG and only scanty tubular deposits of C3 (i.e. pauci-immune glomerulonephritis).

Such studies therefore support the view that ANCA are themselves pathogenic, although as mentioned in previously, care must be taken when extrapolating animal studies to human disease states.
How do ANCA cause vasculitis?

The most accepted model of pathogenesis proposes that ANCA activate cytokine-primed neutrophils, leading to bystander damage of endothelial cells and escalation of inflammation with recruitment of mononuclear cells (Harper and Savage, 2000). ANCA bind with their target antigen on the surface of neutrophils after the cells have been primed. This priming involves neutrophil stimulation with an agent such as lipopolysaccharide (LPS) or the cytokine tumour necrosis factor alpha (TNF-α) at a concentration that does not of itself cause full functional activation, but rather results in the translocation of antigen from intracellular granules to the cell surface (Csemok, Ernst and others, 1994). It has been demonstrated that patients with active SWV have circulating neutrophils in the primed state, with enhanced expression of surface proteinase-3 (Muller Kobold, Mesander and others, 1998; Muller Kobold, Kallenberg, and Tervaert, 1998) and increased expression of the activation markers CD66b, CD63, and CD64 (Muller Kobold, Mesander and others, 1998; Muller Kobold, Kallenberg, and Tervaert, 1998). Subsequent studies have confirmed these data and show that resting neutrophils do not express proteinase-3 or myeloperoxidase (Yang, Tuttle and others, 2000). Furthermore primed circulating neutrophils not only have enhanced proteinase-3 expression but also have increased basal superoxide production (Harper, Cockwell and others, 2001).

In addition to neutrophil priming by LPS or proinflammatory cytokines, in vitro studies have suggested that low concentrations of PR3–ANCA can themselves prime neutrophils
for activation (Hattar, Sibelius and others, 2001). The priming effect appears to be
dependent on the intact ANCA IgG molecule. TNF-α priming and ANCA activation of
neutrophils lead to a respiratory burst and degranulation, generating potential for
endothelial injury.

The binding of ANCA to antigens expressed on the surface of primed neutrophils is not
enough in itself to cause full neutrophil activation: binding of Fc receptors is also
necessary (Mulder, Heeringa and others, 1994). The intracellular signaling events that
lead to the functional response of typical of activated neutrophils are currently
incompletely defined, but are likely to involve tyrosine kinases and protein kinase C, and
G-proteins (Savage, Harper, and Holland, 2002).

After neutrophil stimulation by ANCA, numerous cytotoxic mediators are released,
including reactive oxygen species, chemokines, cytokines, proteolytic enzymes, and
nitric oxide (NO) (Savage, Harper, and Holland, 2002). The release of NO is independent
of nitric oxide synthase (NOS) (Tse, Williams and others, 2001), but whether this source
of nitric oxide is protective or damaging to endothelium is unclear.

If neutrophils are the cause of endothelial injury, they must be brought into close
proximity with endothelial cells. Studies in a flow model using a platelet monolayer as a
surrogate vessel wall have demonstrated that neutrophil “rolling adhesion” on the platelet
monolayer can be converted to firm stationary adhesion by either PR-3–ANCA or MPO-
ANCA (Savage, Harper, and Holland, 2002). This conversion from rolling adhesion to firm adhesion is blocked by antibodies against neutrophil CD11b or FcyRIIa receptors.

Firm adhesion of activated neutrophils to endothelial cells would be predicted to result in endothelial cell damage, and perhaps recruitment of other inflammatory cells including monocytes and T cells. Interestingly, endothelial cell damage by PR3 is mediated (in part) by endothelial cell apoptosis via a mechanism involving PR3 internalization into the endothelial cell independent of its proteolytic enzyme function (Yang, Kettritz and others, 1996; Yang, Preston and others, 2001).

Following neutrophil activation by ANCA, neutrophils are driven down an accelerated but aberrant apoptotic pathway (Harper, Ren and others, 2000). Thus, the neutrophils will develop the morphologic features of apoptosis, but there is failure of cell surface changes such as bi-lipid cellular membrane phosphatidylserine (PS) externalisation (Harper, Ren and others, 2000; Zwaal and Schroit, 1997), which is normally an early feature of all apoptotic cells. The externalization of PS allows apoptotic cells to be recognized and safely removed by phagocytes so that inflammation is not provoked by release of damaging cell contents (in other words, non-phlogistic cell death). After ANCA stimulation, as a result of the lack of PS externalization, phagocytes are less able to process the apoptotic neutrophils in a non-phlogistic fashion, explaining the well-characterised finding of leucocytoclasis often seen in the vasculitic lesions of certain vasculitic syndromes.
T cells and monocytes may also contribute to vascular injury in ANCA associated vasculitides

ANCA associated vasculitic damage is accompanied by T cell and monocytic recruitment (Jennette, 2002). It has been demonstrated that peripheral blood T-cells from patients with either active or quiescent SSV proliferate in response to PR3 or MPO (King, Brooks and others, 1998). The observation that T cell activation continues after disease remission was confirmed by Christensson et al (Christensson, Pettersson and others, 2000), who showed reduced CD28 and increased CD69 (an early T-cell activation marker, also known as the activation induction molecule) on CD3 T cells, and CD38 on CD8-positive T-cells (CD38 is present on T cells stimulated with mitogen, and furthermore CD38⁺ populations of T cells in the mouse are capable of producing high levels of interleukin-4). Increased levels of soluble interleukin-2 receptor during clinical remission were also found. These studies suggest that T cells may contribute to the remitting and relapsing nature of SVV.

Lastly, it has been proposed that stimulation of T and/or B cells by superantigens may result in the proliferation of autoreactive B cells capable of ANCA production (Cohen Tervaert, Popa, and Bos, 1999). This speculative theory will be discussed in more detail in section 1.3.

Tissue expression of antigen targeted by ANCA

Although the antigens targeted by ANCA are mainly distributed on the neutrophil, there is also evidence that monocytes express PR3 and MPO (Preston, Yang and others, 2002).
Moreover, ANCA-induced monocyte activation can result in the production of interleukin-8 (Ralston, Marsh and others, 1997), contributing to the potentially injurious pathways involved in ANCA associated SVV. ANCA-mediated monocyte activation is accompanied by increased expression of CD14, a lipopolysaccharide receptor, and CD18 (one of the members of the β2 family of integrin receptors) (Nowack, Schwalbe and others, 2000). Thus ANCA-activated monocytes may contribute to tissue injury (Preston, Yang and others, 2002). Interestingly glomerular epithelial cells also are reported to express proteinase-3 mRNA, raising the possibility of direct glomerular epithelial injury by ANCA (Schwarting, Hagen and others, 2000).

**Genetic predisposition to ANCA associated vasculitides**

Several investigators have examined potential genetic factors predisposing to vasculitis, including vasculitic syndrome associated with ANCA (Kallenberg, Rarok, and Stegeman, 2002). Since no clear association with HLA alleles has been found attention has focused on genes that control the neutrophil inflammatory response. These will be considered in turn.
α-1-antitrypsin deficiency and ANCA associated vasculitides

An association between PR3-ANCA and the deficient α-1-antitrypsin PiZZ phenotype has been described (Esnault, Testa and others, 1993; Esnault, 1997). Analysis of the incidence of antibodies against neutrophil components in PiZZ-deficient sera has shown that there is an increased incidence of antibodies against α granules and human leukocyte elastase, but not against other classical ANCA antigens such as PR3, MPO, lactoferrin, or bactericidal permeability-increasing protein (BPI protein). It thus appears that α-1-antitrypsin deficiency is not sufficient in itself to cause ANCA-positive vasculitides, but may act as an amplifying factor (Audrain, Sesboue and others, 2001). To support this theory, PiZ heterozygosity in patients with PR3 ANCA vasculitis is associated with a poor prognosis, disseminated disease, and increased mortality compared with non-carriers (Segelmark, Elzouki and others, 1995).

Proteinase-3 polymorphisms and ANCA associated vasculitides

Any genetic predisposition to altered PR3 processing, potentially resulting in abnormal surface expression of PR3 on neutrophils, would be an attractive mechanism explaining the observation of persistent PR3 expression on neutrophils in patients with ANCA associated vasculitides, and the increased risk of vasculitis in individuals who constitutively express higher levels of surface PR3 (Witko-Sarsat, Lesavre and others, 1999). Such observations have provoked analyses of PR3 polymorphisms. Of 10 identified polymorphisms, an association with WG was demonstrated for the A-564G polymorphism in the proteinase-3 promoter (Gencik, Meller and others, 2000a). This site
contains a possible transcription factor-binding site, which could affect the level of surface proteinase-3 expression.

\textit{β2-integrins and ANCA associated vasculitides}

There may be a role for β2 neutrophil integrins in ANCA-mediated activation (Reumaux, Vossebeld and others, 1995; Radford, Savage, and Nash, 2000). A restriction fragment-length polymorphism (RFLP) in exon 11 of the CD18 gene was associated with MPO-ANCA SSV, although no relevant polymorphisms were identified in the vicinity of genes for other cell adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), E-selectin, CD11b, or human urokinase plasminogen activator receptor gene (Gencik, Meller and others, 2000b).

\textit{Fcy receptors and ANCA associated vasculitides}

The Fcy receptors FcγRIIa and FcγRIIIb are constitutively expressed on neutrophils, and polymorphisms have been studied in the context of ANCA associated vasculitides. No association has been found between FcγRIIa receptor polymorphism and SVV (Tse, Abadeh and others, 1999), although there may be an association between the functionally more active NA1 allele of FcγRIIIb in patients with WG and renal involvement (Tse, Abadeh and others, 2000). Patients with WG homozygous for the FcγRII-R131 and FcγRIIIa-F158 may however be more susceptible to disease relapse (Dijstelbloem, Scheepers and others, 1999).
Other genetic polymorphisms and ANCA associated vasculitides

Proinflammatory cytokines such as TNF-α and interleukin-1 may be involved in the development of vasculitic lesions. One study has failed to identify associations among TNFα and interleukin-1β restriction fragment length polymorphisms, and WG (Huang, Giscombe and others, 2000). However, the same investigators did show an association with a polymorphism of CTLA-4 (cytotoxic T lymphocyte–associated antigen-4) and WG. CTLA-4 is a co-stimulatory molecule, which suppresses antigen-specific immune responses by suppressing the CD28 pathway. The investigators found that the prevalence of the shortest CTLA-4 allele was decreased in patients with WG compared with healthy individuals, and proposed that genetic polymorphism of this molecule could contribute to the pathogenesis of WG by allowing an increased T cell activation by antigen.

ANCA from the paediatric perspective

In comparison with the extensive work relating to ANCA and vasculitis in adults, work in children has been limited. Undoubtedly, the ANCA associated vasculitides occur in children, as previously discussed, and a number of reports have described typical C-ANCA and P-ANCA patterns in children with vasculitis, predominantly Wegener's granulomatosis, microscopic polyangiitis and renal crescentic glomerulonephritis (Nash and Dillon, 1997; Ellis, Wood, and Berry, 1995; Vogt, Kim and others, 1994). Generally speaking clinical experience suggests that findings in adults with these conditions can be extrapolated to children.
ANCA have also been reported in other chronic inflammatory disorders in children, including inflammatory bowel disease, SLE, and cystic fibrosis (Nash and Dillon, 1997; Schultz, Csernok and others, 2000; Sediva, Bartunkova and others, 1998). However, in these disorders the antibodies are directed against a variety of epitopes, and there is little correlation with organ involvement or disease activity (Wong, Shah, and Dillon, 1995). The significance of ANCA in these chronic inflammatory conditions in childhood is unclear. ANCA are not present in haemolytic uraemic syndrome (Fitzpatrick, Shah and others, 1992); IgG and IgM ANCA are not found in children with HSP, a relatively common systemic vasculitis of childhood (Nash and Dillon, 1997). The prevalence and significance of IgA isotype ANCA in Henoch-Schönlein purpura is a matter of debate (O'Donoghue, Nusbaum and others, 1992).

Some studies have suggested ANCA are found in acute Kawasaki disease, with an elevated titre and atypical pattern on IIF (Savage, Tizard and others, 1989). Later studies using only samples taken before administration of IVIG therapy did not confirm this, and the same IIF pattern was seen in children with fever resulting from acute infection as in those with Kawasaki disease (Nash, Shah and others, 1995). This is consistent with the experience in adults in which an atypical pattern can be seen in a range of acute and chronic inflammatory conditions, and suggests that this pattern may be due to non-specific binding of antibody secondary to generalised immune activation and polyclonal B cell activation.
Thus although the ANCA associated vasculitides do occur in children, these forms of vasculitis are less common than ANCA negative vasculitides such as HSP and KD. Most of the paediatric practice in dealing with the ANCA associated vasculitides is based on data derived from adult studies, and many assumptions are made (rightly or wrongly) regarding similarities in the clinical management and pathogenesis of ANCA associated vasculitides in children and adults.

**Conclusion**

ANCA-associated systemic vasculitis may develop as a result of a “two-hit” process by the interplay of exogenous and endogenous factors. As regards the genetic contribution, immune response genes are likely to be involved (as in other autoimmune diseases). For example, in PR3-ANCA-associated vasculitis, genetic polymorphism in the PR3 molecule altering its expression on the neutrophil may influence induction and clinical course of WG.

Whilst much is now known about the downstream events mediating ANCA associated vascular injury, relatively little is known about the factors which predispose to the loss of immunological tolerance to neutrophil enzymes. Significant advances in the understanding of the genetic influences that may predispose to ANCA associated vasculitides are anticipated.
1.2.4.2 Anti endothelial cell antibodies

Anti endothelial cell antibodies (AECA) are antibodies that react with endothelial cell (EC) structures (Praprotnik, Blank and others, 2001). They were first reported in the early 1970s in sera from patients with various rheumatic diseases, including systemic lupus erythematosus (SLE) and scleroderma (Lindqvist and Osterland, 1971; Tan and Pearson, 1972). Since then AECA have been demonstrated in a wide variety of inflammatory diseases, sometimes inconsistently. AECA bind to different structures on endothelial membranes, mainly through the F(ab)2 portion of the immunoglobulin, and IgG, IgM, and IgA isotypes of those antibodies have been reported. The endothelial epitopes for AECA have not yet been defined, but it is clear that they are likely to be more than one (Praprotnik, Blank and others, 2001). It is now becoming apparent that AECA may have preferential specificity for blood vessels of different sizes, presumably as a result of differential antigen expression on endothelial cells derived from vessels of different size (Praprotnik, Blank and others, 2001). Thus, sera positive for AECA have been shown to display a broad reactivity against endothelial cells obtained from different human anatomic sources: from large arterial (aorta) or venous (umbilical cord vein, saphenous vein) vessels as well as from small vessels such as renal, skin, omental, and brain microvasculature (Shoenfeld, 2002). Interestingly, AECA are not species specific, since they cross-react with human, bovine, and murine endothelial cells. In this respect, AECA may be non-specific antibodies that are directed against ubiquitously expressed endothelial antigens (Shoenfeld, 2002).
Based on studies showing that AECA derived from patients with large vessel diseases such as Takayasu disease (TD) bind to and activate macrovascular human umbilical vein endothelial cells (HUVEC) in vitro, and not microvascular endothelial cells (Blank, Krause and others, 1999; Shoenfeld, 2002), Shoenfeld et al proposed that AECA from different sources recognise different types of endothelial cell target molecules, which may correlate with the predominant vessel size involved in individual diseases. They therefore have suggested a classification system for AECA based on microvascular or macrovascular endothelial cell binding propensity (Praprotnik, Blank and others, 2001; Shoenfeld, 2002).

A Pitfall in the detection of AECA: methods of detection have variable sensitivity and specificity

Non-standardised methods of detection at least in part explain some of the inconsistencies in the literature relating to the prevalence of AECA in different disease states (Meroni PL and Youinou P, 1996). The use of indirect immunofluorescence permitted the original description of AECA in rodent tissue. Despite its high specificity, this method is limited by low sensitivity (Meroni PL and Youinou P, 1996). Subsequent reports documented a similar reaction using whole human endothelial cells maintained in culture or membrane-coated plates. Enzyme-linked immunosorbent assay (ELISA) is the method most widely employed for the detection of AECA. Whole endothelial cells from different sources (mainly HUVEC) or enriched endothelial cell membranes are used as substrate (Meroni PL and Youinou P, 1996). Although ELISA is easy to perform, false-positive results are possible, probably due to autoantibodies reacting with cytoplasmic or nuclear
components that contaminate the membrane preparation (Meroni PL and Youinou P, 1996). Additionally, few reports have compared the detection of AECA on fixed and unfixed endothelial cells, possibly leading to considerable discrepancies in the results. Recently, it has been suggested that endogenous antibodies reacting to fetal calf serum protein from cell culture medium may give rise to false positivity in a cyto-ELISA for AECA (Revelen, Bordron and others, 2000).

Flow cytometry can be used to detect AECA, but this technique requires a large number of endothelial cells in suspension (Praprotnik, Blank and others, 2001). Moreover, detachment of EC grown in vitro by enzymatic treatment in order to create such endothelial cells suspensions may result in contamination of endothelial cells surface proteins with nuclear and cytoplasmic components exposed upon disruption of the of endothelial cells (Praprotnik, Blank and others, 2001).

Therefore, it is clear that different methodologies can produce different results making the interpretation of studies that employ different techniques problematic. That said, it is now generally accepted that if appropriate cells are used as substrate, ELISA may display the highest specificity and sensitivity for the detection of AECA.

**AECA disease associations**

Many autoimmune diseases have been associated with AECA. These will be considered in turn.
Systemic vasculitides and AECA

The largest disease group in which AECA are detected includes patients with systemic vasculitides. Several investigative groups have reported the occurrence of AECA in both WG and MPA sera (Meroni PL and Youinou P, 1996). Some authors found that the presence of AECA in WG reflected the activity of the disease and may represent an early marker predictive of disease relapse (Gobel, Eichhorn and others, 1996). Prospective analysis showed that the rise in AECA titers preceded the development of relapses in ANCA-negative WG patients, and the persistence of AECA after remission was associated with a highly increased risk of relapses (Gobel, Eichhorn and others, 1996).

In vitro studies support a pathogenetic role for AECA in systemic vasculitis. AECA from the sera of patients with WG are able to activate endothelial cells (Del Papa, Guidali and others, 1996): HUVEC incubated with AECA IgG from WG patients can up-regulate the expression of cell adhesion molecules, and can support leukocyte adhesion to HUVEC. In addition, once bound to HUVEC AECA also induce an increase in production of proinflammatory cytokines by the HUVEC including IL-1, IL-6, IL-8, and monocyte chemotactic protein. These observations could provide pathogenic mechanisms for vascular injury mediated by AECA.

Further evidence for the pathogenicity of AECA comes from animal studies. Vasculitic lesions are associated with murine AECA induced by immunizing naive mice with the IgG fraction from the sera of WG patients (Blank, Tomer and others, 1995). Following the injection of the human WG IgG fraction (displaying both PR3-ANCA and AECA
activity), naive mice produced both murine ANCA and AECA accompanied by histopathological signs of renal and pulmonary vasculitis. The investigators went on to immunize mice with IgG fraction containing only AECA (i.e. deplete of PR3-ANCA). Again, the mice developed murine AECA followed by glomerular vasculitis (Blank, Tomer and others, 1995). This experimental model, therefore, suggests that AECA potentially are involved in the pathogenesis of vasculitic lesions.

Conflicting data have been reported relating to the prevalence of AECA in KD. Kaneko et al studied the sera from patients with respect to binding activity to HUVEC using a cellular based ELISA technique (Kaneko, Savage and others, 1994). They detected IgM AECA in 16/22 sera, and IgG AECA in 19/22 sera. This result was significantly different when compared to sera obtained from healthy control children. In addition, in that study 8/16 sera showed complement-dependent cytotoxicity against HUVEC, which was further enhanced by pre-treatment of the HUVEC with TNF-α. IgM AECA titres measured by ELISA were positively correlated with cytotoxicity. The authors concluded that AECA worked synergistically with TNF-α to mediate complement-dependent endothelial damage in some patients with KD.

In contrast, Nash et al from the same group repeated the study of AECA in KD patients using the same ELISA technique, but this time compared the results with sera obtained from febrile control children (Nash, Shah and others, 1995). They found no difference between KD patients and febrile controls in terms of levels of IgM AECA when total IgM had been taken into account. The authors concluded that the previously observed
difference in IgM AECA between KD and afebrile controls was attributable to differences in total IgM levels, and suggested that AECA were an epiphenomenon related to a non-specific acute phase response.

Other vasculitic diseases where AECA have been detected include Behçet’s disease where the prevalence of AECA was 50% in one study (Cervera, Navarro and others, 1994). Moreover, the prevalence was higher in patients with active disease. Importantly, however, no febrile control group was included in this study and hence this observation may again have represented a non-specific epiphenomenon related to increased total circulating immunoglobin.

Other AECA disease associations

AECA have also been described in association with SLE and antiphospholipid syndrome, rheumatoid arthritis, systemic sclerosis, inflammatory bowel disease, organ transplantation, hyperprolactinaemia, thrombotic thrombocytopenic purpura (TTP), heparin induced thrombocytopenia, and in preparations of pooled human immunoglobulin (Meroni PL and Youinou P, 1996).

Conclusion

AECA would appear to be a logical and obvious pathogenetic mechanism for systemic vasculitis. Definitive proof that AECA are involved in the pathogenesis of vasculitis are lacking, however. Moreover many studies relating to AECA have been hampered by
methodological artefacts, and lack of appropriate disease control groups. Moreover, potential AECA epitopes are currently poorly characterised.
1.2.5 Immune complexes and vasculitis

Immune complexes have already been considered in the context of the association between HCV and cryoglobulinaemia, but some expansion on this pathogenetic mechanism is required. For many years immune complexes have been a recognised association with vasculitis (Dixon FJ, VasquezJJ, and Weigle WO, 1958). Acute serum sickness, which is the prototypic model of immune complex-mediated disease, was one of the first animal models of vasculitis to be described. Our current understanding of the cellular and molecular mechanisms responsible for the inflammation and tissue damage that occurs in immune complex-mediated diseases, such as in SLE or hypersensitivity vasculitis, is based to some extent on knowledge gained from the elucidation of the immunopathogenesis of acute serum sickness in animals.

In this model, which is essentially an example of a type III hypersensitivity reaction, following the injection of a large dose of bovine serum albumin (BSA), rabbits develop antibodies to the foreign protein, with the subsequent formation of circulating BSA-anti-BSA immune complexes. Deposition of those immune complexes in various tissues, including the glomerulus, synovium, and the walls of blood vessels then occurs. The first component of complement, Clq, binds to the Fc portion of IgG or IgM antibodies in the deposited immune complexes and activates the classical pathway of complement activation. Activated complement components (C5a) with chemotactic properties are generated, which attract neutrophils to the area and activate them. The activated neutrophils bind to the deposited immune complexes through their Fc and complement receptors, and attempt to phagocytose the complexes, but are unable to do so. As a
consequence of this "frustrated" phagocytosis, the neutrophils degranulate and release lytic enzymes. When this sequence of events occurs in blood vessels it causes an acute, necrotizing, leukocytoclastic vasculitis (Cochrane and Koffler, 1973). Left untreated, the involved blood vessels in animals with acute serum sickness develop fibrinoid necrosis of the media, and prominent mononuclear cell infiltrates around all layers of the blood vessel walls (Neild, Ivory, and Williams, 1984a; Neild, Ivory, and Williams, 1984b). Treatment of rabbits with acute serum sickness with cyclosporin inhibits the development of mononuclear infiltrates in or around vessels, but does not prevent necrosis. These data suggest that the formation and maintenance of mononuclear cell infiltrates in acute serum sickness model is a T cell- dependent phenomenon (Neild, Ivory, and Williams, 1984a; Neild, Ivory, and Williams, 1984b).

The deposition of virus-anti-virus immune complexes in vessel walls appears to play a central role in the pathogenesis of many virus-associated vasculitides, including the vasculitis that occurs in patients infected with HBV and HCV (see section 1.2.2.1). Immune complex deposition is also central to the pathogenesis of most types of leukocytoclastic vasculitis. The post-capillary venule is an important site of initiation of damage. Immune complexes, formed in a state of antigen excess, circulate in the vascular compartment and precipitate in the walls of blood vessels. The trapping of immune complexes in the microvasculature initiates the cascade of events described above, causing small vessel vasculitis (Bagga and Dillon, 2001).
1.2.6 Cryoglobulins and vasculitis

Cryoglobulins have been mentioned previously in the context of HCV infection (section 1.2.2.1). Cryoglobulins are immunoglobulins that persist in the serum, precipitate with cold temperature, and resolubilize when rewarmed (Cacoub, Costedoat-Chalumeau and others, 2002). Cryoglobulins are classified according to Brouet et al. (Brouet, Clauvel and others, 1974): type I cryoglobulins are single monoclonal immunoglobulins; types II and III are mixed cryoglobulins, composed of different immunoglobulins, with a monoclonal component in type II and only polyclonal immunoglobulins in type III. Type I cryoglobulins are always associated with haematological malignancy. Mixed cryoglobulins (type II or III) are associated with connective tissue disease, malignant haematological disorders, or infections (including HCV, Epstein-Barr virus, HIV, cytomegalovirus, Treponema, and Leismaniasis) (Cacoub, Costedoat-Chalumeau and others, 2002). When mixed cryoglobulins are found in the absence of well-defined disease, the syndrome has been designated “essential mixed cryoglobulinemia”.

The syndrome of mixed cryoglobulinemia is characterised by the clinical triad of purpura, arthralgia, and asthenia associated with type II or type III mixed cryoglobulins. The disorder represents the consequence of an immune complex-type vasculitis (see section 1.2.5) with depressed levels of complement and deposition of immunoglobulins and complement in lesions. Cryoglobulinemic vasculitis may involve many organs, particularly the peripheral nervous system and the kidneys. Cryoglobulinaemic vasculitis is rare in childhood, but should be considered in any child with small vessel vasculitis, and/or membranoproliferative glomerulonephritis (Bagga and Dillon, 2001).
1.2.7 *The endothelium as a target and amplifier of inflammation in vasculitis*

An emerging concept is that the endothelium is not only the prime target for many of the pathogenetic mechanisms described above, but is also a protagonist of the inflammatory cascade associated with vasculitis, and may play an active role in focusing the inflammatory process at vascular sites by mechanisms which will include antigen presentation, cell adhesion molecules, and cytokine production. In addition to amplification of the inflammatory response, the vascular response to inflammation results in vascular remodelling and repair—ultimately leading to vessel occlusion and end organ damage.

1.2.7.1 *The endothelial cell as a target for injury in vasculitis*

As will now be appreciated from the preceding description of the various pathogenetic mechanisms involved in vasculitis, the endothelial cell is a target for injury by infectious agents, malignant processes, ANCA and AECA mediated vascular injury, and immune complex-mediated injury. But it now appears that endothelial cells are not just "innocent bystanders"; and as will be discussed, actively participate in the inflammatory process.

1.2.7.2 *The endothelial cell amplifies inflammation*

Endothelial cells can amplify inflammation by 4 main mechanisms: adhesion molecule expression, cytokine production, angiogenesis, and as will be discussed in considerable
detail in subsequent sections, may play a role in antigen or superantigen presentation to T cells.

**Endothelial cell adhesion molecules**

Numerous studies have described upregulated soluble adhesion molecule expression in serum, on circulating leukocytes, and in lesional tissue of vasculitis syndromes (Sundy and Haynes, 2000; Cid, 2002). Adhesion molecules influence the binding of neutrophils and mononuclear cells to endothelial cells. Adhesion molecule expression in lesional tissue of vasculitis syndromes may reflect the type of cellular infiltrate. For example, vascular cell adhesion molecule-1 (VCAM-1), the ligand for lymphocytic VLA-4, is expressed on vessels in lymphocytic cutaneous vasculitic lesions, whereas E selectin (CD62E) expression is upregulated in leukocytoclastic vasculitic lesions characterised by neutrophil infiltration (Bradley, Lockwood, and Thiru, 1994; Burrows, Molina and others, 1994).

Passage of inflammatory cells from the vascular space into inflamed vascular tissue requires adhesion molecule-mediated, leukocyte-leukocyte, and leukocyte-endothelial cell interactions. Adhesion molecule expression on leukocytes and endothelial cells is influenced by local cytokine production and adhesion molecule-ligand interactions of leukocytes and endothelial cells in turn influence cytokine production from both leukocytes and endothelial cells (Bradley, Lockwood, and Thiru, 1994; Sundy and Haynes, 2000).
Constitutive adhesion molecule expression on endothelial cells is modulated by regional factors such as vessel location and sheer forces (Cines, Pollak and others, 1998). Thus, adhesion molecule expression in the healthy state varies from organ to organ, and by vessel size. For example, lung endothelium uniquely expresses Lu-ECAM-1 in humans, whereas endothelial cell venules in Peyer's patches constitutively express Mad-CAM-1. This heterogeneous pattern of adhesion molecule expression may, in part, explain the propensity of different vasculitis syndromes to affect vessels of a particular size or location (Sundy and Haynes, 2000).

Studies on cell adhesion molecule expression in vasculitis

Lesional endothelial adhesion molecule expression to date has been studied in patients with cutaneous leukocytoclastic vasculitis, KD, PAN, and GCA (Leung, Cotran and others, 1989; Sais, Vidaller and others, 1997; Coll-Vinent, Cebrian and others, 1998; Cid, Cebrian and others, 2000). Common findings in these syndromes were upregulation of E-selectin, VCAM-1 and ICAM-1 by endothelial cells. In glomerular lesions of WG and MPA VCAM-1 and ICAM-1 expression can be observed in the glomerulus, tubular epithelial cells, and peritubular capillaries (Cid, 2002). In GCA adhesion molecule expression occurs in neovessels at the adventitia and within the inflammatory lesions suggesting that the infiltrating leukocytes come from the vasa vasorum (Cid, Cebrian and others, 2000).

It is likely that interactions mediated by adhesion molecules are pathogenetically important in vasculitis. In the study by Chakravorty and others (Chakravorty, Howie and
others, 1999) peripheral blood T cells adhered to intraglomerular, periglomerular, and tubulo-interstitial regions of the cortex of kidney sections taken from patients with renal vasculitis. In that study, blocking monoclonal antibodies against tissue-expressed ICAM-1, VCAM-1, and the CS-1 domain of fibronectin (CS-1Fn) differentially attenuated T cell adhesion. Additionally, in a mouse model of vasculitis induced by immunisation against mycobacterium butyricum, the administration of blocking monoclonal antibodies demonstrated the important participation of interactions mediated by selectins and by α4 integrins in leukocyte adhesion and transmigration (Johnston, Issekutz, and Kubes, 1996).

**Endothelial cells, cytokines and vasculitis**

Endothelial cells may contribute to the inflammatory response in vasculitis by the production of a variety of cytokines and growth factors. Notably, they can produce IL-1α and IL-6 which may contribute to the acute phase response observed in vasculitis (Cid, 2002). Through the production of colony-stimulating factors, they may prolong the survival and promote the proliferation of leukocytes in the perivascular compartment, thus perpetuating the chronic vascular inflammation (Schliesser, Pralle, and Lohmeyer, 1992). Several chemokines including IL-8, and RANTES (regulated upon activation, normal T cell expressed and secreted) can be produced by endothelial cells (MacKay, 2001). Chemokines selectively attract leukocytes, thus endothelial cells may actively contribute to tissue targeting in systemic vasculitis, and by attracting additional leukocytes may perpetuate and amplify vessel inflammation.
Thus, there is now a considerable body of evidence that would support the notion that cytokine and adhesion molecule-mediated pathways may contribute to the inflammatory vascular response to injury by focusing and perpetuating leukocyte infiltration into the vascular and perivascular compartments.

**Therapy of vasculitis aimed at modulation of cytokine and adhesion molecule function**

Therapies designed to modify aberrant cytokine production and/or cell adhesion molecule expression may be a step towards the therapeutic “Holy Grail” or “magic bullet” therapy for vasculitis. As discussed in section 1.1, treatment of vasculitis requires non-specific and aggressive cytotoxic immunosuppressive regimens. This has implications in terms of toxicity, which is of particular concern for paediatric patients (Brogan and Dillon, 2000b). Evidence relating to the role of cytokines and adhesion molecules in vasculitis offers the prospect of therapies that block key pathogenic cytokine or adhesion molecule pathways. This is an approach that has been used with considerable success in rheumatoid arthritis, for example (Emery and Buch, 2002).

The use of anti-TNF-α monoclonal antibody and soluble TNF receptor for the treatment of rheumatoid arthritis has validated the rationale for disrupting cytokine pathways in treating inflammatory disease. Since TNF-α is ubiquitously upregulated in vasculitis, TNF-α blockade may be an obvious and logical therapeutic choice. Anecdotal evidence suggests efficacy in treating rheumatoid vasculitis, temporal arteritis, and WG (Sundy and Haynes, 2000). Recently, a randomised, placebo-controlled multi-centre trial in WG
using etanercept (a TNF-α receptor p75 fusion protein) has started in the USA. The principal aim of the trial is to test the efficacy of etanercept as add-on therapy to standard maintenance treatment to maintain disease remissions (Stone JH, 2000).

Caution must be the order of the day, however, since such a “logical approach” resulted in deterioration in multiple sclerosis in response to TNF-α blockade (Wiendl and Hohlfeld, 2002). The experience in MS demonstrates that theoretically promising agents may paradoxically increase disease activity.

Other potential cytokine therapy targets in the vasculitides include inhibition of signaling by IL-1 and IL-6 by administration of IL-1 receptor antagonist and soluble IL-6 receptor; and IL-10 (which downregulates both Th1 and Th2 immune responses) (Sundy and Haynes, 2000).

Finally, drugs that inhibit the adhesion of leukocytes to endothelial cells including antibodies to CD18, intercellular adhesion molecule type 1 (ICAM-1) antisense, and anti-ICAM-1 antibodies may prove useful in the treatment of vasculitis in the future (Cohen Tervaert, Stegeman, and Kallenberg, 2001; Griffin, Chapman and others, 1999). Lockwood et al. recently reported the use of a humanized monoclonal anti-CD18 antibody in patients with vasculitic tissue injury that resulted in ulceration or limb/digital infarction with incipient gangrene (Griffin, Chapman and others, 1999; Lockwood, Elliott and others, 1999). Remarkable prompt clinical healing of ulceration and the restoration of limb function was noted in four out of five patients.
Angiogenesis and inflammatory amplification

Angiogenesis is part of the vascular response to inflammation in vasculitis, and is particularly relevant to GCA (Cid, 2002). Angiogenesis plays a dual role: on the one hand it presumably constitutes a protective response to organ ischaemia as a result of vascular injury, but on the other hand may contribute to the amplification of the inflammatory vascular response by providing new sites for leukocyte entry into the vascular tree, and also by providing a new source of cytokines, chemokines, and growth factors (Cid, 2002).

Angiogenesis is controlled by a balance between the influx of angiogenic and anti-angiogenic factors, and the control of the expression of relevant receptors. Several molecules may promote angiogenic activity. These include growth factors, chemokines, thymosins, acute-phase proteins and extracellular matrix protein fragments (Carmeliet and Jain, 2000; Carmeliet and Collen, 2000). Several angiogenic factors have been detected in temporal artery lesions from patients with GCA, although their functional relevance is incompletely understood (Cid, 2002).

1.2.8 T cells and vasculitis

T cells are involved in the pathogenesis of vasculitis, but their role in different vasculitis syndromes is less clearly defined. There seems to be no doubt that they play a role in the formation of granulomas of WG (Sundy and Haynes, 1995), and the response of ANCA-negative patients with MPA to monoclonal antibody therapy to T cells provides
compelling evidence that they are involved in the pathogenesis of that entity (Mathieson and Oliveira, 1995).

1.2.8.1 T cells and Giant cell arteritis

Much progress relating to the pathogenesis of GCA points to a central role for T cells in this disorder (Weyand and Goronzy, 1996; Mohan and Kerr, 2000). Non-specific immunological abnormalities include decreased circulating CD8+ lymphocytes, increased circulating soluble IL-2 receptors, circulating soluble adhesion molecules, and cytokines such as IL-6, all support the concept that immune mechanisms play an important role (Weyand and Goronzy, 1996; Mohan and Kerr, 2000).

Arterial wall T cells in GCA have been found to have identical sequences at the third complementary determining region (CDR3) of their T-cell receptors (Weyand, Schonberger and others, 1994). This supports the hypothesis that GCA lesions may represent an immune response toward an antigen present in the arterial wall. The inciting antigen has not yet been identified, however. T cells enter the involved artery through the vasa vasorum in the adventitial layer, and produce INF-γ. A potent activator of macrophages, IFN-γ is crucial for the development of granulomas and giant cells.

1.2.8.2 T cells and small vessel vasculitides

T cell-mediated immunity is thought to contribute to pathogenesis of SSV, and several previous studies have documented the ability of peripheral blood T-cells from patients with either active or quiescent SSV to proliferate in response to PR3 or MPO (Brouwer,
Stegeman and others, 1994; Griffith, Coulthart, and Pusey, 1996; King, Brooks and others, 1998). As previously discussed, circulating T cells are continuously activated, even when small vessel vasculitides are in clinical remission (section 1.2.4.1.4).

To unequivocally establish a true effector role for antigen-specific T-cells, it would be necessary to demonstrate the presence of such cells in lesional vasculitic tissue, and this has not yet been achieved. Immuno-phenotyping of monocytic infiltrates in lesional tissue in ANCA associated vasculitides has demonstrated CD4-positive and CD8-positive T cells (Savage, Harper, and Holland, 2002). Moreover, many of the cell adhesion molecules and cytokines that are required for T cell recruitment are upregulated in lesional vasculitic tissue (section 1.2.7.2).

Cell-mediated immunity, including that of T-cells, macrophages, and fibrin in the absence of immunoglobulin, has been demonstrated in the kidneys of patients with pauci-immune glomerulonephritis (Cunningham, Huang and others, 1999). In that study (of 15 patients) CD3-positive T cells, CD45RO-positive T cells, macrophages, fibrin, and endothelial-associated tissue factor were all demonstrated to be prominent in glomeruli. These mediators were absent in a group of 12 patients with thin basement membrane disease, and only occasionally observed in a group of eight patients with "humorally mediated" (non-crescentic) glomerulonephritis. Thus, these data would suggest that pauci-immune crescentic nephritis is most likely a manifestation of T cell-directed immune injury.
Further support for a Th1 bias rich in INF-γ and poor in IL-4 is provided by the study by Csemok et al (Csemok, Trabandt and others, 1999). This was an interesting study because it examined multiple body compartments to determine whether a specific cytokine pattern (Th1 or Th2) predominates in Wegener's granulomatosis (WG), by evaluating INF-γ and IL-4 expression in biopsied nasal mucosal tissue, bronchoalveolar lavage fluid, and peripheral blood, and compared the findings with those in disease and healthy control subjects. Patients with WG and chronic rhinitis were found to share in situ production of messenger RNA (mRNA) specific for IFN-γ (Th1). Only 2/13 patients with WG expressed IL-4 in nasal mucosa, whereas IL-4 mRNA PCR products were found in inflamed nasal mucosa of all the disease control patients. The granuloma-derived T cells of WG patients produced only IFN-γ, while CD4+ and CD8+ T cells from bronchoalveolar lavage fluid and peripheral blood produced mainly IFN-γ. The authors concluded that a Th1 cytokine pattern predominates in the granulomatous inflammation in patients with WG.

1.2.8.3 T cells and Kawasaki disease

Perhaps the most compelling evidence that T cells are involved in the pathogenesis of vasculitis relates to the observations in KD. While there is currently much debate regarding a conventional antigen versus superantigen aetiopathogenesis, most authorities agree that T cells are central to the pathogenesis. This argument, including an in-depth review of the evidence for and against the role of superantigens in KD and other vasculitides, will be the subject of the next section and will be the main theme followed throughout the subsequent result chapters of this thesis.
1.2.9 Concluding remarks relating to the pathogenesis of vasculitis

Many mechanisms have been described to explain the vascular injury associated with vasculitis syndromes. Most of these are downstream events, and although important do not explain aetiology in most cases. For the purposes of description, the various mechanisms have been described in isolation. It is likely however that different mechanisms will operate together (perhaps synergistically), with varying contributions from each mechanism in individuals with vasculitis.
1.3 Superantigens and vasculitis

1.3.1 Introduction

The discussion from previous sections has established that there is considerable heterogeneity among the various vasculitis syndromes that can affect children and adults. In addition, many different infectious and immune processes can interact on the background of host predisposing factors to culminate in vascular injury. It would be thus overly simplistic to hypothesise that a single aetiopathogenetic factor, such as superantigens, would be the cause of all childhood vasculitic syndromes. A more realistic question would be to ask if there is any evidence that superantigens could be involved at some level in certain vasculitis syndromes, either as the main precipitating factor, or by amplifying an inflammatory cascade already set up by other factors; and secondly to ask what mechanisms superantigens might utilize to cause vascular injury.

This section of the thesis will describe what a superantigen is, outline how they are processed by T cells at a molecular level, and describe the evidence for and against the role of superantigens in KD and other vasculitides.

1.3.2 What is a superantigen?

Superantigens (SAgs) are a class of immuno-stimulatory proteins of bacterial or viral origin with the ability to activate large fractions (5-30%) of the T cell population (Li, Llera and others, 1999; Muller-Alouf, Carnoy and others, 2001), and are responsible for human toxic shock syndrome and some forms of gastroenteritis. By contrast,
conventional antigens stimulate 0.01-0.0001% of T cells (Muller-Alouf, Carnoy and others, 2001). SAg activation of T cells requires interaction with a specific Vβ segment of the T cell receptor (TCR) and with major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells (figure 1.8) (Muller-Alouf, Carnoy and others, 2001). The complete responding Vβ repertoire has not been identified for many of the known SAgs, and some SAgs will bind to more than one Vβ family (for example staphylococcal enterotoxin B [SEB] which typically activates T cells bearing Vβ3, Vβ12, and Vβ17 as major-responding Vβ families; and Vβ14, Vβ15 and Vβ 20 as lesser-responding families) (Cohen Tervaert, Popa, and Bos, 1999).

Contrary to the view of some authors who have suggested that SAgs only stimulate CD4 T cells (Janeway CA, Travers P and others, 1999; Mingari, Cambiaggi and others, 1996), SAgs can activate CD4, CD8 and sometimes γδ T cells (Herrmann, Baschieri and others, 1992; Muller-Alouf, Carnoy and others, 2001). The consequence of T cell stimulation by SAgs is massive release of T cell derived cytokines such as INF-γ, TNF-α and TNF-β (Li, Llera and others, 1999; Choi, Lafferty and others, 1990; Choi, Kotzin and others, 1989; Fraser, Arcus and others, 2000; Kappler, Kotzin and others, 1989), following which the stimulated T cells are generally deleted or become anergic (see below). Since SAg-producing organisms such as Staphylococcus aureus and Streptococci are ubiquitous among the population (including the paediatric population (Lindberg, Nowrouzian and others, 2000)), there is considerable interest in SAgs as initiators or modifiers of autoimmune disease states (Fraser, Arcus and others, 2000; Schiffenbauer,
Figure 1.8: Conventional versus Superantigen presentation to T cells

1 in $10^6$ T lymphocytes activated

1 in 10 T Lymphocytes activated, but only those bearing particular TCR $V_\beta$ sub-units
Soos, and Johnson, 1998; Torres and Johnson, 1998), including the systemic vasculitides (Cohen Tervaert, Popa, and Bos, 1999).

1.3.2.1 Conventional antigen versus superantigen presentation to T cells

Conventional antigens are processed (by intracellular degradation) by classical antigen presenting cells (APC) into peptides that are presented in association with MHC class II molecules expressed at the surface of APC. The peptide-MHC molecules are then specifically recognized by T cells through the groove formed by the α and β variable domains of the TCR. Therefore, each antigen-processed peptide is destined to be recognized in the context of self-MHC molecules by a specific cognate TCR structure (Muller-Alouf, Carnoy and others, 2001). As a result of this when an antigen is introduced into the host the number of T cells that would have the TCR that can recognise the appropriate peptide epitope of the antigen is in the order of 1-100 cells per million.

MHC class II molecules are expressed on the cell surface as α and β heterodimers. After synthesis in the endoplasmic reticulum, MHC class II molecules bind to the invariant chain (Ii) to form a complex between 3 α and β heterodimers, and an Ii trimer (Shoukry, Lavoie and others, 1997). The Ii protects the peptide-binding groove of MHC class II molecules during their transport in the golgi from loading with endogenously derived peptides. Furthermore, the Ii acts as a chaperone that guides properly folded MHC class II molecules to endosomal vesicles, where loading with antigenic peptide occurs. In the
endosomes, the Ii is cleaved, leaving behind a small peptide known as CLIP (class II-associated invariant chain peptide) and thereafter the MHC class II molecule promotes the release of CLIP (Shoukry, Lavoie and others, 1997). MHC class II molecules are then loaded with antigenic peptides and reach the cell surface to be presented to T cells (Shoukry, Lavoie and others, 1997).

MHC class II molecules are also required for the presentation of SAgs. SAg presentation to T cells requires MHC class II molecules and TCR cross-linking, as demonstrated by the formation of TCR:SAg:MHC trimers in solution (Seth, Stem and others, 1994). Thus SAgs differ from conventional antigens in that the former do not require intracellular processing. Whereas the stimulation induced by the MHC class II and conventional antigen complex in the periphery serves to activate and alert T cells against the presence of foreign antigens, SAg stimulation results in early activation followed by anergy and deletion (figure 1.9). From an evolutionary point of view, this would have survival advantages for the SAg-producing organism since depletion of the host immune response to that organism would almost certainly be the result. Multiple factors such as the distribution of the SAg to generative lymphoid tissues or presentation by an inappropriate antigen presenting cell (APC) may contribute to this effect (Baccala, Vandekerckhove and others, 1993). The major differences between SAg and conventional antigen processing are summarised in table 1.3 (adapted from Shoukry et al 1997) (Shoukry, Lavoie and others, 1997).
Figure 1.9: Fate of a T cell following SAg stimulation

Key:  
APC = Antigen presenting cell  
T Ly = T lymphocyte  
= Superantigen

![Diagram of T cell fate](image-url)
Table 1.3 Conventional antigen versus superantigen presentation to T cells

<table>
<thead>
<tr>
<th></th>
<th>Superantigens</th>
<th>Conventional peptide antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal processing</td>
<td>Do not require processing</td>
<td>Internally processed in vesicles</td>
</tr>
<tr>
<td>Binding to MHC class II</td>
<td>Bind outside the antigen groove Half-life association with class II molecules minutes to hours</td>
<td>Bind in the peptide groove Virtually irreversibly associated with class II molecules</td>
</tr>
<tr>
<td>Interaction with the TCR</td>
<td>Does not necessarily require the cognate recognition of the MHC molecule by the TCR</td>
<td>Presentation involves cognate recognition between the TCR and the MHC</td>
</tr>
<tr>
<td>Functional outcome</td>
<td>Activation and proliferation, followed by anergy or apoptosis</td>
<td>Activation and proliferation under conventional circumstances</td>
</tr>
</tbody>
</table>

HLA-DR alleles differ in their ability to present SAgS to T cells

A study by Herman et al demonstrated that different human MHC class II proteins differed in their ability to bind staphylococcal enterotoxins and stimulate murine and human T cells (Herman A, Croteau G and others, 1990). Since the HLA-DR proteins share a common α chain, these results indicate that the polymorphic β chain plays an important role in SAg binding and presentation to T cells. Of the different HLA DR alleles tested, HLA-DR w53 was the poorest enterotoxin presenter. The overall MHC class II hierarchy for supporting SAg stimulation, however, favoured HLA DR over HLA-DQ and HLA DP: (highest to lowest ability: HLA-DR > HLA-DQ> HLA-DP) (Herman A, Croteau G and others, 1990).

Mycoplasma SAgS are “partially Vβ restricted”: SAgS masquerading as conventional antigens

The work of Hodtsev et al has important implications for our understanding of how SAgS are presented to T cells, and in the interpretation of data generated from studies examining T cell Vβ skewing (the “immunological footprint” left by a SAg) (Hodtsev AS, Choi Y and others, 1998).
*Mycoplasma arthritidis*, which induces a chronic form of arthritis in rodents resembling human rheumatoid arthritis, produces a 25-kDa superantigenic toxin called MAM (*Mycoplasma arthritidis* mitogen). MAM, which was first described in the early eighties strongly activates murine T cells bearing Vβ6 and Vβ8 (Hodtsev AS, Choi Y and others, 1998). MAM also stimulates human T cells through the Vβ17 region, but to a lesser extent compared to its activation of murine T cells, or to the human T cells activated by the staphylococcal enterotoxins (Cole and Atkin, 1991). Like Yersinia superantigenic toxins, MAM does not have significant homology with staphylococcal and streptococcal SAgS (Cole, Knudtson and others, 1996).

The binding domains recognized by MAM on the TCR and MHC class II molecules have been extensively characterised (Hodtsev AS, Choi Y and others, 1998). Unlike other superantigenic toxins, MAM recognises two distinct domains on the TCR. It interacts with the Vβ region of the TCR, but it also binds to the complementary-determining region 3 (CDR3) of the β chain, which is central to the conventional peptide antigen recognition by the TCR (Hodtsev AS, Choi Y and others, 1998). During conventional antigen peptide recognition, Vβ and Vα domains of the TCR form contacts with MHC class II, and the complex is stabilised by CDR3-peptide interactions. Similarly, recognition of MAM is Vβ-dependent and is also apparently stabilised by direct contacts with the CDR3-β region.
Not all human Vβ17+ T cells respond to MAM. The response is limited to those cells expressing two appropriate residues at the base of the CDR3-β loop (Hodtsev AS, Choi Y and others, 1998). This suggests that T cell activation by MAM is dependent on TCR junctional diversity, thus limiting the number of potential MAM-reactive T cell clones.

The frequency of T cells responding to this superantigen therefore are significantly lower than to other superantigens (for example SEB), but significantly higher than the T cell response to conventional antigens. Thus, MAM represents a new type of ligand for TCR, distinct from both conventional peptide antigens and other known superantigens: effectively a SAg masquerading as a conventional antigen. Whether this characteristic is limited to MAM or is a feature of other (as yet) poorly characterised SAgS is unknown.

The effect of SAgS can be blocked by peptide SAg antagonists

Any consideration of the biology of SAgS would be incomplete without some mention of the exciting work of Arad et al. This group have developed a peptide antagonist of SEB, which additionally has cross reactivity with other SAgS (Arad G, Levy R and others, 2000).

The investigators synthesised peptides containing SEB residues 150-161, which are conserved among superantigens, yet are not known to be involved in binding to either to the TCR or MHC class II molecules. In addition they synthesised two variants of this sequence (numbers in parentheses represent amino-acid positions in SEB), pl2(150-161) and pl0(152-161). When present in molar amounts 100- to 200-fold higher than SEB,
none of these peptides had substantial SEB agonist activity, as shown by their inability to induce expression of mRNA encoding for the Th1 cytokines IL-2 and IFN-γ in human peripheral blood mononuclear cells (PBMCs). The dodecapeptide p12(150-161) (YNKKKATVQELD) was a particularly active antagonist, inhibiting expression of IL-2 mRNA by 18-fold and that of IFN-γ mRNA by 10-fold. This peptide is a variant of the natural SEB sequence in pSEB(150-161) (TNKKKVTAQELD).

Furthermore, the investigators went on to test the ability of p12 to protect mice from lethal challenge with various staphylococcal and streptococcal SAggs, including SEB. Although 100% of control mice exposed to a lethal dose of SEB were killed within about 48 hours, all survived lethal challenge with SEB when p12 was administered intravenously 30 minutes before challenge, and 70% survived when it was given intraperitoneally. Mice exposed to p12 alone stayed fully viable and showed no detectable side effects. Moreover, p12 not only was protective when given before SEB challenge but also was able to rescue mice undergoing lethal shock even when injected 3 hours after the toxin.

To determine whether the antagonist peptide provided broad-spectrum protective activity, they next studied its effect during lethal challenge with the streptococcal toxin SPEA (streptococcal pyrogenic exotoxin A), and with TSST-1 (which has only 6% overall sequence homology with SEB). p12 was also protective against these toxins. Moreover, the surviving mice rapidly developed protective antibodies against SAg that rendered them resistant to further lethal challenges, even with different superantigens.
Thus, the lethal effect of superantigens can be blocked with a peptide antagonist that inhibits their action at the beginning of the toxicity cascade, before activation of T cells takes place. It is also interesting to note that this group (from Israel) are now developing the p12 technology to protect against possible germ warfare attacks (Kaempfer, Arad and others, 2002).
1.3.2.2 Superantigens and vasculitis

SAgs are one of the environmental factors that have been proposed to modulate a number of autoimmune diseases, including vasculitis. The most compelling evidence for involvement of SAg in the pathogenesis of vasculitis relates to KD, but at the same time this hypothesis has provided the most controversy (Barron, 2002). This section will review the evidence for and against SAgs in the pathogenesis of KD, and other vasculitides.

Superantigens and Kawasaki disease

There are striking similarities between the clinical and immunological features of KD and the superantigen toxin mediated staphylococcal and streptococcal toxic shock syndrome (TSS) and scarlet fever (Curtis, Zheng and others, 1995; Leung, Meissner and others, 1995a; Leung, Giorno and others, 1995; Leung, Meissner and others, 1995b; Leung, Meissner and others, 1995c). The difficulty in distinguishing these diseases has been extensively documented (Hansen, 1983; Raimer, Tschen, and Walker, 1981; Hall, Hoyt and others, 1999), and the exclusion of staphylococcal disease has been one of the criteria for the diagnosis of KD. The similarities between these diseases led to the suggestion that KD is also caused by a bacterial superantigen toxin (Furukawa, Matsubara, and Yabuta, 1991; Levin, Tizard, and Dillon, 1991).

The epidemiology of KD is characteristic of an infectious disease (see section 1.1.3.3) and suggest that KD is caused by an infectious agent to which everyone is exposed and to which most acquire immunity in childhood. Those individuals who succumb to KD may
be genetically predisposed. Despite extensive research into different possible infectious
aetiological agents, none have been conclusively proven to be the cause of KD.

Bacteria producing superantigen toxins are ubiquitous amongst the commensal flora of
the respiratory and gastrointestinal tract (Lindberg, Nowrouzian and others, 2000).
Despite the frequency with which individuals carry these organisms, disease caused by
these commensals is rare. Pre-existing immunity is believed to be a major factor in
determining whether such toxin-producing bacteria cause disease, although other host
and bacterial factors are likely to be involved. These are likely to include SAg dose (high
doses causing TSS, lower doses causing vasculitis in experimental animals (Leung DYM,
Meissner HC and others, 1995); age of the patient - in the rabbit model shock occurs in
mature rabbits, whereas immature rabbits less than 6 months old are more resistant to
shock induced by TSST-1 (Leung DYM, Meissner HC and others, 1995); and perhaps
other poorly-defined immunological and/or genetic factors (Leung DYM, Meissner HC
and others, 1995).

Infiltration of activated T cells expressing HLA-DR in skin biopsies and coronary
vascular lesions at autopsy has been reported (Terai, Kohno and others, 1990). However,
there are conflicting reports regarding whether peripheral blood T cells are activated in
acute KD, as some reports have provided evidence of peripheral blood T cell activation
(Leung, Chu and others, 1983; Barron, DeCunto and others, 1988), whereas other reports
have suggested that there is only a low level of activation of peripheral blood T cells
during acute KD (Furukawa, Matsubara, and Yabuta, 1992).
Perhaps some of the inconsistency relating to studies of T cell activation in peripheral blood in KD relates to the fact that this compartment may not be the optimum site to sample, with higher sensitivity possibly associated with sampling lesional tissue. Another explanation will undoubtedly relate to the timing of the blood sample in relation to disease onset (Leung, Meissner C, and Schlievert, 1997). Nonetheless, these data do indicate an important role for T cells in KD.

The study by Abe et al in 1992 was the first to describe selective expansion of V\(\beta\)2 and V\(\beta\)8.1 T cells in KD (Abe, Kotzin and others, 1992), indicating T cell V\(\beta\) skewing-the hallmark of a SAg-mediated process. Since then, many similar studies have examined T cell V\(\beta\) repertoires in KD using a variety of techniques (mainly flow cytometry or semi-quantitative polymerase chain reaction), or examined the prevalence of serological conversion or colonization with SAg-producing organisms. These studies are summarised in table 1.4: the top half of the table (in white) are studies which support the SAg hypothesis; the studies in the bottom half of the table (in grey) refute the SAg theory.

Clearly the jury is still out regarding whether or not SAg are involved in the aetio-pathogenesis of KD.
Table 1.4: Studies for and against the SAg theory in KD

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>Controls</th>
<th>FACs study and Vβ Abs used</th>
<th>PCR study</th>
<th>Main findings in KD patients</th>
<th>Other comments and microbiological/histological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abe, Kotzin, et al. 1992</td>
<td>19</td>
<td>12 healthy adults; 8 healthy children; 10 febrile control children</td>
<td>5.1, 5.2/5.3, 6.7, 8.1/8.2, 12</td>
<td>Semi-quantitative PCR of 22 Vβ families</td>
<td>Increased mean Vβ2 &amp; Vβ 8.1</td>
<td></td>
</tr>
<tr>
<td>Abe, Kotzin, et al. 1993</td>
<td>23</td>
<td>13 healthy children; 8 healthy adults</td>
<td>2, 5.1, 8.1/8.2, 12</td>
<td>CDR3 regions sequenced, but not divided into CD4 or CD8 subsets</td>
<td>Increased mean Vβ2 and 8.1: extensive CDR3 junctional diversity i.e. polyclonal Vβ family expansion</td>
<td></td>
</tr>
<tr>
<td>Leung, Meissner, et al. 1993</td>
<td>16</td>
<td>15 healthy children</td>
<td>no</td>
<td>no</td>
<td>SAg-producing bacteria isolated from 13/16 KD patients, but only 1/15 controls. TSST-1 isolated from 11/13 toxin positive patients; SPEB &amp; SPEC were cultured from the other 2 patients</td>
<td>Sites cultured were throat, rectum, axilla, and groin</td>
</tr>
<tr>
<td>Curtis, Zheng, et al. 1995</td>
<td>21</td>
<td>22 healthy adults; 28 febrile control children</td>
<td>2.5,8,12,19</td>
<td>no</td>
<td>Increased mean Vβ2 overall; 2 patients had significantly lower Vβ2; did not confirm increased Vβ8.1 suggested by Abe</td>
<td></td>
</tr>
<tr>
<td>Leung, Giorno, et al. 1995</td>
<td>1</td>
<td>nil</td>
<td>1,5,1,8,1</td>
<td>Examined CDR3 in Vβ2 T cells</td>
<td>Increased Vβ2 in peripheral CD4 &amp; CD8 T cells; found to be polyclonal expansion based on CDR3 diversity; increased Vβ2 T cells infiltrating myocardium and coronary artery</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Patients</td>
<td>Controls</td>
<td>FACs study and Vβ Abs used</td>
<td>PCR study</td>
<td>Main findings in KD patients</td>
<td>Other comments and microbiological/histological findings</td>
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</tr>
<tr>
<td>Yamashiro et al 1996</td>
<td>12</td>
<td>8 children with food intolerance</td>
<td>2,5a, 5b, 5c, 6a, 8a, 12a</td>
<td>no</td>
<td>No skewing of peripheral blood T cell Vβ repertoire</td>
<td>Increased infiltration of Vβ2 T cells in the small intestine but not the jejunum</td>
</tr>
<tr>
<td>Leung, Sullivan, et al. 1997</td>
<td>3</td>
<td>nil</td>
<td>2, 8, 1, 12</td>
<td>no</td>
<td>Serial measurement of Vβ repertoire: increased Vβ2 in 2 patients which returned to normal in convalescence; low Vβ2 in one patient; all had coronary artery lesions</td>
<td></td>
</tr>
<tr>
<td>Masuda, Takei, et al. 1998</td>
<td>45</td>
<td>24 healthy children; 13 disease control children</td>
<td>no</td>
<td>no</td>
<td>Investigated peripheral T cell responses to SAg; found T cell anergy (lower IL2 production and proliferation) in KD in response to SPEC, but SPEA or TSST-1. Did not examine for evidence of infection with SPEC producing organisms. The T cell response normalised within 1 year.</td>
<td></td>
</tr>
<tr>
<td>Nomura, Masuda, et al. 1998</td>
<td>10</td>
<td>10 healthy children</td>
<td>no</td>
<td>Reverse transcription polymerase chain reaction</td>
<td>25 Vβ families analysed: Deletion of Vβ9 and Vβ15 observed</td>
<td>Authors suggest a relationship between deletions observed and KD</td>
</tr>
<tr>
<td>Yoshioka T et al 1999(Yoshioka, Matsutani, et al. 1999-42 /id)</td>
<td>22</td>
<td>10 healthy adults; 14 febrile control children</td>
<td>no</td>
<td>Adaptor ligation polymerase chain reaction</td>
<td>Mean peripheral Vβ2 or Vβ6.5 T cells in acute KD higher than in the convalescent phase. This expansion was polyclonal because CDR3 DNA sequences were different</td>
<td>Serum antibodies to SPEC were higher in KD patients than control groups</td>
</tr>
<tr>
<td>Burgner et al 2001 (Burgner, Curtis, et al. 2001 6327 /id)</td>
<td>87</td>
<td>57 healthy children</td>
<td>no</td>
<td></td>
<td>Similar nasal S. aureus colonisation rates (25%) between KD patients and controls</td>
<td>29% of isolates from KD patients were mitogenic compared with 7% of controls</td>
</tr>
</tbody>
</table>
Table 1.4: Studies for and against the SAg theory in KD

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>Controls</th>
<th>FACs study and Vβ Abs used</th>
<th>PCR study</th>
<th>Main findings in KD patients</th>
<th>Other comments and microbiological/histological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nomura Y et al 2001</td>
<td>15</td>
<td>22 age matched infants; 40 healthy adults</td>
<td>no</td>
<td>no</td>
<td>Higher TSST-1 and SEB antibody titres in KD</td>
<td>Mothers of KD patients had lower antibody titres to TSST-1 than their affected offspring. Authors conclude that that maternal antibody for TSST-1 may protect from KD</td>
</tr>
<tr>
<td>Suzuki H et al 2001</td>
<td>54</td>
<td>nil</td>
<td>Vβ2 and Vβ6.5; rest not stated</td>
<td>Adaptor-ligation polymerase chain reaction</td>
<td>74% of patients showed polyclonal expansion of Vβ2 and/or Vβ6.5 T cells</td>
<td>22/35 paired KD samples showed higher IgM to SPEC than in convalescent phase</td>
</tr>
<tr>
<td>Pietra et al 1994</td>
<td>28</td>
<td>12 healthy children</td>
<td>2, 5, 8.1, 8.2, 12</td>
<td>No</td>
<td>No Vβ expansions detected, nor changes in HLA-DR, IL2-R, but increased CD45RO in CD8 population</td>
<td></td>
</tr>
<tr>
<td>Sakaguchi et al 1995</td>
<td>20</td>
<td>18 healthy children; 20 healthy adults</td>
<td>2, 8.1</td>
<td>No</td>
<td>No Vβ expansions or deletions detected</td>
<td></td>
</tr>
<tr>
<td>Marchette NJ 1995</td>
<td>150</td>
<td>96 healthy children; 129 healthy adults</td>
<td>no</td>
<td>No</td>
<td>No serological evidence of TSST-1 in KD patients</td>
<td></td>
</tr>
<tr>
<td>Tristani-Firouzi et al 1995</td>
<td>7</td>
<td>3 healthy children; 14 healthy adults</td>
<td>no</td>
<td>Reverse transcriptase polymerase chain reaction</td>
<td>No selective expansion of Vβ2 or Vβ8.1</td>
<td>Immunoglobulin production in response to polyclonal stimulants was suppressed in the acute phase of KD, and normalised in convalescence (?evidence of TH1 response)</td>
</tr>
<tr>
<td>Abe et al 1995</td>
<td>20</td>
<td>20 febrile controls</td>
<td>no</td>
<td>Semi-quantitative PCR</td>
<td>Could not confirm that TSST-1 is specifically associated with KD, or that the changes in the Vβ repertoire are due to the SAGs isolated from KD patients</td>
<td>Authors postulate that the changes in Vβ2 occur via an undefined immunological process, not related to SAg</td>
</tr>
<tr>
<td>Study</td>
<td>Patients</td>
<td>Controls</td>
<td>FACs study and Vβ Abs used</td>
<td>PCR study</td>
<td>Main findings in KD patients</td>
<td>Other comments and microbiological/histological findings</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------</td>
<td>-----------------------------------</td>
<td>-----------------------------</td>
<td>-----------</td>
<td>-------------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>Nishiyori A et al 1995</td>
<td>20</td>
<td>18 healthy children; 20 healthy adults</td>
<td>2, 8.1</td>
<td>no</td>
<td>No expansion of Vβ2 or Vβ8.1; No increase in antibodies to TSST-1 in KD (acute or convalescent)</td>
<td>Authors conclude that the pathological or aetiological role of a new TSST-1 secreting S. aureus clone in patients with KD was not confirmed. Group A Streptococci could not be isolated from either KD patients or controls.</td>
</tr>
<tr>
<td>Todome et al 1995</td>
<td>127</td>
<td>17 disease control children; 6 healthy controls</td>
<td>no</td>
<td>no</td>
<td>No noticeable differences between S. aureus strains from KD patients and control children in the production of staphylococcal exotoxins A-E</td>
<td>In KD patients IgG seroconversion rates to TSST-1, SEA, SEB, and SEC were 10%, 15%, 21% and 16%, respectively.</td>
</tr>
<tr>
<td>Terai et al 1995</td>
<td>26</td>
<td>22 healthy children</td>
<td>no</td>
<td>no</td>
<td>Culture supernatants of bacterial isolates from KD patients did not support involvement of SAg-producing bacteria</td>
<td>Found no evidence of an S. aureus strain or TSST-1 sequence uniquely associated with KD</td>
</tr>
<tr>
<td>Deresiewicz et al 1996</td>
<td>6</td>
<td>nil</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.4: Studies for and against the SAg theory in KD

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>Controls</th>
<th>FACs study and V(\beta) Abs used</th>
<th>PCR study</th>
<th>Main findings in KD patients</th>
<th>Other comments and microbiological/histological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morita et al 1997 (Morita, Imada, et al. 1997 716 /id)</td>
<td>50</td>
<td>nil</td>
<td>no</td>
<td>no</td>
<td>found a very low frequency of detection of anti-superantigen antibodies by ELISA and no marked IgG seroconversion to several streptococcal SAgS, indicating the absence of a serological relationship between toxin-producing streptococcal infection and the onset of KD</td>
<td></td>
</tr>
<tr>
<td>Choi et al 1997 (Choi, Chwae, et al. 1997 657 /id)</td>
<td>26</td>
<td>13 children with seizures or pre-operative admissions</td>
<td>2,8</td>
<td>2 stage PCR to estimate the CDR3 size profile among T cells expressing V(\beta)1, 2, 4, 5, 8, 14, 16, 17, 18, and 20 chains. Several clonal expansions were found mainly in the CD8+ T cells that disappeared during the long term follow-up period; only 2 patients had increased T cells expressing V(\beta)8; no difference in V(\beta)2.</td>
<td>Authors suggested that conventional Ags rather than a SAg were involved in the pathogenesis of acute KD</td>
<td></td>
</tr>
<tr>
<td>Mancia L et al 1998 (Mancia, Wahlstrom, et al. 1998 916 /id)</td>
<td>25</td>
<td>24 healthy children</td>
<td>2, 3, 5(\alpha), 5(\alpha)2, 5(\alpha)3, 6(\alpha), 6(\alpha)7(\alpha)8(\alpha)1/8(\alpha)2, 9, 11, 13(\alpha)1, 13(\alpha)5, 14, 16, 17, 18, 20, 21(\alpha)3, 22, 23</td>
<td>no</td>
<td>No abnormal usage of any V(\beta) family found in KD.</td>
<td>Did observe increase IL2R-expressing CD4 T cells</td>
</tr>
</tbody>
</table>
Superantigens and other vasculitides

There are limited data examining this hypothesis, and most of it relates to vasculitides affecting adults.

Simpson *et al* studied T cell Vp repertoires in 28 adults with miscellaneous vasculitides (Simpson, Skinner and others, 1995). These included 12 with MPA, 10 with WG, and 6 with unclassifiable vasculitis. Controls comprised 28 age and sex-matched healthy adults. The majority of the vasculitis patients had inactive disease at the time of sampling, and only 8 patients had active disease. Using a reverse transcriptase PCR technique they analysed Vp mRNA levels for 20 different Vp families. Overall they found increased Vp2.1 mRNA expression in the vasculitis patients, and this was most marked for patients with MPA. Moreover, this expansion seemed to be polyclonal, because there was little conservation of the of the TCR Vp2.1 junctional region. The authors concluded that their data could be compatible with a SAg mediated immunopathogenesis. Interestingly, the increased Vp2.1 usage appeared to be unaffected by disease activity.

Giscombe *et al* studied 11 patients with necrotizing vasculitides (10 with WG; 1 with PAN) (Giscombe, Grunewald and others, 1995). 7 of the patients had active disease. Controls comprised 16-57 healthy adults. This study used monoclonal antibodies and flow cytometry to analyse 10 different Vp families. They found a higher number of T cell Vp expansions within (predominantly) the CD4 population of T cells in the vasculitis group. In long-term studies of the T cell expansions (for up to 18 months), there was no obvious correlation to clinical features such as disease activity or treatment. Moreover,
the expansions were heterogeneous, i.e. did not affect any single Vβ family predominantly. The authors concluded that their observations may, in the future, lead to the identification of unknown antigens, but did not go as far as speculating on a SAg-mediated immunopathogenesis.

Popa et al studied T cell Vβ repertoires by flow cytometry in 27 patients with WG (Popa, Stegeman and others, 1998). 11 had active disease, and 16 inactive disease. Controls comprised 16 healthy adults. They observed Vβ expansions in 21/27 patients, and 4/16 controls. Expansions were present in the CD4 and CD8 T cell populations. Again, the expansions appeared to be heterogeneous, although the authors did not state which individual Vβ families were affected. Interestingly, the senior author of that study (which was only published in abstract form) went on to state in a subsequent review that no SAg-related Vβ expansions could be detected in their WG population (Cohen Tervaert, 2002).

Stegeman et al studied nasal carriage of Staphylococci in 57 patients with WG (Stegeman, Tervaert and others, 1994). Thirty-six of the 57 patients were found to be chronic nasal carriers of S. aureus. Nasal carriers of staphylococcus were nearly 8 times more likely to relapse with their disease. The same group went on to confirm this observation in a further 63 patients with WG, and moreover pin-pointed the risk to those strains of Staphylococcus aureus capable of producing TSST-1 (Popa ER, van der Meer B and others, 2002).
1.3.2.3 Concluding remarks regarding SAgs and vasculitis

There are data suggesting a role for the SAgs in the aetio-pathogenesis of the vasculitides. This is strongest (and most controversial) for KD. It should be borne in mind that SAg versus conventional antigen processes are not mutually exclusive: i.e. both could be important, perhaps at different stages of the disease process. How SAgs could contribute to vascular injury is a different question, and largely a matter of speculation so far. This issue will be considered in chapter 4.
1.4 Aims of this thesis

The aims of this thesis were:

1. To examine the hypothesis that SAgS could be involved in the aetiopathogenesis of vasculitis syndromes of the young.

2. To investigate whether endothelial cells can promote SAg-driven T cell responses.

3. To investigate the consequence of endothelial cell mediated SAg-driven T cell activation.
Chapter 2: Materials and methods

2.1 Introduction

2.2 Subjects, classification of vasculitic syndromes, and assessment of disease activity

2.3 Materials

2.4-2.6 Methods
2.1 Introduction

This section contains predominantly materials and methods that are used in more than one chapter. Methods applicable to one chapter only, or those that have been developed or adapted specifically for this study will be discussed in detail in the appropriate section.

2.2 Subjects, classification of vasculitic syndromes, and assessment of disease activity

2.2.1 Subjects and classification of vasculitic syndromes

A detailed description of patients is made in the relevant chapters, but some comment regarding the classification of vasculitic syndromes used in this thesis is required. The main classification system used in chapters 3 and 5 was that described by the Chapel Hill consensus (Jennette, Falk and others, 1994), although in chapter 3 children were classified using both the Chapel Hill criteria and the American College of Rheumatology (ACR) classification criteria (Hunder, Arend and others, 1990). Children were classified using both systems because there is controversy over the most reliable classification system for the childhood vasculitides (Hunder G, 1998; Ozen, Besbas and others, 1992; Petty RE and Cassidy JT, 2001a; Besbas, Ozen and others, 2000) and there is a considerable degree of "polyangiitis overlap" in the childhood vasculitides (Brogan and Dillon, 2000a; Brogan, Davies and others, 2002; Leavitt RY and Fauci, 1986) such that neither system is absolutely sensitive or specific (Jennette and Falk, 2000). Moreover, neither system has been formally validated in children (Petty RE and Cassidy JT, 2001a). Classification of vasculitic syndrome is particularly important when considering aetio-
pathogenesis, however, and hence in Chapter 3 (which examines a possible aetio-pathogenetic mechanism of systemic vasculitis in childhood) both systems were used to allow comparison with previously published data, which may have used either system. Since Chapter 5 examines a marker for endothelial injury (which occurs in all vasculitis syndromes irrespective of classification) only the Chapel Hill criteria were used. It should be emphasised, however, that neither of these systems comprehensively address vasculitis in childhood, and are thus of limited usefulness. The Chapel Hill and ACR criteria are shown in Appendix 1.

2.2.2 The assessment of vasculitic disease activity

The assessment of vasculitic disease activity was performed using the Birmingham Vasculitis Activity Score (BVAS) (Luqmani, Bacon and others, 1994). This is a tool which can provide uniform assessment of current disease activity in groups of patients with different forms of vasculitis, and is not specifically designed for any single vasculitic syndrome. It thus has obvious advantages when considering patients with different vasculitic syndromes, or overlapping features of different vasculitic syndromes. The BVAS is weighted towards objective and categorical or biochemical evidence of active vasculitis. Furthermore, objective evidence of organ involvement (e.g. gut or kidneys) is weighted more heavily than symptoms that have a subjective element (e.g. myalgia, arthralgia). Laboratory markers have attractions but also present problems- they may be affected by chronic changes such as hypertension, or by concurrent infection. Anti-neutrophil cytoplasmic antibody (ANCA) titres may be able to predict relapses of
disease (Savage, 2001), but many patients are ANCA-negative (especially children) (Brogan and Dillon, 2000a; Brogan, Davies and others, 2002).

In the BVAS scoring system, only recent (i.e. within the previous month) activity is measured. The observer must only record the presence of an abnormality if has occurred afresh or deteriorated within the previous month and it is concluded that the abnormality is caused by active vasculitis. If a particular statement is recorded as being true, the score for that statement contributes towards the value for its relevant section. The score is calculated as the total sum of the values obtained from each of the nine sections. Each section can only contribute a maximum score as indicated.

The main limitation of the BVAS is that it has never been formally validated in children. Moreover, although it is designed to score different vasculitis syndromes, KD was not included in the original series of patients in which the BVAS was validated. Nonetheless, with this limitation taken into account, the criteria within the BVAS are entirely appropriate and relevant to the paediatric vasculitides (including KD) with the possible exception of the laboratory reference ranges quoted (which are adult ranges). The BVAS proforma is shown in Appendix 2.
2.3 Materials

2.3.1: Fluorochrome-conjugated antibodies for flow cytometry

The fluorochrome-conjugated monoclonal antibodies used are given in table 2.1. All antibody dilutions were made with 0.01 M phosphate buffered saline with 0.1% sodium azide, and checked by plotting a dilution curve against median fluorescence index, the relevant dilution for each antibody corresponding to the shoulder of the curve. Each antibody was checked against an appropriate isotype-control antibody with the same protein concentration, as per manufacturer recommendation.
Table 2.1: Fluorochrome-conjugated antibodies for flow cytometry.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Isotype</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Mouse IgG1</td>
<td>Phycoerythrin (PE)</td>
<td>SK7</td>
<td>1:20</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>CD4</td>
<td>Mouse IgG1</td>
<td>Quantum Red™ (QR)</td>
<td>Q4120</td>
<td>1:20</td>
<td>Sigma</td>
</tr>
<tr>
<td>CD8</td>
<td>Mouse IgG2a</td>
<td>QR</td>
<td>UCHT-4</td>
<td>1:20</td>
<td>Sigma</td>
</tr>
<tr>
<td>Vβ1</td>
<td>Rat IgG1</td>
<td>FITC</td>
<td>BL37.2</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ2</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>MPB2D5</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ3</td>
<td>Mouse IgM</td>
<td>FITC</td>
<td>CH92</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ5.1</td>
<td>Mouse IgG2a</td>
<td>FITC</td>
<td>IMMU157</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ5.2</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>36213</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ7</td>
<td>Mouse IgG2a</td>
<td>FITC</td>
<td>ZOE</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ8.1/8.2</td>
<td>Mouse IgG2a</td>
<td>FITC</td>
<td>56C5</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ11</td>
<td>Mouse IgG2a</td>
<td>FITC</td>
<td>C21</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ12</td>
<td>Mouse IgG2a</td>
<td>FITC</td>
<td>VER2.32.1</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ13.1</td>
<td>Mouse IgG2b</td>
<td>FITC</td>
<td>IMMU222</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ13.6</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>JU-74</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ14</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>CAS 1.1.3</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ16</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>TAMAYA</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ17</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>E17.5F3</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ20</td>
<td>Mouse IgG</td>
<td>FITC</td>
<td>ELL 1.4</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Vβ21.3</td>
<td>Mouse IgG2a</td>
<td>FITC</td>
<td>IG125</td>
<td>1:10</td>
<td>Immunotech</td>
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<td>Vβ22</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>IMMU 546</td>
<td>1:10</td>
<td>Immunotech</td>
</tr>
<tr>
<td>CD69</td>
<td>Mouse IgG2a</td>
<td>PE</td>
<td>CH/4</td>
<td>1:20</td>
<td>Insight Biotechnology Ltd</td>
</tr>
<tr>
<td>CD25</td>
<td>Mouse IgG1 heavy chain and κ light chain</td>
<td>FITC</td>
<td>Tu39</td>
<td>1:20</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>HLA-DR, DP, DQ</td>
<td>Mouse IgG2a κ</td>
<td>Cy-chrome</td>
<td>HA58</td>
<td>1:20</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD54</td>
<td>Mouse IgG1 κ</td>
<td>PE</td>
<td>51-10C9</td>
<td>1:20</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD106</td>
<td>Mouse IgG1 κ</td>
<td>Cy-chrome</td>
<td>68-5H11</td>
<td>1:20</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD62E</td>
<td>Mouse IgG1 κ</td>
<td>PE</td>
<td>AC1.2</td>
<td>1:100</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD62P</td>
<td>Mouse IgG1 κ</td>
<td>PE</td>
<td>SN6</td>
<td>1:15</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD105</td>
<td>Mouse IgG1</td>
<td>PE</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3.2 Cytokines and protein reagents

The following cytokines were used: recombinant TNF-α (Sigma, used at a concentration of 100 ng/ml); recombinant INF-γ (Genzyme, used at a concentration of 1000 U/ml). The following SAGs were used: staphylococcal toxic shock toxin type 1 (TSST-1; Sigma, used at a concentration of 10 ng/ml); staphylococcal enterotoxin type B (SEB; Sigma, used at a concentration of 100 ng/ml). The following fluorochrome-conjugated proteins were used: annexin-V (FITC; Annexin V-FITC kit, Bender MedSystems, 1:100 dilution); Ulex europaeus I Agglutinin (UEA-I FITC; ICN Biomedicals, 1:20 dilution).

2.3.3 Tissue culture media

The following tissue culture media were used: RPMI-1640 culture medium containing 2 mM L-glutamine (Life Technologies, Gibco BRL) and supplemented with antibiotics (1% penicillin/streptomycin/amphotericin); MCDB-131 with 10 mM L-glutamine (Life Technologies, Gibco BRL) and antibiotics (1% penicillin/streptomycin/amphotericin); foetal calf serum (heat inactivated at 56° C for 60 minutes to destroy complement).

2.4 Methods

2.4.1: Isolation of peripheral blood mononuclear cells and T cells from whole blood

5-40 ml of blood from a single healthy adult donor was collected into sterile 20ml Universal bottles containing 40 microlitres of preservative-free heparin (Monoparin, CP Pharmaceuticals Ltd, 1000 U/ml). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Lymphoprep™ (Nycomed) centrifugation as follows. All
reagents, tubes and the centrifuge were pre-cooled to 4°C to minimise the activation of T
cells. The blood sample was diluted 1:1 with RPMI-1640 medium containing 2 mM L-
glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The diluted sample was
slowly and carefully overlaid, onto an equal volume of lymphocyte separation medium
(Lymphoprep™) in an appropriate number of 15ml conical tubes using a 10ml pipette.
The tubes were centrifuged at 2,000 rpm (1,110 g) with the brake off for 20 minutes at
4°C. A wide tipped Pasteur pipette was used to aspirate PBMCs from the interface into a
fresh 30 ml Universal container containing 10 ml RPMI to wash the cells. To allow
efficient sedimentation of cells, it was ensured that the interphase was diluted at least 3:1.
After further centrifuging at 1500 rpm (500 g) for 10 min at 4°C, the supernatant was
discarded by inversion of the tube and the cell pellet resuspended by gentle ‘flicking’ of
the container. The cells were then washed again in 10 or 20 ml RPMI followed by
centrifugation at 1200 rpm (280 g) for 10 min at 4°C. The cell pellet was resuspended in
5-20 ml of RPMI-1640 medium (supplemented with 10% heat-inactivated FCS, 2 mM L-
glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin), aiming to give a
concentration above that required. PBMC were either used immediately in experiments,
or frozen suspended in FCS with 10% dimethyl sulfoxide (DMSO, Sigma).

2.4.1 Preparation of purified CD3⁺ T lymphocytes from PBMCs

T cells (CD3⁺ lymphocytes) were further purified from the PBMC population by using a
T cell negative isolation kit (Dynal) as per manufacturer recommendations. This system
utilises an antibody mix containing anti-HLA class II DR/DP, anti-CD16 (a and b), anti-
CD56, and anti-CD14 and depletion magnetic beads thus removing B cells, natural killer
(NK) cells, activated T cells, monocytes, and granulocytes (if present). The purified T cell population routinely contained greater than 98% CD3^+ cells, and 0.2% MHC class II (DP/DQ/DR) as determined by flow cytometry (figures 2.1 and 2.2).

2.4.2 Calculation of cell number and cell viability

10 μl of resuspended cells were removed and mixed with 10 μl of trypan blue to differentiate dead from live cells. The number of cells was counted using a Neubauer cell haemocytometer. The cell viability (%) was calculated as 100x (live cells)/ total cells (live and dead). The resuspended cells were then diluted further, based on the live cell count, to give the desired concentration (usually 1 x 10^6 /ml).

2.4.3 Storage of cells

Unless cells were required fresh for immediate use, they were frozen at -70°C. Cells for freezing were aliquoted in 2 ml flat microtubes (Sarstedt) at a concentration of 10^7
Figure 2.1: Purity of CD3 lymphocyte population after T cell negative isolation using magnetic beads
Figure 2.2: Percentage of contamination MHC Class II positive cells in the purified CD3 T cell population

0.32%

0.8%
PBMCs per 1.0 ml in FCS containing 10% DMSO. The cells were cooled stepwise to prevent formation of ice crystals (4°C for 30 minutes, -20°C for 30 minutes, then to -70°C). Cells for storage for periods greater than one month were stored in 2 ml cryogenic vials (Nalgene) and transferred to liquid nitrogen after initial freezing to -70°C.

2.4.4 Flow cytometry of PBMC or T cells

3-colour FACS analysis of PBMCs or T cells from experiments was performed as follows. Cells were plated onto U-bottomed 96-well plates at a concentration of $1 \times 10^6$ cells per ml, and incubated for 30 minutes at 4°C using fluorochrome-conjugated monoclonal antibodies to CD4 (Quantum Red™), or CD8 (Quantum Red™), CD69 (phycoerythrin), and one of the 17 FITC-conjugated T cell Vβ families antibodies. Following incubation for 30 minutes, cells were washed 3 times with 0.01 M phosphate buffered saline with 0.1% sodium azide, then fixed in buffer containing 10% formaldehyde and 1% azide (CellFIX, Becton Dickinson). Flow cytometry was performed on a FACScalibur flow cytometer (Becton Dickinson) using CellQuest software with optimal compensation set for green, orange, and far-red fluorescence. T cells were identified by gating for forward and light-scatter characteristics, CD4+ or CD8+ populations, and the different Vβ families. Percentages of T cells (CD3+CD4+ or CD3+CD8+) expressing different Vβ gene products were subsequently calculated using quadrants set on dot-plots, with markers for positivity defined using isotype-control antibodies. 20-40 thousand gated events were stored for each Vβ family analysis. Vβ-specific activation was determined in some experiments by gating on the individual Vβ family sub-population (CD4+ or CD8+ and Vβ double-positives) and then plotting a
histogram of CD69 fluorescence, with determination of the CD69 median fluorescence index (MFI) for each Vβ family in the CD4^ and CD8^ sub-population of T cells. The plate plans for determination of the Vβ repertoire are shown in Appendix 3, and for determination of Vβ-specific CD69 expression in Appendix 4. Flow cytometry instrument settings for PBMCs/T cells are given in Appendix 5.

2.4.5 In vitro technique for validation of effect of SAgs on T cell Vβ repertoire skewing and T cell activation

The flow cytometry technique and antibodies were validated by examining the changes in PBMCs from 2 healthy adults in response to 8 days incubation with the SAgs TSST-1 or SEB. The technique used was the same as that described by Curtis et al (Curtis, Zheng and others, 1995). PBMCs from a healthy adult donor were cultured at a density of 2 x 10^6/ml in RPMI medium (Gibco) and 10% fetal calf serum (heat inactivated) in the presence or absence of 10 μg/ml anti-CD3, 10 ng/ml TSST-1, or 10 ng/ml SEB. The cells were cultured at 37°C and were stimulated with 10 units/ml of recombinant human interleukin-2 (IL-2) (Recombinant Human IL-2; R &D Systems) on days 3 and 7. The cells were then harvested on day 8 and the percentage of each of the Vβ families within the CD4 and CD8 populations, and the expression of T cell activation markers was analysed as described above.

Stimulation of PBMCs from healthy adults (n=2) with TSST-1 after 8 days resulted in an increased percentage of T cells expressing Vβ2 as compared with control (resting PBMC
i.e. cultured but unstimulated for 8 days). This was true of both the CD4 and CD8 populations of T cells (figure 2.3a and 2.3b). In contrast, stimulation with SEB resulted in an increased percentage of T cells expressing Vβ3; again this was true of both the CD4 and CD8 T cell populations (figure 2.4a and 2.4b). There was also a decrease in Vβ2 observed in response to SEB within the CD4 T cell population, presumably reflecting true “skewing” of the Vβ repertoire (figure 2.4a). In addition, incubation of PBMC with SAg (TSST-1 or SEB) resulted in T cell activation as determined by upregulation of CD69 and CD25. This was true for both CD4 and CD8 populations of T lymphocytes (figure 2.5a-d).
Figure 2.3: Stimulation of healthy adult (n=3) PBMC with TSST-1

Figure 2.3a: CD4 Vb response to TSST-1 (mean, SEM)

Figure 2.3b: CD8 Vb response to TSST-1 (mean, SEM)
Figure 2.4: Stimulation of healthy adult (n=3) PBMC with SEB

Figure 2.4a: CD4 Vb response to SEB (mean, SEM)

Figure 2.4b: CD8 V-beta repertoire

Figure 2.4a: CD4 Vb response to SEB (mean, SEM)
Figure 2.5: T cell activation in response to Sags (mean CD69 MFI, SEM of 3 experiments)

**Figure 2.5a: CD4CD69**

- **Figure 2.5b: CD8CD69**

- **Figure 2.5c: CD4CD25**

- **Figure 2.5d: CD8CD25**
2.4.6 Endothelial cell culture

Endothelial cells can be isolated and cultured from a wide variety of sources, including different anatomical sites and different species. Each type requires specific techniques for isolation and growth. They may require various growth factors and special media. In general, endothelial cells require media which are rich in amino acids and various sugar moieties, and also require high quality FCS at concentrations up to 20%.

Human umbilical vein cells (HUVEC) are an ideal source of endothelial cells since they are derived from a plentiful and renewable resource that would otherwise be discarded. The basic method employed is as described in 1973 (Jaffe, Nachman and others, 1973).

2.4.7 HUVEC culture media

The media used are listed in section 2.3.3. RPMI 1640 medium, containing 10mM L-glutamine, 80μg gentamicin, 100 units penicillin/streptomycin was used for collection and storage of umbilical cords, and kept at 4° C in autoclaved polypropylene bottles. Washing of umbilical cords was performed with RPMI with same supplements as above but containing 5% FCS (HUVEC wash medium). Collagenase type II solution (0.1%) comprised of 1g collagenase dissolved in 1L of RPMI-1640 which was then passed through a 0.2 micron tissue culture filter and stored in aliquots at -20° C until required. Primary and secondary cultures used a specialised media for growth of endothelial cells: MCDB-131, supplemented with 10mM L-glutamine, 100 U/ml penicillin/streptomycin, amphotericin B, and 20% FCS.
2.4.8 Foetal calf serum

The quality and batch of FCS was found to be a critical determinant of yield of HUVEC from either primary culture or growth in secondary cultures. For this reason, batches of FCS were tested for quality. Specific batches, which proved to be most effective at sustaining growth of HUVEC, were ordered in large quantities and were used solely for HUVEC culture. All FCS was heat inactivated at 56° C for 60 minutes and stored at -20° C in aliquots until required.

2.4.9 Protocol for isolation of HUVEC

Only fresh, intact cords were used, always within 48 hours of collection (and usually within 24 hours of collection). Cords that were heavily meconium stained were discarded. All the procedures described below were performed in a Class II safety cabinet using aseptic technique. Autoclaved glassware and metal instruments were used. Culture and wash media, and enzymes were pre-warmed to 37° C in a water bath.

1. The cords were inspected for damage such as cuts, or needle punctures from cord blood sampling. Suitable cords were then sprayed with 70% IMS, and blood expressed into a collection pot. Washed cords were then placed in a sterile Duran bottle containing RPMI-1640 medium with antibiotics as described above.
2. Single cords were then cut at least 1 cm from both ends. One end was clamped with an artery forceps. The other end was then inspected and the vein identified. The vein was dilated with blunt forceps, and cannulated with a sterile plastic filling tube (Kwill). The filling tube was secured with sterile suture and finally a small artery forceps.

3. The vein was then infused with warmed RPMI-1640 medium with 5% FCS (HUVEC wash medium). Sites of leakage were clamped as necessary.

4. This process was repeated for a maximum of three cords at one time.

5. The vein was flushed with wash medium to remove excess blood, and then filled with 0.1% Collagenase II solution.

6. The cord was then incubated at 37° C in 5% C02 for 15 minutes.

7. After incubation, the digest was removed from the cord into sterile Duran bottles, and flushed through with equal volume of wash medium. This was transferred to a sterile, 50ml conical tube.

The digest was centrifuged at 200g for 7 minutes at room temperature. The supernatant was discarded, and the pellet resuspended in MCDB-131 medium with 10 mM L-glutamine, 20 % FCS and antibiotics (HUVEC culture medium).

2.4.10 HUVEC primary culture and sub-culture

The digest was transferred to tissue culture flasks. These were always surface modified, polystyrene flasks designed for culture of adherent cells. 25 or 75 cm² flasks were used depending on requirements. In general, digest from a large cord (enough to take 20 to 30
ml of collagenase solution) was resuspended in 10 ml of culture medium and transferred to 25 cm² flask. These were incubated at 37°C and 5% CO₂. Cells were washed in fresh culture media the following day and inspected under phase contrast microscopy.

HUVEC could be identified as small clusters of oval adherent cells. Cultures were inspected each day for growth. When cells were approaching confluence (which usually occurred after 72 hours culture), they were sub-cultured. Cells that grew poorly, or were contaminated with smooth muscle or fibroblastic cell overgrowth were discarded.

HUVEC that were near-confluent were then washed three times in warmed PBS to remove non-adherent cells and protein in FCS. Cells were washed once in 0.5 ml Trypsin-EDTA solution (per 25-cm² flask) which was then removed and a further 0.5-ml Trypsin-EDTA solution added. Cells were inspected under phase-contrast microscopy, and when rounding and becoming dislodged (usually after 30 seconds), the flask was tapped against the bench top to aid removal. The cells were quickly resuspended in warmed HUVEC culture medium and transferred to a 50-ml sterile conical tube. These were then seeded into 48 or 24 well flat-bottom tissue culture plates. Tissue culture plates were then incubated at 37°C in 5% CO₂. Details of specific conditions used to grow HUVEC for various experimental procedures are mentioned in the relevant sections in subsequent chapters. HUVEC morphology and phenotype, especially in terms of induction of cell adhesion molecules in response to pro-inflammatory stimuli may alter after serial passages. The cells used throughout this study were therefore always first to
third sub-culture passage. Generally, cells were used within one week of passaging. Medium was replaced every 3 days.

2.4.11 Identification of HUVEC

Endothelial cells were inspected under phase-contrast microscopy prior to use for characteristic cobblestone appearance (figure 2.6). In particular, care was taken to avoid cultures where there was significant overgrowth of smooth muscle-like cells (which are characteristically spindle-shaped and can be seen to arch over endothelial monolayers). Additionally, cells were identified by the characteristic forward and side scatter of HUVEC, and by the binding of high levels of FITC-conjugated UEA-I by flow cytometry, as shown in Figure 2.7.

2.4.12 Protocol for detection of cell adhesion molecule expression by flow cytometry

Single colour FACS analysis of HUVEC was performed as follows. HUVEC from experiments were re-suspended in 0.01 M phosphate buffered saline with 0.1% sodium azide and 5% FCS, and plated onto U-bottomed 96-well plates. The cells were then incubated for 30 minutes with fluorochrome-conjugated antibodies to MHC class II, ICAM-1 (CD54), VCAM-1 (CD106), E-selectin (CD62E), or P-selectin (CD62P). Following 3 washes (with PBS supplemented with 5% FCS; centrifuged at 200g for 5
Figure 2.6: Phase contrast light microscopy of resting HUVEC (figure 2.6a) and HUVEC following stimulation with 1000U/ml of INF-γ for 48 hours (figure 2.6b)
Figure 2.7: Forward and side scatter characteristics of HUVEC, and characterization by binding of UEA-I-FITC

- Control
- UEA-I FITC: 99%
minutes and the supernatant discarded by flicking and blotting the plate), FACS analysis was performed immediately by gating on the typical forward and light scatter characteristics of HUVEC, and histograms plotted for each antibody to determine the median fluorescence index. In addition, percentage positivity for each antibody was determined using isotype control antibodies with a cut-off for positivity at 2%. Endothelial cells were analysed on a FACScalibur flow cytometer using CellQuest software. 5000 events within endothelial gate were collected. Flow cytometer instrument settings for endothelial cells are given in Appendix 5.

2.4.13 Assay for endotoxin contamination of HUVEC

When considering experiments involving the activation of HUVEC, an important consideration (as with all tissue culture) is the possibility of endotoxin contamination. Certainly this was a complication that was encountered when the initial HUVEC culture and sub-culture was being set up. The offending agent was found to be a batch of endothelial cell attachment factor (ECAF) which was used in early tissue culture experiments, but eventually abandoned because of this risk.

To assess for the possibility of endotoxin contamination, a timed-gel formation endotoxin kit was utilised (Sigma; product number TGF-1). This kit is designed for the rapid determination of endotoxin levels in foetal bovine serum, but can be used for any tissue culture reagent. This kit provides semi-quantitative results and as such should not be used to determine exact endotoxin levels. The Timed Gel Formation test is based on the observation that higher levels of endotoxin will shorten the incubation time required for
gel formation. The test relies on predetermined gel formation times for samples containing known amounts of endotoxin as determined by the LAL gel clot method and is based on Control Standard Endotoxin.

**Test Protocol:** Contact/contamination of the dilution tubes was avoided.

1. Labelled one baked dilution tube and two LAL single test vials for each sample to be tested. Placed LAL vials in ice bath until ready for incubation.
2. Dispensed 1 ml of endotoxin-free water into each dilution tube.
3. Added 0.5 ml of test sample to the labelled dilution tube containing endotoxin-free water.
4. Vortexed and placed tubes in boiling water for 5 minutes. Exercised care to avoid contamination of tubes.
5. Removed tubes from boiling water and allowed heated samples to cool to touch [30°C]. Dispensed 0.2 ml of cooled test sample through septum cap into labelled LAL vials using 0.5 ml syringe. Swirled gently to dissolve LAL completely. Care taken not to agitate vials vigorously.
6. Placed vials upright in 37°C water bath and began timing.
7. Incubated the test samples for the time period indicated on the Endotoxin Standard Chart or Curve (see product information), sufficient to detect the expected endotoxin level. (Comment: care should be taken not to disturb vials during the incubation period since this may interfere with formation of the gel resulting in a false negative. Once removed from the water bath, a sample should not be returned to incubation).
8. At the indicated time, gently removed vials from water bath and slowly inverted 180°. A solid gel that did not slide down the side of the vial was a positive result. A liquid, cloudy or soft gel, or a gel that slid down the vial when inverted was considered a negative result.

9. A positive result indicated an endotoxin concentration greater than or equal to the indicated level on the Endotoxin Standard Chart or Curve.

Possible sources of error included:

1. Incomplete solubilization at start of test.
2. Mixing too vigorously.
3. Disturbing the vial during incubation.
4. Disturbing a formed gel during inversion of the LAL vial at the end of incubation.
5. Incorrect incubation temperature.
6. Incorrect incubation time.
7. Neglecting to boil serum samples (if they are the sample to be tested, to denature proteases which may impair gel formation).
2.5 Isolation and analysis of whole blood cellular and platelet microparticles

2.5.1 Preparation of platelet poor plasma for microparticle analysis

1.4-5 mls of whole blood was collected into bottles containing 3.2% trisodium citrate (Becton Dickinson). Platelet poor plasma was obtained by immediate centrifugation of the whole blood at 5000G for 5 minutes twice. Plasma was then stored at −70° Celcius until use.

2.5.2 Isolation of microparticles from platelet poor plasma

Platelet poor plasma was defrosted in a water bath at 37 ° Celcius. Exact volumes of plasma (400-800 microlitres) were then centrifuged at 17000 G for 60 minutes and the supernatant decanted to obtain the microparticle (MP) pellet. The MP were then reconstituted in 350 microlitres of annexin V buffer (Bender Medsystems), and divided into ten 35-microlitre aliquots plated onto the first 10 wells of a 96 well U-bottomed plate.

2.5.3: Labelling of microparticles with annexin V and monoclonal antibodies

The labelling and quantification of MP was achieved as follows. 5 microlitres of a 1 in 10 dilution of FITC-conjugated annexin V in buffer (Bender Medsystems) was added to every well. In addition, antibodies against platelet or endothelial surface markers conjugated to red (PE) or far-red (PERCP or CYC) fluorochromes were used to
differentiate MP of platelet or endothelial origin. Platelet markers examined were the constitutively expressed platelet marker CD42a (mouse IgG1 anti-human CD42a-PERCP, Becton Dickinson), and the platelet activation marker P-selectin (mouse anti-human CD62P-PE, BD PharMingen). Endothelial surface markers examined were E-selectin (mouse IgG1 anti-human CD62E-CYC, BD PharMingen); CD105 ("endoglin"; mouse anti-human IgG1 CD105-PE, Serotec); ICAM-1 (mouse IgG1 anti-human CD54-CYC, BD PharMingen); and VCAM-1 (mouse IgG1 anti-human CD106-PE, BD PharMingen). 10 microlitres of each antibody (diluted 1 in 3 with RPMI medium with 5% fetal calf serum) was added to individual wells. The final dilution of the annexin V-FITC was 1 in 100, and 1 in 15 for each of the conjugated antibodies. MP were incubated with the labelled antibodies and annexin V for 10 minutes at room temperature with gentle shaking. The incubation was then terminated by adding 200 microlitres of annexin V buffer to each well, and the samples transferred to tubes prior to flow cytometry.

2.5.4: Flow cytometric analysis of microparticles

All analysis was performed on a FACScalibur flow cytometer (Becton Dickinson). To obtain optimal forward and side scatter instrument settings for MP, 0.8 micrometer and 3 micrometer latex beads (Sigma) were run and logarithmic forward and side scatter plots obtained (Combes, Dignat-George and others, 1997). Gates were then set to include particles less than approximately 1.5 micrometers, but to exclude the first forward scatter channel containing maximal noise. Optimal compensation was set for green, red and far-red fluorescence (Appendix 5). Specific binding for each antibody was determined using
isotype control antibodies with equal protein: fluorochrome ratios, and at the same final dilution as per manufacturer recommendation. Since annexin V is a protein and not an antibody (and hence no isotype control antibody exists), the threshold for annexin V binding was determined by using the fluorescence threshold established for MP in the absence of labelled annexin V. Particles less than 1.5 micrometers in size and binding annexin V were then gated, and histograms obtained for this gated population for binding to individual monoclonal antibodies to determine the cell of origin of the MP. The gate was checked by examining MP derived from supernatants taken from monolayers of HUVEC stimulated with 100 ng/ml of TNF-α (Sigma) at 24 hours. MP samples were run at medium flow rate with a cut-off time of 1 minute, which resulted in capture of approximately 5000 gated bead events.

2.5.5 Determination of absolute microparticle number per ml of plasma

To convert flow cytometer counts to an estimate of the number of MP per ml of plasma, a predetermined number (always 200000, calculated as per manufacturer recommendations, Appendix 6) of 3 micrometer latex beads (Sigma) were run concurrently with the microparticle samples. The absolute number of annexin V binding microparticles per ml of plasma was then determined by using the proportion of beads counted and the exact volume of plasma from which the microparticles were analysed, as described by Combes et al in 1997(Combes, Dignat-George and others, 1997). The following equation was thus derived to convert flow cytometer counts to an estimate of the number of MP per ml of plasma (figure 2.8):
Since samples from each individual were run 10 times, microparticle counts from individual subjects were expressed as the mean number per ml of plasma, with standard error of the mean based on 10 measurements. To determine the absolute number of MP derived from different cellular populations (i.e. platelet, or endothelial) the absolute number of total MP derived from the above equation was multiplied by the percentage positivity for that particular marker. A representative set of flow cytometric plots, and the gating protocol are shown in figure 2.9.
Figure 2.9: Flow cytometry gating protocol for microparticles

Annexin V binding microparticles

3μm latex beads

Gate

Control Antibody
E selectin Antibody

24% E selectin + M1
2.5.6 Validation of the technique for the analysis of microparticles

Whilst the technique used to extract MP from whole blood was adapted from a previously validated and published technique (Combes, Dignat-George and others, 1997), considerable time was spent modifying and validating the technique described to extract and analyse microparticles in this thesis.

2.5.6.1: Reproducibility of MP analysis

Since the sample from individual subjects was divided into 10 wells, an estimate of the total number of MP was derived 10 times, and standard error of the mean thus derived from 10 measurements for each subject. Using this approach, blood from a healthy adult donor was divided into 3, and the microparticle analysis performed in triplicate (i.e. 3 X 10 wells). The results are shown in figure 2.10 (mean, SEM) and provide an estimate of the reproducibility of the technique.

2.5.6.2: Effect of different centrifugation protocols on microparticle analysis

Different centrifugation protocols for the preparation of platelet poor plasma and extraction of MP from plasma were examined, and were found to influence the absolute number of MP measured, and the intra-subject reproducibility of the MP count. There is a trade-off between fast centrifugation protocols which would remove platelets more effectively from whole blood but in theory could cause red cell or platelet fragmentation (and thus create artefactual phosphatidylserine exposure with spuriously high MP
Figure 2.10: Reproducibility of MP analysis

The figure shows the MP number in millions per milliliter for different analyses. There are three analyses: Analysis 1 (solid bars), Analysis 2 (hatched bars), and Analysis 3 (open bars). The x-axis represents different markers: average total, CD42a, P-selectin, ICAM-1, CD105, VCAM-1, and E-selectin. The y-axis represents the MP number in millions per milliliter, ranging from 0 to 0.6.
counts), and slower centrifugation protocols which run the risk of unacceptably high platelet contamination and/or incomplete MP isolation from plasma potentially making the MP analysis inaccurate. Four different whole blood centrifugation protocols were investigated (Table 2.2).

**Table 2.2: Centrifugation protocol for isolation of MP from whole blood.**

| Protocol 1: | 5000G for 5 minutes; supernatant then spun at 17000G for 60 minutes (discard supernatant and analyse MP pellet). |
| Protocol 2: | 5000G for 5 minutes; supernatant then spun at 15000G for 60 minutes (discard supernatant and analyse MP pellet). |
| Protocol 3: | 1500G for 15 minutes; supernatant then spun at 17000G for 15 minutes (discard supernatant and analyse MP pellet). |
| Protocol 4: | 5000G for 5 minutes; supernatant then re-spun at 5000G for 5 minutes; supernatant then spun at 17000G for 60 minutes (discard supernatant and analyse MP pellet). |

The results (mean total MP count, SEM of 10 measurements) for each protocol are shown in figure 2.11. As demonstrated, the least accurate reproducibility was that associated for protocol 3, with little to choose from between the remaining protocols. The protocol used in this thesis was protocol 4, since it appeared that this was reproducible and was the least likely to be associated with platelet contamination since two sequential centrifugations were employed to prepare the platelet poor plasma.
Figure 2.11: Effect of different centrifugation protocols on MP count
2.5.6.3: Effect of freeze-thawing on microparticle analysis

The effect of 24 hours of freezing at \(-70^\circ\text{C}\) followed by a rapid defrost (water bath at 37 \(^{\circ}\text{C}\) for 1 minute) on the intra-subject reproducibility of mean total MP number (SEM of 10 measurements) was assessed. The result is shown in figure 2.12. MP number was unaffected by freeze-thaw.
Figure 2.12: Effect of freeze-thaw on MP number (mean, SEM)
2.5.6.4: Effect of delayed separation of plasma from whole blood on microparticle analysis

To assess the affect of delayed separation of the plasma from whole blood, the blood sample taken from a healthy adult was left at room temperature, and the MP profile analysed from aliquots of the sample at 0, 1, 2, 4, and 24 hours. The results are shown in figure 2.13. Data for VCAM-1 MP are omitted because they were undetected at any time point. From the figure it can be seen that delay in separation of the blood sample for longer than 2 hours profoundly affected the whole MP profile, but in particular the total MP count and platelet MP expressing CD42a, presumably the result of platelet activation and ultimately fragmentation with time. Hence in this thesis, all samples for MP analysis were separated from whole blood within an hour, and usually within 10 minutes.

2.5.6.5: Properties of latex beads which affect microparticle analysis

Uniform latex particles were first discovered in 1947 (Sigma product information). Since then they have been utilized in a wide variety of applications including electron microscopy and cell counter calibration, antibody mediated agglutination diagnostics, and phagocytosis experiments.

Polystyrene microparticles are negative charge-stabilized colloidal particles, supplied as aqueous suspensions with small amounts of surfactant to prevent agglutination. Particles can be diluted with deionized water. However, some particles, especially at very dilute
Figure 2.13: Effect of delayed plasma separation from whole blood on MP profile

- Total number of AnV+ MPs
- CD42a MPs
- Psel MPs
- ICAM-1 MPs
- CD105 MPs
- Esel MPs

MP number (Millions/ml of plasma) vs. Time (hours)
concentrations, may require additional surfactant to prevent clumping and uneven dispersion. An early observation was that if the latex beads were left longer than 10 minutes or so, the number counted by the flow cytometer would fall very quickly. Since the bead number is critical in the conversion of flow cytometer counts and appears as a denominator in the conversion equation (figure 2.8), a falsely low bead count would result in a falsely elevated MP count. In this thesis, 6 µL of neat beads was added to 2 mls of 0.2 µm filtered de-ionized water. 10 µL of the dilute beads thus contained 200 000 beads as determined using the equation provided by sigma (Appendix 7). Beads were made afresh every 5 minutes as required. Using a medium flow rate on the flow cytometer (with a cut-off time of 60 seconds or until 5000 bead events were collected) resulted in 5000 bead events counted usually after approximately 58 seconds. Diluted beads that were left for longer than an hour prior to use resulted in counts 2 or even 3-fold less, profoundly affecting the MP calculation.
2.6 Statistics

A number of parametric and non-parametric tests were used in this thesis, and a
description of individual tests and their application is given in the relevant chapters.
Parametric tests were used wherever possible (i.e. when the data were normally
distributed, or could be easily transformed to normality). If the data could not be easily
transformed (e.g. Chapter 5), non-parametric tests were performed.

Parametric tests used were one-way analysis of variance (ANOVA) with Bonferroni and
Tamhane post-hoc tests; the F test of variance; and the 2-sample test of proportion. In
chapter 3, all p values quoted are adjusted for multiple comparisons using the Bonferroni
technique. Non-parametric tests used were the Kruskall-Wallis test, the Wilcoxon signed-
ranks test, and Spearman rank correlation coefficients. Normality of the data was
assessed by plotting histograms, and by the Kolmogorov-Smirnov and the Shapiro-Wilks
tests of normality.

In addition, for assessment of the diagnostic test characteristics for endothelial
microparticles in Chapter 5, sensitivity, specificity, negative and positive predictive
value, and likelihood ratios were calculated (Gilbert R and Logan S, 2000). In addition, a
receiver operator characteristic curve (ROC curve) was plotted (Gilbert R and Logan S,
2000).
All graphs were plotted using Microsoft Excel 97, with the exception of column dot-plots, which were plotted using Graphpad Prism version 1.03. All statistics were calculated using Microsoft Excel 97, and SPSS versions 8 and 10.0.
Chapter 3: T cell activation and Vβ repertoires in childhood vasculitides

3.1 Summary
3.2 Introduction
3.3 Patients and methods
3.4 Results
3.5 Discussion
3.1 Summary

Introduction: Superantigens (SAgs) are potent stimulators of T cells bearing specific Vβ T cell receptors (TCR) and may play a role in the aetio-pathogenesis of systemic vasculitis, although this remains contentious.

Aims: To investigate the possible aetiological role of SAgs, this study examined peripheral blood T cell activation, and Vβ repertoires in children with systemic vasculitis.

Methods: FACS analysis of 17 different peripheral blood T cell Vβ families was performed in 20 healthy control children; 27 disease control children with non-vasculitic inflammatory disease; 25 children with primary systemic vasculitis; 6 patients with KD; and 6 patients with Henoch-Schönlein purpura (HSP). T cell activation markers (CD69 and CD25) in the CD4 and CD8 T cell subsets were also examined in these patients, and in an additional 8 patients with PAN (5 with active disease, 3 with inactive disease), and an additional 5 disease controls with viral sepsis.

Results: Children with active vasculitis had higher CD4 and CD8 peripheral blood T cell CD69 expression than those with inactive vasculitis and healthy controls, but not disease controls. No difference in CD25 expression in either the CD4 or CD8 T cell populations was observed between active and inactive vasculitis groups, however. There was a significantly increased variance of CD4 Vβ12, and Vβ17 and CD8 Vβ1 in the primary systemic vasculitis group as compared to control and disease controls. Moreover, 80% of the primary systemic vasculitis children had one or more CD4 Vβ expansions or deletions, as compared with 30% of controls (p<0.002), and 37% of the disease controls (p<0.002). In the KD group, the mean % of CD4 Vβ2 T cells was higher than in controls.
or disease controls. In the HSP group, there was no consistent skewing of the T cell Vβ repertoire.

**Conclusion:** The observation of peripheral T cell activation in systemic vasculitis of the young may support a role for T cells in the pathogenesis of these disorders. Moreover, there are differences in the T cell Vβ repertoire in children with vasculitis over and above those observed in disease controls. Whilst these data provide impetus for further research into this contentious field, they do not unequivocally resolve the question of the role of SAgS in childhood vasculitic syndromes.
3.2 Introduction

As described, systemic vasculitis is characterised by the presence of inflammation in and around a blood-vessel wall (Petty and Cassidy, 2001a). In recent years, there has been considerable interest in the role of superantigens (SAgs) in the aetio-pathogenesis of several autoimmune diseases, including the systemic vasculitides (Cohen Tervaert, Popa, and Bos, 1999; Giscombe, Grünewald and others, 1995; Schiffenbauer, Soos, and Johnson, 1998; Simpson, Skinner and others, 1995; Torres and Johnson, 1998).

Following SAg activation, T cells rapidly proliferate resulting in T cell Vβ "expansions" (Choi, Kotzin and others, 1989; Fraser, Arcus and others, 2000; Kappler, Kotzin and others, 1989; Shoukry, Lavoie and others, 1997). This is followed by T cell Vβ-restricted deletion from the peripheral circulation- a process mediated by Fas-Fas ligand (Fraser, Arcus and others, 2000; Mingari, Cambiaggi and others, 1996; Webb, Hutchinson and others, 1994). Thus the characteristic "immunological footprint" left by SAgs are the presence of T cell Vβ expansions and deletions in the peripheral circulation of subjects exposed to these toxins.

Abnormal expansions and deletions of T cells bearing particular Vβ gene products have been found in the peripheral blood of adults with primary systemic necrotizing vasculitis (microscopic polyangiitis, Wegener's granulomatosis, giant cell arteritis, and polyarteritis nodosa) (Cohen Tervaert, Popa, and Bos, 1999; Giscombe, Grunewald and others, 1995; Popa, Stegeman and others, 1998; Simpson, Skinner and others, 1995), providing indirect evidence of superantigenic involvement in the aetio-pathogenesis of these vasculitides.
Furthermore, an important observation in Wegener's granulomatosis in adults is that those with the disease who are nasal carriers of superantigen-producing staphylococci are significantly more likely to have relapses of vasculitis compared with non-carriers (Cohen Tervaert, Stegeman and others, 1998; Cohen Tervaert, Popa, and Bos, 1999).

In some studies, children with KD also show non-clonal expansion of peripheral T cells bearing Vβ2 (Abe, Kotzin and others, 1992; Curtis, Zheng and others, 1995; Leung, Meissner C, and Schlievert, 1997), although other workers have not confirmed this observation, and argue against a SAg-mediated pathogenesis (Choi, Chwae and others, 1997; Morita, Imada and others, 1997; Pietra, De Inocencio and others, 1994; Rowley, Shulman and others, 2001; Terai, Miwa and others, 1995). Furthermore, T cells infiltrating the walls of coronary arterial aneurysms (Leung, Giomo and others, 1995), and the intestinal mucosa of patients with KD show a skewed T cell Vβ profile, with increased numbers of cells expressing Vβ2 (Yamashiro, Nagata and others, 1996). These results have led to speculation that SAgS play an important aetio-pathogenic role in this vasculitic illness. Similar studies on vasculitis in children, apart from KD, are however lacking.

To investigate the possible aetiological role of SAgS this study examined peripheral blood TCR Vβ repertoires in children with primary systemic vasculitis, Kawasaki disease, and Henoch-Schönlein purpura.
3.3 Patients and methods

3.3.1 VASCULITIS PATIENTS

37 children with vasculitis at various stages of disease activity and treatment had Vβ repertoires studied. The diagnosis of vasculitis was established in all on the basis of clinical features of vasculitis in addition to suggestive selective visceral angiography and/or tissue biopsy. The vasculitis was subsequently classified using the American College of Rheumatology (ACR) 1990 classification criteria (Lightfoot, Jr., Michel and others, 1990), and the Chapel Hill consensus (Jennette, Falk and others, 1994). Children were classified using both systems because there is controversy over the most reliable classification system for the childhood vasculitides (Besbas, Ozen and others, 2000; Hunder G, 1998; Ozen, Besbas and others, 1992; Petty RE and Cassidy JT, 2001a), and there is a considerable degree of "polyangiitis overlap" in the childhood vasculitides (Brogan and Dillon, 2000a; Leavitt RY and Fauci, 1986a), such that neither system is absolutely sensitive or specific (Jennette and Falk, 2000b). Neither system, however, has been formally validated in children (Petty and Cassidy, 2001a).

25 children were classified as primary systemic vasculitis. The mean age was 10.1 years (range 2.5-15.6 years), and the male to female ratio was 2.1:1. Of this group, using the ACR classification criteria, 19 had polyarteritis nodosa (PAN), 1 had Wegener's granulomatosis (WG), 2 had hypersensitivity vasculitis, and 3 had unclassifiable vasculitis. Using the Chapel Hill consensus 16 had PAN, 6 had microscopic polyangiitis
(MPA), and 3 had unclassifiable vasculitis. Clinical details of these patients are summarised in table 3.1. Additionally, longitudinal studies were performed on paired samples collected from patients before and after induction of remission of vasculitis (n=6). Remission was defined as patients with a Birmingham vasculitis activity score of zero (Luqmani, Bacon and others, 1994) (Appendix 2).
Table 3.1 Clinical features of patients with primary systemic vasculitis
<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex/age (yrs)</th>
<th>Clinical features</th>
<th>Selective visceral angiography</th>
<th>Biopsy</th>
<th>ANCA (IIF)</th>
<th>Active disease at time of sampling</th>
<th>Vasculitis classification, and system used for classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/11.4</td>
<td>Fever, purpura, crescentic nephritis, arthritis, intestinal inflammation</td>
<td>Renal &amp; hepatic perfusion defects; aneurysms of medium and small hepatic arteries</td>
<td>Skin biopsy -ve for vasculitis; 33% crescentic nephritis, pauci-immune</td>
<td>neg</td>
<td>N</td>
<td>1. PAN (ACR) 2. Microscopic polyangiitis (Chapel Hill consensus)</td>
</tr>
<tr>
<td>2</td>
<td>M/2.7</td>
<td>Periodic fever, failure to thrive, testicular infarction, pulmonary vasculitis</td>
<td>Small aneurysms and caliber variation of℠A and its branches</td>
<td>Lung: medium sized artery perivascular &amp; vascular mononcytic and granulocyto infiltrate; spleen: non-specific inflammatory changes; testic: infarction only (no vasculitis demonstrated)</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>3</td>
<td>F/13.6</td>
<td>Fever, arthralgia, orbital granuloma, microscopic haematuria</td>
<td>Not done</td>
<td>Orbital granulomatous inflammation compatible with WG</td>
<td>neg</td>
<td>Y</td>
<td>1. WG (ACR) 2. Unclassified (Chapel Hill consensus)</td>
</tr>
<tr>
<td>4</td>
<td>F/7.5</td>
<td>Fever, myalgia, weight loss</td>
<td>Renal perfusion defects; SMA aneurysms; &quot;corkscrew&quot; changes of medium and small-sized hepatic arteries</td>
<td>Not done</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>5</td>
<td>F/6.2</td>
<td>Fever, myalgia, livedo reticularis, brain stem infarction, hypertension, renal impairment</td>
<td>Large aneurysms affecting predominantly medium-sized renal arteries</td>
<td>Skin biopsy -ve for vasculitis</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>6</td>
<td>F/14.5</td>
<td>Fever, myalgia, intestinal inflammation, arthralgia</td>
<td>Not done</td>
<td>Brain biopsy: non-specific reactive changes only</td>
<td>neg</td>
<td>Y</td>
<td>Unclassified using either ACR or Chapel hill consensus criteria</td>
</tr>
<tr>
<td>7</td>
<td>F/9.8</td>
<td>Fever, weight loss, livedo reticularis, testicular pain</td>
<td>Small aneurysms and caliber variation of medium-sized renal arteries; pruning of small renal arteries; perfusion defects of kidneys, tortuous small hepatic arteries</td>
<td>Indeterminate upper and lower gastrointestinal inflammation</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>8</td>
<td>M/15</td>
<td>Fever, weight loss, livedo reticularis, myalgia</td>
<td>Normal</td>
<td>Skin biopsy: vasculitis affecting medium and small arteries</td>
<td>neg</td>
<td>N</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>9</td>
<td>M/14.1</td>
<td>Fever, urticaria, palpable purpura, myalgia, Raynaud's</td>
<td>Not done</td>
<td>Skin- leucocytoclastic vasculitis</td>
<td>neg</td>
<td>N</td>
<td>1. Hypersensitivity vasculitis (ACR) 2. Microscopic polyangiitis OR cutaneous leucocytoclastic angiitis (Chapel Hill consensus)</td>
</tr>
<tr>
<td>10</td>
<td>M/14.1</td>
<td>Fever, weight loss, livedo reticularis</td>
<td>Caliber change and beading of medium and small renal arteries</td>
<td>Not done</td>
<td>neg</td>
<td>N</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>11</td>
<td>M/14.1</td>
<td>Fever, myalgia, weight loss, livedo reticularis</td>
<td>Small aneurysms and caliber variation of medium and small renal arteries and branches of SMA</td>
<td>Not done</td>
<td>neg</td>
<td>N</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
</tbody>
</table>

*ACR: American College of Rheumatology; #SMA: superior mesenteric artery; $IMA: inferior mesenteric artery; †ANCA: cytoplasmic anti-neutrophil cytoplasmic antibody; ‡ANCA: perinuclear anti-neutrophil cytoplasmic antibody.*
<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex/age (yrs)</th>
<th>Clinical features</th>
<th>Selective visceral angiography</th>
<th>Biopsy</th>
<th>ANCA (RF)</th>
<th>Active disease at time of sampling</th>
<th>Vasculitis classification, and system used for classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>F/9.7</td>
<td>Fever, anaemia, myalgia, arthritis, weight loss</td>
<td>Small aneurysms affecting SMA and branches; small aneurysms and stenoses of medium to small renal arteries</td>
<td>Not done</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>14</td>
<td>M/14</td>
<td>Fever, weight loss, testicular pain, myalgia, intestinal inflammation, subcutaneous nodules</td>
<td>Small aneurysms of SMA branches</td>
<td>Skin: perivascular monocytic infiltrate in small arteries</td>
<td>neg</td>
<td>N</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>15</td>
<td>M/11.2</td>
<td>Fever, weight loss, myalgia, deranged liver function, no urinary sediment</td>
<td>Not done</td>
<td>Liver: perivascular granulomatous inflammation</td>
<td>pANCA</td>
<td>N</td>
<td>1. Unclassified (ACR) 2. Unclassified (Chapel Hill consensus)</td>
</tr>
<tr>
<td>16</td>
<td>F/10.9</td>
<td>Fever, arthralgia, muscular rash, nephritis-nephrotic</td>
<td>Not Done</td>
<td>&gt;50% crescentic glomerulonephritis</td>
<td>pANCA</td>
<td>N</td>
<td>1. Unclassified (ACR) 2. Microscopic polyangiitis (Chapel Hill consensus)</td>
</tr>
<tr>
<td>17</td>
<td>M/13.1</td>
<td>Fever, heavy proteinuria, myalgia, weight loss, urticaria, pericarditis, negative streptococcal serology</td>
<td>Small aneurysms and caliber variation of medium and small renal arteries and SMA</td>
<td>Renal biopsy normal</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. Microscopic polyangiitis (Chapel Hill consensus)</td>
</tr>
<tr>
<td>18</td>
<td>M/2.9</td>
<td>Fever, weight loss, intestinal inflammation, bullous rash, arthritis</td>
<td>Small aneurysms and caliber change affecting SMA &amp; IMA branches, small aneurysms and pruning of medium and small renal arteries</td>
<td>Skin: perivascular monocytic infiltration of small arteries</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. Microscopic polyangiitis (Chapel Hill consensus)</td>
</tr>
<tr>
<td>19</td>
<td>M/6.2</td>
<td>Fever, myalgia, abdominal pain, weight loss</td>
<td>Small aneurysms and caliber variation with cut-off of medium and small renal arteries and SMA</td>
<td>Upper GI biopsy normal</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>20</td>
<td>F/2.5</td>
<td>Fever, weight loss, myalgia, Polymorphic rash, peeling of extremities, conjunctival injection-chronic symptoms persisting for &gt;12 months; coronary arterial aneurysms on echocardiography</td>
<td>Small aneurysms and caliber variation of medium and small renal and mesenteric arteries</td>
<td>Not done</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) - initial classification of KD changed to PAN due to multiple relapses and chronicity of symptoms 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>21</td>
<td>F/15.6</td>
<td>Fever, weight loss, myalgia</td>
<td>Small aneurysms affecting medium and small renal and hepatic arteries</td>
<td>Not done</td>
<td>neg</td>
<td>N</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>22</td>
<td>M/8.2</td>
<td>Fever, myalgia, weight loss, hypertension, livedo reticularis</td>
<td>Small aneurysms affecting medium and small renal and hepatic arteries</td>
<td>Not done</td>
<td>neg</td>
<td>N</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>23</td>
<td>M/12</td>
<td>Fever, myalgia, polymorphous rash, lymphadenopathy, diarrhoea, conjunctival injection, weight loss, abdominal pain</td>
<td>Not done</td>
<td>Skin: leukocytoclastic vasculitis</td>
<td>neg</td>
<td>Y</td>
<td>1. Hypersensitivity vasculitis (ACR) 2. Microscopic polyangiitis (Chapel Hill consensus)</td>
</tr>
<tr>
<td>24</td>
<td>M/13.9</td>
<td>Fever, weight loss, oral ulceration, myalgia, abnormal liver function</td>
<td>Renal perfusion defects and renal arterial pruning; small aneurysms of medium-sized hepatic arteries</td>
<td>Liver: non-specific inflammatory changes</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
<tr>
<td>25</td>
<td>M/12.5</td>
<td>Fever, palpable purpura, myalgia</td>
<td>Small aneurysms and caliber variation affecting branches of SMA and medium and small hepatic arteries</td>
<td>Skin: perivascular lymphocytic infiltrate in small arteries, but not arterioles</td>
<td>neg</td>
<td>Y</td>
<td>1. PAN (ACR) 2. PAN (Chapel Hill consensus)</td>
</tr>
</tbody>
</table>
6 children (5 boys) fulfilled completely the diagnostic criteria for KD (Dajani, Taubert and others, 1993; Petty RE and Cassidy JT, 2001c). The mean age was 1.7 years (range 0.25-5.5 years). 4 had coronary arterial aneurysms, and one had axillary arterial aneurysm formation in addition to coronary arterial aneurysms. All patients were in the second week of the illness, and 4 had already received intravenous immunoglobulin (IVIG) at the time of blood sampling. The patients with KD were described separately from those with primary systemic vasculitis since KD differs from these latter vasculitides in that it has a distinct clinical phenotype with unique association with the muco-cutaneous lymph node syndrome (Brogan and Dillon, 2000a; Brogan, Bose and others, 2002) (although there are overlapping clinical features with classical "infantile PAN", perhaps now regarded by some as a severe form of KD), is usually self-limiting (unlike PAN, WG, and MPA), and has epidemiological features suggestive of an infectious aetiology, unlike PAN, WG or MPA.

6 children (2 boys) were classified as Henoch-Schönlein purpura (HSP) using both ACR (Lightfoot, Michel and others, 1990) (Appendix 2) and Chapel Hill consensus criteria. The mean age was 11.1 years (range 7-15 years). 4 had renal biopsy evidence demonstrating typical glomerulonephritis with IgA-predominant immune deposits, and one had skin biopsy again demonstrating typical IgA-dominant immune deposits in a small arterial wall.
3.3.2 CONTROLS AND DISEASE CONTROLS

Blood samples were obtained from 20 control children undergoing venepuncture for pre-operative assessment prior to minor orthopaedic procedures. Children with syndromic diagnoses, underlying inflammatory disease, on regular medication, or with intercurrent infection were excluded from this group. The mean age was 10.6 years (range 0.5-19 years), and the male to female ratio was 1.5:1.

Disease controls comprised 27 children with bacterial or viral sepsis (n=10), autoimmune disease without vasculitis (n=6: one ulcerative colitis, one juvenile dermatomyositis, one CINCA syndrome (chronic idiopathic neurological cutaneous and articular syndrome), one Sneddon syndrome, one transient post-viral anti-phospholipid syndrome, and one focal and segmental glomerulosclerosis); or recipients of renal allografts (n=16, 8 children with dysplastic kidneys; 8 children with posterior urethral valves). These recipients of renal allografts were included since they were on a similar spectrum of immunosuppressive therapy (prednisolone, azathioprine, and cyclosporin) as the primary systemic vasculitis group, but did not have a prior history of vasculitis or other autoimmune disease and hence would control for any effect on the T cell repertoire mediated by immunosuppressive drugs. The mean age of this group was 11.3 years (range 4.5-17.2 years), with male to female ratio of 1.5:1.

Positive controls comprised 2 children with the toxic shock syndrome (TSS). The first was a 3-week-old baby who developed the staphylococcal TSS secondary to a post-operative wound infection following repair of gastroschisis. The organism responsible
was a Staphylococcus aureus producing the superantigens TSST1 and staphylococcal enterotoxins A, G, and I. Blood was obtained for T cell analysis from this patient approximately 10 days into the illness. The second patient was a 2-year-old boy who developed streptococcal TSS secondary to an infected gastrostomy site. The responsible organism was a Lancefield group A Streptococcus, although this was not sent for toxin typing. Blood was obtained from this patient 12 hours into the illness.

In addition to the above patients who had Vβ repertoire studies performed, T cell activation markers were studied in the above patients plus another 8 patients with PAN (5 with active disease, 3 with inactive disease), and 5 patients with viral infections (upper respiratory tract infection).

Written, informed consent was obtained from all parents, and older children involved in the study. The study was approved by the local hospital research ethics committee.

3.3.3 ANALYSIS OF PERIPHERAL BLOOD T CELL ACTIVATION MARKERS AND T CELL Vβ REPERTOIRES

This was performed as described in Chapter 2, sections 2.4.1, 2.4.4 and Appendix 3. In addition to the analysis of CD4 and CD8 T cell Vβ repertoires, the T cell activation markers CD69 and CD25 (table 2.1) were analysed within the CD4 and CD8 T cell populations.
3.3.4 IN VITRO TECHNIQUE FOR VALIDATION OF EFFECT OF SAGs ON T CELL Vβ REPertoire SKewing

In addition to the *in vitro* validation technique described in Chapter 2 (section 2.4.5), analysis of the T cell Vβ repertoire was performed in 2 children with TSS (described above) to check the validity of the technique *in vivo*.

3.3.5 STATISTICS

Comparison of group demographics was performed using the Kruskall-Wallis test. T cell activation between the groups was assessed using the Kruskall-Wallis and Mann-Whitney tests.

Assessment of skewing of the T cell Vβ repertoire between the groups of children was performed by comparison of mean percentages for each Vβ family using ANOVA, and all p values adjusted for multiple between-group comparisons using the Bonferroni method when the groups had equal variance, or the Tamhane method when significant differences in the variance for individual Vβ families was found. In addition, to further protect from the effect of multiple comparisons, biological significance was only inferred from the statistical results when the difference in the vasculitis group was consistent for a particular Vβ family between both the control and disease control groups. Vβ skewing was also assessed by comparison of the variance of each Vβ family between the groups using the F test, with significance adjusted for 17 comparisons (i.e. the number of Vβ families examined). To check the validity of the F test, normality of the data was assessed
using the Kolmogorov-Smirnov and the Shapiro-Wilks tests, and no evidence for non-normality was demonstrated in any Vβ family.

For comparison, T cell Vβ skewing was also assessed using previously published but arbitrarily-defined definitions of T cell Vβ expansions and deletions, with the proportion of children in each group having an expansion or deletion compared using the two-sample test of proportion. Thus, a Vβ expansion was defined as a value more than the control group mean plus 2 standard deviations for an individual Vβ family, and a deletion was a value less than the control group mean minus 2 standard deviations (Popa, Stegeman and others, 1998; Wedderburn LR, Maini MK and others, 1998).

Paired data derived from the longitudinal study of 6 of the vasculitis patients before and after induction of remission were analysed using the Wilcoxon signed-rank test. Statistical significance was defined at p<0.05 (adjusted for multiple comparisons), and is implied in the results wherever differences are highlighted. All statistics were performed using Microsoft Excel 97 and SPSS 10.0 for Windows.

3.4 Results

3.4.1 PATIENT DEMOGRAPHICS

Children with KD were younger than children with other vasculitides, controls, and disease controls (p=0.001). Otherwise there were no significant age or sex differences between the groups of children. The median time of blood sampling from disease onset
was 12 months (range: 0.6-72 months) in the primary systemic vasculitis group, and 1.5 months (range 0.5-6 months) in the HSP group. All the patients in the KD group were sampled in the second week of the illness.

3.4.2 T CELL ACTIVATION IN PATIENTS AND CONTROLS

Since there is a linear pattern of CD69 staining in CD4 and CD8 T cells the level of expression was determined by analysis of both the median fluorescence index (MFI), and by determining the percentage of cells with “high” CD69 expression (using an arbitrary cut-off between CD69 “high” and CD69 “low” expression).

Overall, patients with active vasculitis (all types of vasculitis; mean BVAS 9.4; range 3-20) had higher levels of peripheral CD4+ T cell activation as determined by CD69 expression compared with those with inactive vasculitis (BVAS=0 by definition), (p=0.01 for CD69 MFI; p=0.005 for percentage of high CD69); and healthy controls (p=0.04 for CD69 MFI; p=0.007 for percentage of high CD69). There was no difference in CD4+ CD69 expression between the active vasculitis group and the disease controls (p=0.4 for CD69 MFI; p=0.37 for percentage of high CD69). These data are summarised in figure 3.1.
Figure 3.1: CD4 T cell CD69 Expression

- Active vasculitis
- Inactive vasculitis
- Healthy controls
- Disease controls

CD4 CD69 MFI

% of CD4 cells expressing high CD69

p=0.4
p=0.04
p=0.01
p=0.7
p=0.001
P=0.37
P=0.007
P=0.005
P=0.53
P=0.003
Regarding the CD8 T cell population, patients with active vasculitis (all types of vasculitis) had higher CD8+ CD69 expression than those with inactive vasculitis (p=0.04 for CD69 MFI; p=0.01 for percentage of high CD69). CD69 expression was only higher in the active vasculitis compared with healthy controls when the results were expressed as percentage of high CD69 (p=0.007), and not when the result was expressed as CD69 MFI (p=0.11). There was no difference in CD8+ CD69 expression between the active vasculitis group and disease controls (p=0.5 for CD69 MFI; p=0.3 for percentage of high CD69). These data are summarised in figure 3.2.

To examine T cell activation profiles within different types of vasculitis, the vasculitis patients were sub-divided into a primary systemic vasculitis group (inclusive of the necrotizing vasculitides PAN, MPA, and WG); HSP; and KD. There were several missing data points from these latter 2 groups, and so no statistical analysis was performed for these sub-categories. Again, there was higher CD4+CD69 expression (MFI data only shown) in the active primary systemic vasculitis group compared with inactive vasculitis (p=0.007); and healthy controls (p=0.02); but there was no difference between active vasculitis and disease controls (p=0.92). These data and the data regarding the HSP and KD patients are summarised in figure 3.3. CD8+CD69 expression was higher in the active primary systemic vasculitis group than in the inactive group (p=0.042), but no difference was observed between the active primary systemic vasculitis group and the healthy controls or disease controls (p=0.13 and p=0.4 respectively). These data are summarised in figure 3.4.
Figure 3.2: CD8 T cell CD69 expression

- Active vasculitis
- Inactive vasculitis
- Healthy controls
- Disease controls

Comparisons and p-values:  
- P=0.5
- P=0.11
- P=0.04
- P=0.007
- P=0.007
- P=0.01
- P=0.007
- P=0.007
- P=0.05
- P=0.007
Figure 3.3: CD4 T cell CD69 expression by vasculitis type

- PSV active
- PSV inactive
- Healthy controls
- Disease controls

- HSP active
- HSP inactive
- Healthy controls
- Disease controls

- KD active
- KD inactive
- Healthy controls
- Disease controls
Figure 3.4: CD8 T cell CD69 expression by vasculitis type

- PSV active
- PSV inactive
- Healthy controls
- Disease controls

- HSP active
- HSP inactive
- Healthy controls
- Disease controls

- KD active
- KD inactive
- Healthy controls
- Disease controls
There was no difference in CD4+CD25 or CD8+CD25 expression between the active vasculitis and inactive vasculitis groups (figures 3.5 and 3.6). CD25 expression was not measured in the controls or disease control groups.

3.4.4 T CELL Vβ REPERTOIRES FROM PATIENTS AND CONTROLS: COMPARISON OF MEANS

Figures 3.7a and 3.71b summarise the mean percentage of CD4 and CD8 T cells expressing each of the individual Vβ gene products for controls, disease controls, and children with vasculitis (PSV, KD, and HSP). For comparison, the T cell Vβ repertoire from the 2 positive control children with TSS compared with controls and disease controls is shown in figures 3.8a and 3.8b.

3.4.4.1 PRIMARY SYSTEMIC VASCULITIS

For CD4 T cells, the mean percentage of Vβ13.1 was higher in the primary systemic vasculitis group than controls (p=0.02) but not disease controls (p=0.2). For the CD8 T cells, the mean percentage of Vβ2 was lower in the disease controls versus the controls (p=0.03), although there was no difference between vasculitis and controls or disease controls. The mean percentage of CD8 Vβ7 T cells was higher in the primary systemic vasculitis group than the controls (p=0.01) but not the disease controls (p=0.1). The mean percentage of CD8 Vβ17 T cells was lower in the primary systemic vasculitis group than controls (p=0.03) but not disease controls (p=0.1).
Figure 3.5: CD4 CD25 expression

- CD4 CD25 MFI
  - Active vasculitis
  - Inactive vasculitis

- % of CD4 cells expressing high CD25
  - Active vasculitis
  - Inactive vasculitis

- p-values:
  - CD4 CD25 MFI: p=0.48
  - % of CD4 cells expressing high CD25: p=0.71
Figure 3.6: CD8 CD25 expression

Figure: Scatter plot showing the comparison of CD8 CD25 expression between active and inactive vasculitis.

- **CD8 CD25 MFI (Median Fluorescence Intensity):**
  - Active vasculitis: Lower MFI values (around 5) compared to inactive vasculitis (around 15).
  - p-value: 0.25

- **% of CD8 cells expressing high CD25:**
  - Active vasculitis: Higher percentage (up to 90%) of CD8 cells expressing high CD25 compared to inactive vasculitis (up to 70%).
  - p-value: 0.53
Figure 3.7a and 3.7b: T cell Vβ repertoires in study groups

Mean (SEM) CD4 and CD8 Vβ repertoire from healthy control children, disease control children, and children with vasculitis (primary systemic vasculitis, Kawasaki disease, and Henoch Schonlein purpura). All comparisons were performed using ANOVA, and all p values adjusted to account for multiple comparisons (see text).
Figure 3.7a and 3.7b: Mean percentage of CD4 and CD8 T cells expressing individual Vβ receptors for controls, disease controls, and the 3 groups of children with vasculitis.
**Figure 3.8a and 3.8b:**

CD4 and CD8 Vβ repertoire from 2 children with toxic shock syndrome. The first patient with staphylococcal TSS shows an expansion of CD4 and CD8 Vβ2 T cells. The second patient with streptococcal TSS shows an expansion of CD4 Vβ5.1 T cells.
Figure 3.8a and 3.8b: CD4 and CD8 repertoire from 2 children with TSS, compared with controls and disease control children.

Figure 3.8a: CD4 T cell V beta repertoire in TSS patients

Figure 3.8b: CD8 T cell V beta repertoire in TSS patients
3.4.4.2 KAWASAKI DISEASE

In the KD group the mean percentage of CD4 Vβ2 cells was higher than in both the control and disease control groups (p=0.03, and p=0.01 respectively). The mean percentage of CD8 Vβ2 cells was higher in the KD group than in the disease controls (p=0.02) but not the controls (p=0.9). The mean percentage of CD8 Vβ7 T cells was higher in the KD group than both the controls (p=0.008) and disease controls (p=0.03).

3.4.4.3 HENOCH SCHÖNLEIN PURPURA

In the HSP group the mean percentage of CD4 Vβ1 T cells was lower than in both the control (p=0.02), and disease control groups (p=0.02). The mean percentage of CD4 Vβ14 was lower in the HSP group than in the controls (p=0.01) but not the disease controls (p=0.3). The mean percentage of CD8 Vβ1 was lower in the HSP group than in the controls (p=0.007), but not the disease controls (p=0.2).

3.4.5 T CELL Vβ REPERTOIRES: COMPARISON OF VARIANCE

Figures 3.9a and 3.9b summarise the ratios of the variance (F ratio) of each Vβ family for CD4 and CD8 T cells from the primary systemic vasculitis group and the controls and disease controls. F ratios were obtained by dividing the variance ("spread") for each Vβ family in the vasculitis group by the corresponding Vβ family variance in the control, and disease control groups. If the variances are similar, the F ratio is close to 1. If the variance of any Vβ family is greatly increased in the vasculitis group, the corresponding F ratio is much greater than 1. P values for the F ratios thus obtained between the groups were then
Variance ratios (F ratios) of each Vβ family for CD4 and CD8 T cells. F ratios were obtained by dividing the variance ("spread") for each Vβ family in the vasculitis group by the corresponding Vβ family variance in the control, and disease control groups. If the variances are similar, the F ratio is close to 1. If the variance of any Vβ family is greatly increased in the vasculitis group, the corresponding F ratio is much greater than 1 (adjusted p values asterisked).
Figures 3.9a and 3.9b: Variance ratios (F ratios) of each Vβ family for CD4 and CD8 T cells.

**Figure 3.9a: Ratio of variance of CD4 v beta repertoire**

- **Vasculitis vs controls**
- **Vasculitis vs disease controls**

* *p*<0.05 (adjusted for 17 comparisons) for both vasculitis versus controls and vasculitis versus disease controls

**Figure 3.9b: Ratio of variance of CD8 v beta repertoire**

- **vasculitis vs controls**
- **vasculitis vs disease controls**

* *p*<0.05 (adjusted for 17 comparisons) for both vasculitis versus controls and vasculitis versus disease controls
derived using the F test, with alpha adjusted for 17 comparisons. Only when the variance ratio was statistically significantly increased for both vasculitis versus controls and vasculitis versus disease controls was it regarded as biologically significant.

The variance was increased in the vasculitis group compared with both the controls and disease controls for CD4 Vβ12 (vasculitis versus controls adjusted p<0.00001; vasculitis versus disease controls adjusted p<0.00001); CD4 Vβ17 (vasculitis versus controls adjusted p=0.005; vasculitis versus disease controls adjusted p=0.0005); and CD8 Vβ1 (vasculitis versus controls adjusted p=0.005; vasculitis versus disease controls adjusted p=0.0001).

To minimise the effect of patient heterogeneity within the primary systemic vasculitis group the data were reanalysed comparing only the 18 patients with classical PAN as defined by the ACR criteria, and who had diagnostic visceral angiography for PAN (one patient satisfying criteria for PAN with positive skin biopsy had a normal visceral angiogram, and so was excluded from this analysis). Again, the variance was increased in the PAN group compared with both control and disease control groups for CD4 Vβ12 (vasculitis versus controls adjusted p<0.00001; vasculitis versus disease controls adjusted p<0.00001); CD4 Vβ17 (vasculitis versus controls adjusted p=0.001; vasculitis versus disease controls adjusted p=0.0001); and CD8 Vβ1 (vasculitis versus controls adjusted p=0.001; vasculitis versus disease controls adjusted p<0.00001).
In the KD and HSP groups, there were no consistent differences in the variance compared with controls and disease controls.

### 3.4.6 T CELL Vβ EXPANSIONS AND DELETIONS

Using the arbitrary definitions of Vβ expansion and deletion described previously, 80% of the primary systemic vasculitis children had one or more CD4 Vβ expansions or deletions compared with 30% of the controls (95% CI of the difference 21-79%, p<0.002) and 37% of the disease controls (95%CI of the difference 16-70%, p<0.002). 15/18 (83%) of the patients with classical PAN (ACR defined, and confirmed on visceral angiography) had one or more CD4 Vβ expansions or deletions, this percentage being significantly higher than the controls (p< 0.002) and disease controls (p=0.002), although there was no clear relationship between the duration of vasculitis prior to blood sampling and the presence of expansions or deletions. There were no differences in the number of expansions or deletions in the CD8 T cell population between the vasculitis and control/disease control groups.

In the KD group, 67% of the patients had one or more CD4 Vβ expansions or deletions, no different from the controls (95% CI of the difference from controls -7-81%, p=0.1; 95% CI of the difference from disease controls -14-73%, p=0.1). There were no differences in the number of expansions or deletions in the KD CD8 T cell population.

In the HSP group there were no differences in the number of CD4 or CD8 expansions or deletions compared with either the control or disease control groups.
3.4.7 LONGITUDINAL STUDIES ON PRIMARY SYSTEMIC VASCULITIS CHILDREN

Overall, there were no differences in the mean percentage of individual Vβ families in either the CD4 or CD8 population of T cells when paired samples from 6 primary systemic vasculitis pre and post-induction of remission were compared. However, there was a greater variance of certain Vβ families prior to induction of remission: CD4 Vβ12 (F ratio 190, adjusted p=0.00001), CD4 Vβ14 (F ratio 296, adjusted p=0.00005), and CD8 Vβ1 (F ratio 81, adjusted p=0.001). Moreover, 5 out of 6 of the active vasculitis group had 1 or more CD4 T cell Vβ expansions or deletions (4 patients with one or more expansions, and one patient with one expansion and one deletion), compared with 1 out of 6 in the remission group (95% CI of observed difference 4-100%, p=0.02). No difference in the numbers of expansions or deletions in the CD8 Vβ repertoire was observed pre and post induction of remission.

3.5 Discussion

There are limited data suggesting that SAgS play an important role in the initiation and/or subsequent relapse of the systemic vasculitides in adults (Cohen Tervaert, Popa, and Bos, 1999; Cohen Tervaert, 2002). No such data exist for the primary systemic vasculitides (PAN, WG, MPA) in children, although a recent small series of 6 children with HSP nephritis in association with staphylococcus infection demonstrated increased percentages in the peripheral blood of T cells bearing Vβ5.2, Vβ5.3, and Vβ8, suggesting
superantigenic stimulation of the immune system in these patients (Hirayama, Kobayashi and others, 2001).

The response of a T cell to superantigenic stimulation is complex, and the observed "skewing" of the Vβ repertoire, which typically follows superantigenic stimulation (figures 2.3 and 2.4) is dependent on several factors. Firstly, timing of the blood sample from the onset of superantigenic exposure is critical, since initially there is Vβ restricted T cell activation, followed by specific T cell Vβ proliferation, and finally Vβ deletion (Leung, Meissner C, and Schlievert, 1997; Shoukry, Lavoie and others, 1997). It is reported that this peripheral Vβ deletion following superantigenic stimulation is the result of apoptosis of activated T cells, although other possibilities including internalisation of T cell receptors (resulting in a state of anergy) (Fraser, Arcus and others, 2000), or sequestration of specific activated Vβ families into sites of tissue inflammation (Leung, Giorno and others, 1995; Yamashiro, Nagata and others, 1996) may also contribute to removal of certain Vβ families from the peripheral circulation. Other influences such as the HLA type of an individual (Herman A, Croteau G and others, 1990), and the recent description of "partially Vβ-restricted" SAggs (Hodtsev AS, Choi Y and others, 1998) could also theoretically affect the ability to detect peripheral Vβ skewing following SAg exposure in some studies. It is therefore feasible that depending on the timing of blood sampling in relation to SAg exposure, or other SAg or host determinants, peripheral T cell Vβ skewing may not be observed, perhaps explaining some of the conflicting data emerging from studies in KD.
The flow cytometric technique for the analysis of T cell activation and T cell Vβ repertoires used in this study was able to demonstrate the classical response of T cells to SAg stimulation in vitro: there was expansion of CD4 and CD8 T cells expressing Vβ2 for TSST-1, and Vβ3 for SEB. This observation was in agreement with what is known about the major responding Vβ families for these 2 SAgS (Cohen Tervaert, Popa, and Bos, 1999; Curtis, Zheng and others, 1995). Furthermore, there was a high degree of T cell activation in response to SAgS at 8 days, as compared to control PBMCs left cultured but unstimulated for 8 days. Further evidence for the validity of this technique was provided rather serendipitously by the presentation of 2 children with classical TSS (one staphylococcal and one streptococcal; the author wishes to add that both children were treated successfully and made complete recoveries). Analysis of the Vβ repertoires in these children revealed expansions of T cell Vβ families compatible with the staphylococcal and streptococcal toxic shock syndromes (Vβ2 and Vβ5.1 respectively (Watanabe-Ohnishi, Low and others, 1995)).

T cell activation as determined by CD69 expression was higher in patients with active vasculitis (all types) and in those with primary systemic vasculitis compared with those with inactive disease, and healthy controls. This result was in contrast to the findings of Christensson et al who showed that adult patients with ANCA-positive vasculitis show an increased expression of T cell activation markers (CD69) irrespective of immunosuppressive therapy or disease activity (Christensson, Pettersson and others, 2000). The reason for this difference may be related to the nature of the vasculitic syndromes examined - ANCA associated vasculitides comprised the minority of subjects.
in the study presented in this chapter (table 3.1). Another possibility may relate to age-related maturational changes in the immune system. It was interesting to note that there was no difference between the active and inactive vasculitis groups in terms of CD25 expression. Thus, in support of the concept of persistent T cell activation by Christensson et al there was some evidence of high CD25 expression within the CD4 and CD8 T cell populations in a few of the patients with a BVAS of zero. These data would support a role for T cells in the pathogenesis of primary systemic vasculitis affecting children. These results also emphasise, however, that observations in adults with vasculitis may not hold true for vasculitis syndromes affecting children.

Despite the inherent limitations of examining peripheral blood T cell Vβ repertoires in patients with long-standing chronic inflammatory disease for evidence of previous SAg exposure, it was possible to demonstrate changes in the Vβ repertoire of certain vasculitis patients. Thus, in the primary systemic vasculitis group (PAN, WG, MPA, and unclassifiable vasculitis), there were minor deviations in the mean percentages of CD4 and CD8 Vβ families, although no consistent difference in the mean Vβ percentages for vasculitis versus both controls and disease controls was observed. Comparison of the Vβ variance, however, revealed significantly increased variance in the primary systemic vasculitis patients for some Vβ families as compared to controls and disease controls: CD4 Vβ12 and CD4 Vβ17, and CD8 Vβ1. Additionally, significantly more "expansions" and "deletions" were observed in the primary systemic vasculitis group than in the control and disease control groups.
As documented in table 3.1, there was a degree of heterogeneity within the primary systemic vasculitis group. These patients were analysed as one group because evidence for superantigenic involvement has been suggested for adult patients with PAN, MPA, and WG. Moreover, there is a greater degree of polyangiitis overlap in children making classification problematic. Nonetheless we were able to demonstrate skewing of the Vβ repertoire when these patients were compared with controls and disease controls. Furthermore, when the 18 patients satisfying classification criteria for classical PAN inclusive of diagnostic angiography were analysed separately, the result was unchanged suggesting that the observed skewing of the Vβ repertoire is not a consequence of patient heterogeneity.

In the KD group, although patient numbers were small, in accordance with the findings of others (Abe, Kotzin and others, 1992; Curtis, Zheng and others, 1995) there was a significantly increased percentage of CD4 Vβ2 in the second week of the illness as compared to controls and disease controls. Interestingly, there was also a significantly increased percentage in CD8 Vβ7 in the KD group. It is suggested that this observation was unlikely to be the result of the presence of genetic polymorphisms, which have been shown to affect Vβ7.2 (Zhao, Whitaker, and Robinson, 1994), since the antibody clone used in this study (ZOE) recognises Vβ7.1. That said, some cross reactivity between this antibody and Vβ7.2 was not formally excluded in this study. In the HSP group, again although patient numbers were small, CD4 Vβ1 T cells were found to be lower in the peripheral circulation than that observed in control and disease control children, but no differences in variance or numbers of Vβ expansions or deletions were observed.
Lastly, paired samples obtained from 6 vasculitis patients (all with classical PAN) before and after induction of remission of vasculitis showed "normalisation" of the Vβ repertoire following treatment. Thus, the variance of CD4 12, 14, and CD8 Vβ1 was significantly greater in the group prior to treatment as compared to those following induction of remission. Also, there were significantly more Vβ expansions in the pre-treatment vasculitis group than the post-treatment group.

Whilst these observations could be compatible with an effect of SAgs on the immune system in patients with vasculitis, it cannot be concluded from this study that SAgs are directly involved in the initiation or relapse of vasculitis. Indeed, the T cell Vβ skewing observed could be an epiphenomenon in these patients. Potential confounding factors include a non-specific skewing of the Vβ repertoire as a result of chronic inflammation, the use of glucocorticoids and immunosuppressants, and prolonged periods of in-patient care (potentially altering carriage of bacterial commensals) in the vasculitis group. Attempts were made to control for these factors, however, by the inclusion of disease controls who were renal allograft recipients with no prior history of vasculitis or other autoimmune disease, and who were treated with a similar (but not identical) range of glucocorticoid and immunosuppressive therapy as the vasculitis children. These children were treated for lengthy periods of time as in-patients at the same institution as the vasculitis group, and thus theoretically exposed to the same range of microbial commensals. Moreover, the disease control group also included children with miscellaneous non-vasculitic autoimmune disease and sepsis. Even with this (arguably)
over-controlled disease control group, it was possible to demonstrate CD4 Vβ skewing in the children with vasculitis that was not observed in the disease controls, even though the latter group displayed a comparable degree of T cell activation (figures 3.1 and 3.2).

Another interesting observation was the lack of similarity between the CD4 and CD8 Vβ changes observed in the vasculitis patients, since SAgs are known to affect both T cell subsets (as in the patient with staphylococcal TSS, and in the in vitro responses of T cells to SAgs). Whilst this observation may mitigate against SAgs as a major influence in these disease states, it should be borne in mind that the signaling events governing the T cell response to SAgs (including activation, expansion, deletion, anergy or tissue sequestration) may be different for CD4 and CD8 T cells, and could vary with time within these T cell subsets.

In conclusion the data presented in this study support a role for T cells in the pathogenesis of primary systemic vasculitides in childhood. Moreover, there were changes in the T cell Vβ repertoire in children with vasculitis over and above those observed in disease controls (who exhibited significant T cell activation, but no T cell Vβ repertoire skewing). Whilst these data provide impetus for further research into this contentious field, they do not unequivocally resolve the question of the role of SAgs in childhood vasculitic syndromes. The results described in this study did however provide enough "smoking-gun" evidence to prompt further study of the mechanisms that SAgs may utilize to cause vascular injury- the subject of Chapter 4.
Chapter 4: Vβ-restricted T cell adherence to endothelial cells:
a mechanism for superantigen dependent vascular injury.

4.1 Summary
4.2 Introduction
4.3 Materials and methods
4.4 Results
4.5 Discussion
4.1 Summary

Introduction and aims: To investigate the potential for endothelial cells (EC) to operate as a superantigen (SAg)-presenting cell for T cells (TC), and the potential for such an interaction to cause EC activation.

Methods: MHC class II⁺ HUVEC were co-cultured for 4 hours with purified TC and the SAg SEB or TSST-1. After staining with fluorescent conjugated monoclonal antibodies flow cytometric analysis was performed on the HUVEC and TC to examine Vβ-restricted TC adherence to the EC monolayer; Vβ-restricted TC activation (CD69 upregulation); and surface expression of EC activation markers.

Results: Co-culture of purified TC with MHC class II⁺ HUVEC and TSST-1 or SEB resulted in Vβ-restricted CD4 and CD8 CD69 upregulation (Vβ2 activation for TSST-1; Vβ3, Vβ 5.1 and Vβ12 activation for SEB). Additionally, there was CD4 and CD8 TC Vβ-restricted adherence to the HUVEC monolayer at 4 hours, which was partially abrogated by blockade of the integrin VLA-4, but not LFA-1. ICAM-1, E-selectin, and VCAM-1 expression were upregulated on the MHC class II⁺ HUVEC following exposure to SAg in the presence of T cells.

Conclusion: MHC class II⁺ EC operate as competent SAg-presenting cells for CD4 and CD8 lymphocytes in vitro. Dual signalling between the EC and TC results in Vβ-restricted activation and adherence to endothelial monolayers, and EC activation. It is proposed that this mechanism could account in part for the vasculitic injury associated with SAg-mediated diseases.
4.2 Introduction

In Chapter 3 data were presented supporting the concept that T cells are involved in the pathogenesis of vasculitis syndromes affecting the young. Moreover, there were changes observed within the CD4 and CD8 T cell Vβ repertoires over and above those observed in disease controls. These observations could be compatible with a SAg-mediated effect, but by no means confirm this beyond all doubt.

An important observation is that T cells infiltrating the walls of coronary arterial aneurysms and the intestinal mucosa of patients with KD show a skewed T cell Vβ repertoire, with increased numbers of cells expressing Vβ2 (Leung, Giorno and others, 1995; Yamashiro Y, Nagata S and others, 1996).

Whilst there are many observational clinical studies providing indirect evidence of superantigenic involvement in the vascular inflammatory process (Barron, 2002), there are only a limited number examining the mechanisms that SAgs may utilize to cause vascular injury. Proposed mechanisms for SAg-induced vascular injury include: 1. direct activation of autoreactive T cells resulting in vessel wall destruction; 2. activation of autoreactive B cells resulting in production of autoantibodies such as anti-neutrophil Cytoplasmic antibodies (ANCA) or anti-endothelial cell antibodies (AECA), which are known to result in vessel injury; and 3. direct binding of SAg to endothelial cells via charge interactions with subsequent antibody binding and immune complex vasculitis (Cohen Tervaert, Popa, and Bos, 1999).
Although the exact aetio-pathogenesis of most vasculitis syndromes is unknown, it is now clearly established that endothelial cells actively participate in the pathogenesis of systemic vasculitis, both as a target for injury, and as active protagonists of the inflammatory process (Cid, 2002). Limited experimental data exist demonstrating that the endothelial cell, following stimulation with interferon-γ (INF-γ) to upregulate MHC class II, can operate as a SAg-presenting cell for T cells resulting in T cell activation and adherence to endothelial monolayers in vitro (Imanishi, Akatsuka and others, 1995; Kita, Eguchi and others, 1996; Uchiyama, Araake and others, 1992). Furthermore, only one study has demonstrated that a consequence of the interaction between the endothelial cell, CD4 T lymphocytes, and the SAg Staphylococcal enterotoxin type B (SEB) is upregulation of the cell adhesion molecules intercellular adhesion molecule type 1 (ICAM-1), vascular cell adhesion molecule type 1 (VCAM-1), and E-selectin on the endothelial cell (Baum, Yaron, and Yellin, 1998). In that study, it was possible to block the SAg-dependent T cell mediated endothelial cell activation using monoclonal antibodies against tumour necrosis factor-α (TNF-α), thus implicating this cytokine as an important mediator of the observed endothelial activation (Baum, Yaron, and Yellin, 1998). This observation, although important since it demonstrated the significance of the endothelial cell both as a SAg-presenting cell and as an amplifier of the vascular inflammatory response, was limited because it did not demonstrate that the endothelial cell activation was the result of Vβ-restricted T cell activation (the hallmark of a SAg-mediated process) and adherence to the endothelium, and precluded the potential
importance of CD8 T cells in SAg-dependent T cell mediated endothelial activation and injury.

In this study the potential for the endothelial cell to operate as a SAg-presenting cell for T cells, and in particular the effect of such an interaction on the T cell in terms of Vβ-restricted activation and adherence to the endothelium was studied in further detail. Furthermore, the effect on the endothelium was assessed by measuring cell adhesion molecule expression.

4.3 Materials and methods

4.3.1 Antibodies, cytokines, and protein reagents

The monoclonal antibodies used in these experiments are described in detail in table 2.1. Also used were blocking antibodies against the α subunit of the integrin LFA-1 (lymphocyte function associated molecule 1, CD11a; clone 38 [a kind gift from Professor Nancy Hogg, Leukocyte Adhesion Laboratory, Imperial Cancer Research Fund] mouse IgG2a anti-human CD11a, used at a concentration of 1.8 μg/ml), or against the α subunit of VLA-4 (CD49d, very late antigen-4; clone MCA697, Serotec, mouse IgG1 anti-human CD49d, used at a concentration of 10 μg/ml). The control antibody for anti-LFA-1 was a mouse IgG2a antibody (clone 4U, a kind gift from Professor Nancy Hogg, used at a concentration of 1.8 μg/ml), and the control for anti-VLA-4 was a mouse IgG1 antibody (clone MCA928, Serotec, used at a concentration of 10 μg/ml). Again, as previously
described all antibody dilutions were made with 0.01 M phosphate buffered saline with 0.1% sodium azide, and checked by plotting a dilution curve.

4.3.2 Isolation of human umbilical vein endothelial cells (HUVEC)

HUVEC were prepared as described in sections 2.4.6-2.4.13. In experiments where HUVEC were stimulated with INF-γ for 48-72 hours, this cytokine was added when the HUVEC reached approximately 80% confluency, and the medium containing INF-γ removed prior to experiments. All HUVEC used in experiments were first to third passage.

4.3.3 PBMC and T cell incubation with SAg: determination of Vβ-specific activation

To examine the Vβ-specific activation of T cells to SAGs and the requirement of MHC class II⁺ cells for SAg presentation to T cells, PBMCs or purified T cells were incubated in 6 or 24-well tissue culture plates in the presence or absence of either TSST-1 (10ng/ml) or SEB (100ng/ml) for 4 hours at 37°C. These experiments were different from the experiments described in section 2.4.5, which determined changes in the percentages of the T cell Vβ families in response to SAGs after 8 days. Again, the concentrations of SAg used were derived from previous studies (Baum, Yaron, and Yellin, 1998; Curtis, Zheng and others, 1995).

Analysis of Vβ-specific activation was then determined by flow cytometric analysis of CD69 expression within individual Vβ families as described below in section 4.3.5. In some experiments the incubation was continued for 24 hours in order to assess the
relevance of the remaining 0.2% of cells expressing MHC class II in the purified T cell population.

4.3.4 HUVEC-T cell co-culture experiments

Monolayers of HUVEC (with and without pre-treatment with INF-γ 1000 U/ml for 48-72 hours to upregulate MHC class II) were co-cultured in the presence of T cells (1 million/ml), T cells and SAg (TSST-1 10 ng/ml; or SEB 100 ng/ml), or TNF-α 100 ng/ml for 4 hours at 37°C. The HUVEC monolayer was then dispersed using trypsin/EDTA for 1 minute and the HUVEC prepared for flow cytometry as described in section 2.4.12. To examine T cell adherence, the HUVEC monolayer was washed 4 times with PBS to remove non-adherent T cells, and again the HUVEC-adherent T cell monolayer dispersed with trypsin/EDTA and prepared for flow cytometry. Supernatants (including PBS washes) containing the non-adherent T cell population were centrifuged at 500G for 8 minutes and the non-adherent T cell population frozen at -80°C in FCS containing 10% DMSO for further analysis. The cell-free supernatants were also frozen for analysis of endothelial microparticles (see below). The plate plan for these co-culture experiments is shown in Appendix 7.

4.3.5 Flow cytometry of T cells and HUVEC

3-colour FACS analysis of PBMCs or T cells from experiments was performed as previously described. In these experiments, an abbreviated panel of Vβ-specific antibodies was used (Vβ1, Vβ2, Vβ3, Vβ5.1, Vβ8.1, and Vβ12) which included the
known major responding Vβ families to TSST-1 and SEB. As described, T cells were identified by gating for forward and light-scatter characteristics, CD4⁺ or CD8⁺ populations, and the different Vβ families. Vβ-specific activation was then determined by gating on the individual Vβ family sub-population (CD4 or CD8 and Vβ double-positives) and then plotting a histogram of CD69 fluorescence, with determination of the CD69 median fluorescence index (MFI) for each Vβ family in the CD4 and CD8 sub-population of T cells. Single colour FACS analysis of HUVEC was as described in section 2.4.12.

4.3.6 Integrin blockade of SAg-mediated T cell adherence to HUVEC

To investigate potential molecular mechanisms of the SAg mediated Vβ-specific T cell adhesion to HUVEC, MHC class II⁺ HUVEC were again co-cultured with purified T cells, but this time the T cells were pre-incubated for 30 minutes with monoclonal antibodies against the α subunit of the integrin LFA-1 (lymphocyte function associated molecule 1, CD11a; clone 38 [a kind gift from Professor Nancy Hogg, Leukocyte Adhesion Laboratory, Imperial Cancer Research Fund] mouse IgG2a anti-human CD11a, used at a concentration of 1.8 µg/ml), or against the α subunit of VLA-4 (CD49d, very late antigen-4; clone MCA697, Serotec, mouse IgGl anti-human CD49d, used at a concentration of 10 µg/ml). Pre-incubation with isotype control antibodies used at the same concentrations as the monoclonal antibodies against the α integrins served as control conditions. Thus the control antibody for anti-LFA-1 was a mouse IgG2a antibody (clone 4U, a kind gift from Professor Nancy Hogg, used at a concentration of
1.8 µg/ml), and the control for anti-VLA-4 was a mouse IgG1 antibody (clone MCA928, Serotec, used at a concentration of 10 µg/ml). Following the pre-incubation period, the blocking or control antibodies were left in the co-culture, and thus the T cells (at an exact concentration of 10⁶ /ml) plus blocking antibodies were added in the presence or absence of the SAgs TSST-1 or SEB. Vβ-specific T cell adherence after 4 hours incubation was assessed as described above, and the effect of the blocking antibodies was assessed in 4 ways: 1. By phase-contrast light microscopy; 2. By counting the absolute number of total adherent lymphocytes using flow cytometry; 3. By analysis of the adherent Vβ repertoires expression (expressed as the percentage of the CD4 or CD8 populations); and 4. By analysis of Vβ-specific CD69 expression within the adherent population of T cells. The plate plan for these experiments is given in Appendix 8. These sets of experiments were repeated a total of 3 times for LFA-1 blockade: twice for TSST-1, and once for SEB; and once for VLA-4 blockade (TSST-1 only). As such, no statistics were performed on the data generated from these experiments.

**4.3.7 Statistics**

Results from different experimental conditions were compared using the Kruskall Wallis and Mann-Whitney tests, with significance set at p<0.05. All statistics were performed using SPSS version 10.0.
4.4 Results

4.4.1 SAgs activate T cells in a Vβ-specific manner, and require MHC class II expressing cells.

To confirm the ability of SAgs to activate T cells in a Vβ-specific manner, and the requirement of MHC class II-expressing cells to act as SAg-presenting cells for T cells, PBMCs (containing T cells and class II expressing monocytes) or purified T lymphocytes (greater than 98% CD3 positive, and containing approximately 0.2% MHC class II positive cells) were incubated with the SAgs TSST-1 (10 ng/ml) or SEB (100 ng/ml) for 4 hours at 37°C. The CD4 and CD8 T cell populations were then analysed using 3-colour FACS analysis for Vβ-specific activation as determined by expression of the T cell activation induction molecule CD69. Incubation of PBMCs with TSST-1 resulted in upregulation of CD69 on CD4Vβ2 (figure 4.1a) and CD8Vβ2 T cells (figure 4.1b), whereas SEB resulted in upregulation of CD69 on CD4Vβ3 and Vβ12 (figure 4.1a), and CD8Vβ3 and Vβ12 T cells, with a lesser (but reproducible) response in CD8Vβ5.1 (figure 4.1b). Incubation of purified T cells (devoid of MHC class II-expressing cells) with SAg for 4 hours, however, did not result in Vβ-specific CD4 or CD8 T cell upregulation of CD69 (figure 4.2a and 4.2b).
Figure 4.1 PBMC incubation with SAg (TSST-1 or SEB) results in Vβ restricted CD69 upregulation at 4 hours.

Bold arrows indicate major responding Vβ families within the CD4 (figure 4.1a) and CD8 (figure 4.1b) populations of lymphocytes.
Figure 4.1: CD69 expression on lymphocytes after incubation of peripheral blood mononuclear cells (PBMC) with SAg (4 hours)

Figure 4.1A: PBMC incubation with SAg: CD4 T cell CD69 expression

Figure 4.1B: PBMC incubation with SAg: CD8 T cell CD69 expression
Absence of Vβ restricted CD69 upregulation.
Figure 4.2: CD69 expression on lymphocytes after incubation of purified T cells (CD3⁺) with SAg (4 hours)

Figure 4.2A: 4 hour incubation of purified T cells with SAg: CD4 T cell expression of CD69

Figure 4.2B: 4 hour incubation of purified T cells with SAg: CD8 T cell expression of CD69
4.4.2 HUVEC upregulate MHC class II in response to INF-γ and become competent SAg-presenting cells for T cells resulting in Vβ-specific T cell activation and adherence to HUVEC monolayers

Pre-treatment of HUVEC with INF-γ (1000 IU/ml) resulted in upregulation of MHC class II at 48-72 hours (figure 4.3). To further assess the competence of class II-expressing HUVEC to present SAg to T cells, monolayers of HUVEC with and without pre-treatment with INF-γ for 48 hours were co-cultured in the presence or absence of purified T cells for 4 hours with TSST-1 (10 ng/ml) or SEB (100 ng/ml). After the incubation non-adherent T cells were removed by washing 4 times with PBS with 10% FCS. FACS analysis of the adherent and non-adherent T cell populations was then performed specifically examining for CD4 and CD8 Vβ-specific activation, and Vβ-specific adherence to the HUVEC monolayer.

Only HUVEC which had been pre-treated with INF-γ were able to present SAg to T cells resulting in Vβ-specific CD4 and CD8 upregulation of CD69 on the T cells which were adherent to the HUVEC monolayer: for TSST-1 there was activation of CD4Vβ2 (p=0.017) and CD8Vβ2 activation (p=0.028) (figures 4.4a and 4.4b); for SEB there was activation of CD4Vβ3 (p=0.012), CD4Vβ5.1 (p=0.025), CD4Vβ12 (p=0.016), CD8Vβ3 (p=0.004), and CD8Vβ12 (p=0.025) (figures 4.4c and 4.4d). Non-adherent T cells (CD4 or CD8) did not demonstrate Vβ-specific upregulation of CD69 (p= non-significant for all CD4 and CD8Vβ families; figures 4.5a-d).
Figure 4.3: Upregulation of MHC class II on HUVEC by INF-\(\gamma\)

No INF-\(\gamma\) (control)

INF-\(\gamma\) 48 hours

HUVEC MHC class II Phycoerythrin Fluorescence
Co-culture of MHC class II positive HUVEC with T cells in the presence of SAgS TSST-1 (figures 4.4a and 4.4b) or SEB (figures 4.4c and 4.4d) resulted in upregulation of CD69 on CD4Vβ2^ and CD8Vβ2^ cells (TSST-1), and CD4Vβ3, CD4Vβ5.1 and CD4Vβ12, and CD8Vβ3 and CD8Vβ12 (SEB). Bold arrows indicate statistical significance (p<0.05).
Figure 4.4: Vβ specific CD4 and CD8 T lymphocyte activation within the adherent population of T cells (mean, SEM of 4 experiments)

Figure 4.4A: Vβ specific CD4 activation of adherent T cell population in response to TSST-1

Figure 4.4B: Vβ specific CD8 activation of adherent T cell population in response to TSST-1

Figure 4.4C: Vβ specific CD4 activation of adherent T cell population in response to SEB

Figure 4.4D: Vβ specific CD8 activation of adherent T cell population in response to SEB
Co-culture of MHC class II positive HUVEC with T cells in the presence of SAgS TSST-1 (figures 4.5a and 4.5b) or SEB (figures 4.5c and 4.5d) did not result in Vβ specific activation of T cells (CD4 or CD8) within the non-adherent population (p=non-significant for all Vβ families).
Figure 4.5: Vβ specific CD4 and CD8 T lymphocyte activation within the non-adherent population of T cells (mean, SEM of 4 experiments)

Figure 4.5A: Vβ specific CD4 activation of non-adherent T cell population in response to TSST-1

Figure 4.5B: Vβ specific CD8 activation of non-adherent T cell population in response to TSST-1

Figure 4.5C: Vβ specific CD4 activation of non-adherent T cell population in response to SEB

Figure 4.5D: Vβ specific CD8 activation of non-adherent T cell population in response to SEB
Moreover, T cell Vβ-specific adherence to class II expressing HUVEC was observed (again Vβ2 for TSST-1, and Vβ3 and Vβ12 for SEB), and although this was true for both CD4 and CD8 T cells, Vβ-specific adherence was greater for CD4 T cells than CD8 T cells (figures 4.6a-d). In those experimental conditions where there was Vβ-specific adherence to the HUVEC monolayer, there was a concomitant decrease in the corresponding Vβ family in the non-adherent population of CD4 and CD8 T cells. These data are summarised as “Vβ ratios”, i.e. the ratio of the percentage of adherent to non-adherent Vβ families for each experimental condition. Thus, if there was no specific Vβ adherence to the HUVEC monolayer, the Vβ ratio was close to unity; if there was increased adherence to the HUVEC monolayer (and conversely a decrease in the percentage of that Vβ family within the non-adherent population) the Vβ ratio was greater than 1. In response to co-culture of T cells with MHC class II⁺ HUVEC in the presence of TSST-1, the mean Vβ ratio was increased for CD4Vβ2, and CD8Vβ2 and CD8Vβ5.1 (figures 4.7a and 4.7b). In response to co-culture of T cells with MHC class II⁺ HUVEC in the presence of SEB the mean Vβ ratio was increased for Vβ3 and Vβ12 (true for both CD4 and CD8 lymphocytes; Figures 4.7c and 4.7d).
Figure 4.6: Vβ-specific T cell adherence to HUVEC

Figure 4.6A: TSST-1 induced Vβ specific CD4 adherence to HUVEC monolayer

Figure 4.6B: TSST-1 induced Vβ specific CD8 adherence to HUVEC monolayer

Figure 4.6C: SEB induced Vβ specific CD4 adherence to HUVEC monolayer

Figure 4.6D: SEB induced Vβ specific CD8 adherence to HUVEC monolayer
The Vβ ratio was calculated by calculating the ratio of the percentage of adherent to non-adherent Vβ families for each experimental condition. Thus, if there was no specific Vβ adherence to the HUVEC monolayer, the Vβ ratio was close to unity; if there was increased adherence to the HUVEC monolayer the Vβ ratio was greater than 1. Bold arrows indicate statistical significance (all p=0.01). The broken arrow indicates statistical significance (p=0.01) but a lesser-responding Vβ family.
Figure 4.7: CD4 and CD8 Vβ ratios in response to TSST-1 (figures 4.7a and 4.7b) and SEB (figures 4.7c and 4.7d)
4.4.3 SAg-dependent T cell-mediated endothelial cell activation: upregulation of cell adhesion molecules

To assess the effect on the endothelial cell of V\(\beta\) specific T cell activation and adherence to the HUVEC monolayer, HUVEC cell adhesion molecule expression was determined in the different T cell co-culture experimental conditions described above using flow cytometry. Incubation of HUVEC with TNF-\(\alpha\) at 100 ng/ml served as a positive control in these experiments. At 4 hours there was upregulation of E-selectin (4.4-fold), ICAM-1 (1.2-fold), and VCAM-1 (3.3-fold) on HUVEC which had been pre-incubated with INF-\(\gamma\) and co-cultured with SAg (TSST-1 or SEB) and T lymphocytes which was of a comparable or greater magnitude to that observed in response to incubation with 100ng/ml of TNF-\(\alpha\) (figure 4.8- data for TSST-1 shown). No upregulation of P-selectin on HUVEC was observed at 4 hours in any experimental condition (data not shown). No upregulation of E-selectin, ICAM-1 or VCAM-1 was observed on HUVEC that had not been pre-incubated with INF-\(\gamma\). There was a small increase in VCAM-1 expression on HUVEC pre-treated with INF-\(\gamma\) and incubated with T cells alone (1.6-fold), but this was less than the VCAM-1 upregulation that was observed in the presence of T cells and SAg (either TSST-1 or SEB; data for TSST-1 shown only, figure 4.8).
Up-regulation of E-selectin, VCAM-1, and to a lesser extent ICAM-1 (expressed as percentage “high expression” and median fluorescence index [MFI]) only occurred on MHC class II+ HUVEC in the presence of TSST-1 (10 ng/ml) and T cells: thus the cell adhesion molecule expression was TSST-1 dependent and T cell-mediated. Incubation with TNF-α (100 ng/ml) served as a positive control.
Figure 4.8: TSST-1 dependent T cell-mediated endothelial cell activation

**E-selectin**

<table>
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<td>HUVEC (MHC class II-) + T cells</td>
<td>13% MFI 2.1</td>
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<tr>
<td>HUVEC (MHC class II+) + T cells</td>
<td>18% MFI 2.7</td>
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<tr>
<td>HUVEC (MHC class II-) + T cells + TSST-1</td>
<td>12% MFI 1.9</td>
</tr>
<tr>
<td>HUVEC (MHC class II+) + T cells + TSST-1</td>
<td>62% MFI 22.5</td>
</tr>
<tr>
<td>HUVEC (MHC class II-) + TNF-α 100 ng/ml</td>
<td>40% MFI 12</td>
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**ICAM-1**

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<td>HUVEC (MHC class II+) + T cells</td>
<td>37% MFI 14.0</td>
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<td>36% MFI 9.3</td>
</tr>
<tr>
<td>HUVEC (MHC class II+) + T cells + TSST-1</td>
<td>47% MFI 17</td>
</tr>
<tr>
<td>HUVEC (MHC class II-) + TNF-α 100 ng/ml</td>
<td>48% MFI 17</td>
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**VCAM-1**

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<td>27% MFI 8.2</td>
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<td>HUVEC (MHC class II-) + T cells + TSST-1</td>
<td>18% MFI 3.6</td>
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<tr>
<td>HUVEC (MHC class II+) + T cells + TSST-1</td>
<td>53% MFI 19.0</td>
</tr>
<tr>
<td>HUVEC (MHC class II-) + TNF-α 100 ng/ml</td>
<td>30% MFI 9.0</td>
</tr>
</tbody>
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4.4.4 Integrin blockade of SAg-mediated T cell adherence to HUVEC

Pre-incubation of T cells with anti-LFA-1 resulted in a decrease in the absolute number of T cells adherent to the MHC class II⁺ HUVEC monolayer (true for both TSST-1 and SEB), but an increase in the percentage of adherent Vβ2 for TSST-1, or Vβ3 and Vβ12 for SEB. This was true of both the CD4 and CD8 lymphocyte populations and consistent over all three experiments (twice for TSST-1 and once for SEB), and suggested that the integrin LFA-1 (the ligand for ICAM-1) blocked predominantly T cell adhesion caused by HLA mismatch between the T cells and HUVEC, but not the true SAg-mediated adhesion. Figure 4.9 illustrates these results from a single representative experiment using phase-contrast light microscopy (data for TSST-1 shown). Figure 4.10
Legend for figure 4.9: Phase-contrast light microscopy (medium power) demonstrating adherent T cells on a background of HUVEC. The HUVEC appear spindle-shaped because they have been incubated with INF-γ for 48 hours to upregulate MHC class II. Blockade of LFA-1 or VLA-4 by pre-incubation of T cells with monoclonal antibodies against these integrins resulted in decreased T cell adhesion (figure 4.9B and 4.9D), but in the case of LFA-1 blockade the percentage of T cells expressing Vβ2 (for TSST-1) or Vβ3 and Vβ12 (for SEB) increased. This result suggested that LFA-1 blocked non-specific T cell adherence caused by HLA-mismatch and did not affect SAg-mediated T cell adherence.
Figure 4.9: Integrin blockade of TSST-1 mediated T cell adherence to MHC ClassII$^+$ HUVEC (order of pictures corresponds to plate plan given in Appendix 8)

4.9A: MHC class II+ HUVEC, T cells, TSST-1 (10 ng/ml)
4.9B: MHC class II+ HUVEC, T cells, TSST-1 (10 ng/ml), anti-LFA-1 (1.8 mcg/ml)
4.9C: MHC class II+ HUVEC, T cells, TSST-1 (10 ng/ml), control antibody for anti-LFA-1 (1.8 mcg/ml)
4.9D: MHC class II+ HUVEC, T cells, TSST-1 (10 ng/ml), anti-VLA-4 (10 mcg/ml)
4.9E: MHC class II+ HUVEC, T cells, TSST-1 (10 ng/ml), control antibody for anti-VLA-4 (10 mcg/ml)
Figure 4.10: Absolute number of adherent T cells in response to 4 hour co-culture of MHC class II+ HUVEC with SAg (TSST-1 or SEB): effect of LFA-1 blockade.

**Figure 4.10A: Absolute number of adherent T cells:**

**TSST-1**

- □ TSST-1: 4 hours
- □ TSST-1 & LFA-1 blockade: 4 hours
- □ TSST-1 & control antibody: 4 hours

**Figure 4.10B: Absolute number of adherent T cells:**

**SEB**

- ■ SEB: 4 hours
- □ SEB & LFA-1 blockade: 4 hours
- □ SEB & control antibody: 4 hours
demonstrates the absolute adherent T cell counts using flow cytometry and confirms the visual representation of these data given in 4.9 (data for TSST-1 and SEB given, single representative experiments each). Figure 4.11 illustrates the Vβ repertoire of the adherent T cell population (from a single representative experiment). In addition, the T cells which were adherent displayed Vβ-specific activation as determined by CD69 expression (Vβ2 for TSST-1; Vβ 3, Vβ12, and to a lesser extent Vβ5.1 for SEB). Again this was true for both CD4 and CD8 lymphocyte populations. These data are summarised in figure 4.12.

In contrast, pre-incubation of T cells with anti-VLA-4 resulted in a decrease in the absolute number of adherent T cells (CD4 and CD8) in response to TSST-1 (figure 4.13), with no concomitant increase in the percentage of adherent Vβ2 T cells (figure 4.14). In addition, analysis of the Vβ-specific activation profile following VLA-4 blockade revealed a decrease in adherent T cell Vβ2 CD69 expression (figure 4.15). Taken together, these two observations suggested that blockade of VLA-4 resulted in a decrease of both non-specific T cell adherence (occurring as a result of HLA-mismatch) and TSST-1 mediated T cell adhesion.
Figure 4.11: Vβ repertoire of adherent T cells in response to 4 hour co-culture of MHC class II+ HUVEC with SAg (TSST-1 or SEB): effect of LFA-1 blockade.

Figure 4.11A: CD4 V-beta repertoire of adherent T cells

Figure 4.11B: CD8 V-beta repertoire of adherent T cells

Figure 4.11C: CD4 V-beta repertoire of adherent T cell population

Figure 4.11D: CD8 V-beta repertoire of adherent T cell population
Figure 4.12: Vβ-specific CD69 expression of adherent T cells in response to 4 hour coculture of MHC class II+ HUVEC with SAg (TSST-1 or SEB): effect of LFA-1 blockade.

Figure 4.12A: Adherent CD4 CD69 MFI: TSST-1

Figure 4.12B: Adherent CD8 CD69 MFI: TSST-1

Figure 4.12C: Adherent CD4 CD69 MFI: SEB

Figure 4.12D: Adherent CD8 CD69 MFI: SEB
Figure 4.13: Absolute number of adherent T cells in response to 4 hour co-culture of MHC class II+ HUVEC with TSST-1: effect of VLA-4 blockade.
Figure 4.14: Vβ repertoire of adherent T cells in response to 4 hour co-culture of MHC class II+ HUVEC with TSST-1: effect of VLA-4 blockade.

**Figure 4.14A: CD4 V-beta repertoire of adherent T cells**

![CD4 V-beta repertoire graph](image)

**Figure 4.14B: CD8 V-beta repertoire of adherent T cells**

![CD8 V-beta repertoire graph](image)
Figure 4.15: Vβ-specific CD69 expression of adherent T cells in response to 4 hour co-culture of MHC class II+ HUVEC with TSST-1: effect of VLA-4 blockade.

**Figure 4.15A: Adherent CD4 CD69 MFI: TSST-1**

**Figure 4.15B: CD8 CD69 MFI: TSST-1**
4.5 Discussion

The prototypic SAg-mediated disease is the toxic shock syndrome (TSS), a condition first described in 1978 associated with infection with Staphylococcus aureus (Todd, Fishaut and others, 1978). This entity is characterised by acute onset of fever, shock, and multi-organ dysfunction- the consequence of massive T cell activation and cytokine production in response to SAgS. Histological examination of tissue from patients with this disease typically reveals perivascular lymphocytic infiltration (Larkin, Williams and others, 1982; Paris, Herwaldt and others, 1982). More recently, there has been increased interest in SAgS as modifiers of several autoimmune disease states, including the systemic vasculitides. The evidence for this presently remains circumstantial. However, the best evidence has been provided by the observation of peripheral blood and lesional tissue T cell Vβ skewing in KD (Abe, Kotzin and others, 1992; Curtis, Zheng and others, 1995; Leung, Giorno and others, 1995; Leung, Meissner C, and Schlievert, 1997; Yamashiro Y, Nagata S and others, 1996), and peripheral blood T cell Vβ skewing in microscopic polyangiitis (Giscombe, Grunewald and others, 1995; Simpson, Skinner and others, 1995).

The results of the present study outlined in this chapter provide one possible mechanism whereby SAgS may initiate and maintain vascular injury, namely by utilising the endothelial cell as a SAg-presenting cell for T lymphocytes. This mechanism is attractive because it would result in large numbers of T cells becoming activated directly on the
surface of the endothelium, and could explain the observation of upregulated cell adhesion molecules on endothelial cells observed in most vasculitis syndromes, and the persistence of the skewed Vβ T cell infiltrate in the perivascular compartment in lesional tissue from KD patients.

Perhaps under-emphasised in the literature is the role that cytotoxic CD8 T lymphocytes may play in the immune response to SAgs. A common misconception is that CD8 lymphocytes do not respond to SAgs since they classically recognise conventional antigens presented on the surface of cells in association with MHC class I molecules (Janeway CA, Travers P and others, 1999; Mingari, Cambiaggi and others, 1996). This study, in accordance with others (Herrmann, Baschieri and others, 1992) and in keeping with the results presented in Chapter 3, has clearly demonstrated that CD8 lymphocytes do become activated in a Vβ-restricted manner (and also will proliferate in a Vβ-restricted manner- Chapter 3) in response to SAgs, and that this is dependent upon the presence of SAg-presenting cells (monocytes or endothelial cells) expressing MHC class II. This result is worthy of emphasis since some workers have suggested that the presence of activated cytotoxic CD8 lymphocytes, which have been observed in lesional tissue in patients with KD, is evidence of a conventional antigenic-driven process and precludes a superantigenic-driven process (Brown, Crawford and others, 2001). Injection of SEB into mice leads to a strong and transient activation of both CD4 and CD8 lymphocytes (Herrmann, Baschieri and others, 1992). These CD8 lymphocytes are highly cytotoxic towards MHC class II expressing cells between 2 and 3 days after injection of SEB ex
vivo. It is thus now clear that CD8 lymphocytes also play an important role in the immune response to SAgs, and may be responsible in part for the vascular injury associated with SAg-mediated diseases.

The data presented in this chapter also demonstrate that the endothelial cell can operate as a competent SAg-presenting cell following upregulation of MHC class II by INF-γ. Moreover, the consequence of this interaction is CD4 and CD8 T cell Vβ-restricted activation and adherence to the HUVEC monolayer, with subsequent upregulation of HUVEC cell adhesion molecules, a situation which could conceivably result in a state of chronic endothelial cell activation and ultimately vascular injury. Observations in vivo which support such a mechanism include the presence of increased numbers of Vβ2 lymphocytes in lesional tissue in KD (Leung, Giorno and others, 1995; Yamashiro Y, Nagata S and others, 1996), and upregulation of MHC class II on endothelial cells in the lesional tissue of many chronic inflammatory syndromes (Barkley, Allard and others, 1989; Koretz, Momburg and others, 1987), including KD (Naoe S, personal communication).

The results of this study which demonstrate Vβ-restricted T cell adherence to HUVEC are in accordance with a previous in vitro study which demonstrated a preferential adhesion of murine Vβ8.1,2 splenic T cells to a human endothelial cell line (Kita, Eguchi and others, 1996). In that study, the response was mainly within the CD4 population of T cells. This process was dependent on the expression of MHC class II (DR and DQ) on the
endothelial cells, and the authors were able to inhibit the enhanced Vβ-restricted adhesion with monoclonal antibodies directed against human ICAM-1, but not with antibodies directed against human VCAM-1. This result was in contrast to the findings of Siegelman and others who demonstrated that the binding of CD44 on activated lymphocytes to endothelial hyaluronan (HA) mediates primary adhesion under sheer-stress, followed by further firm adhesion involving predominantly the integrin VLA-4 (on T cells activated with SEB) and VCAM-1 interaction, and with only a minor contribution from the integrin LFA-1 (Siegelman, Stanescu, and Estess, 2000).

Because of time constraints only a limited number of experiments examining the blockade of SAg-mediated T cell adhesion were performed in this study. Nonetheless, each single experiment inherently had several built-in controlling conditions since several non-SAg responding Vβ families were examined in each experiment. Thus, although these data would constitute preliminary results, they are worthy of some description and comment. Using monoclonal antibodies against the α integrins LFA-1 (the ligand for ICAM-1, ICAM-2, and ICAM-3), and against the α integrin VLA-4 (the ligand for VCAM-1 and fibronectin) it was possible to block some of the TSST-1-mediated adhesion of T cells to MHC class II+ HUVEC as determined by a decrease in the absolute number of T cells adherent, with little change in the percentage of adherent Vβ2 CD4 or CD8 lymphocytes. Moreover, of those Vβ2 T cells that were adherent, there was a decrease in the CD69 expression of these cells following blockade of VLA-4. That there was no decrease in the percentage of Vβ2 adherent T cells does imply, however, that
blockade of VLA-4 did not completely abrogate the TSST-1-mediated T cell adhesion and points to other mechanisms mediating T cell adhesion caused by SAgS. This result was in contrast to the effect of blockade of LFA-1 which resulted in a decreased absolute number of adherent T cells, but a definite rise in the percentage of SAg-specific Vβ adhesion- a result that was observed in all 3 experiments performed examining this situation (twice for TSST-1, and once for SEB). Thus in summary, these observations would suggest that the integrin VLA-4 (but not LFA-1) on activated CD4 and CD8 T cells interacts with VCAM-1 (or fibronectin) on activated HUVEC and contributes in part to the T cell adhesion mediated by SAgS in this non-flow model.

The data derived from these attempts at integrin blockade of T cell adhesion also emphasise an important limitation of the model described in this study, namely that the T cells and HUVEC co-cultured were not HLA-matched. Thus any results obtained are occurring on the background of a mixed lymphocyte-endothelial cell reaction. That said, all the experiments described in this chapter were designed specifically to control for this important confounding factor. Thus, at 4 hours, the data were relatively “clean” and in keeping with the finding of others (Baum, Yaron, and Yellin, 1998). At 24 hours, however, there was evidence of HUVEC activation and injury that may not have been entirely caused by SAg-dependent T cell mediated mechanisms, and it is likely that some of the “noise” in the model was the result of the mixed T cell-endothelial reaction.
The data presented here are also in keeping with previous work demonstrating that INF-γ upregulates MHC class II on HUVEC thus inferring Staphylococcal enterotoxin A (SEA)-binding activity on the HUVEC for activation of human T cells (Uchiyama, Araake and others, 1992). In that study, antibodies against MHC class II blocked the increased SEA-binding activity on HUVEC in response to INF-γ.

Thus it is now apparent that the interaction between T cells (CD4 and CD8) and endothelial cells in the presence of SAg results in adherence of T cells to the endothelium via complex interactions involving more than one mechanism. So far, some of the previously published experimental data are conflicting although a thorough understanding of the mechanisms of T cell adherence and activation following SAg stimulation may add to the armamentarium of molecular blockade of potential injurious pathways culminating in vascular injury in SAg-mediated disease states. For example, it remains to be seen what contribution the newly-discovered SAg-peptide antagonists (Arad G, Levy R and others, 2000) may have in the blockade of SAg-dependent T cell mediated endothelial activation and endothelial adherence.

In conclusion, based on the results presented in this chapter and accepting important limitations to the in vitro model described, it is proposed that MHC class II-expressing endothelial cells operate as competent SAg-presenting cells for CD4 and CD8 lymphocytes. This results in increased T cell adherence to endothelial monolayers in vitro.
mediated in part by VLA-4/VCAM-1 interactions, and Vβ-restricted CD4 and CD8 T cell activation within the adherent population of lymphocytes. The consequence of this interaction is upregulation of endothelial cell adhesion molecules, although, care must be taken not to overly extrapolate the grossly unphysiological environment of the in vitro model to the in vivo situation.

Whilst this observation is potentially important, the finding of endothelial cell activation may not correlate with endothelial injury, per se. To further investigate this matter further, Chapter 5 considers in further detail the consequence to the endothelial cell following the interaction between SAgs and T cells, namely the generation of a recently described surrogate marker of endothelial injury- endothelial microparticles (EMP).
Chapter 5: Endothelial and platelet microparticles in childhood vasculitides: a window to the activated endothelium in vasculitis of the young.

5.1 Summary

5.2 Introduction and background on EMP

5.3 Methods

5.4 Results

5.5 Discussion
5.1 Summary

Introduction and aims: Microparticles (MP) are released from endothelial cells in response to a variety of injurious stimuli and recently have been shown to be increased in a number of diseases associated with endothelial dysfunction. In chapter 4 it was established that HUVEC activation occurred in response to SAg-T cell interactions. In this chapter, it is demonstrated that endothelial microparticles (EMP) were released from HUVEC into the supernatant as a consequence of SAg-dependent T cell mediated endothelial cell activation. This observation led to the in vivo studies presented in this chapter, which examined EMP and platelet MP (PMP) profiles in children with systemic vasculitis (SV) to test the hypothesis that EMP may provide a tool for the diagnosis and monitoring of disease activity, and could provide insight into the endothelial injury associated with childhood vasculitis syndromes.

Methods: Supernatants from the HUVEC-T cell co-culture experiments described in section 4.3.4 were analysed for EMP. Patients studied were 39 children with SV at various stages of disease activity, 24 febrile disease control children, and a control group of 43 healthy subjects. Supernatants from in vitro experiments, or plasma from subjects was ultracentrifuged at 17000G for 60 minutes, and the MP pellet examined using flow cytometry.

Results: In addition to HUVEC activation in response to co-culture with T cells and SAg at 4 hours, there was increased EMP release from the activated HUVEC which occurred earlier and was of a greater magnitude to that observed in response to TNF-α. Plasma from patients with active SV contained a 8.5-fold elevation of E-selectin positive EMP compared with patients in remission (p=0.000), a 5.6 fold elevation compared with
controls (p=0.000), and a 10.4-fold elevation compared with disease controls (p=0.000).

A similar result was obtained for EMP expressing the marker CD105 (endoglin). EMP correlated with the Birmingham Vasculitis Activity Score and acute phase reactants in the patients with SV, but there was overall a poor correlation between EMP and acute phase reactants in the disease controls. The test characteristics of EMP for the diagnosis of active vasculitis were superior to previously published data regarding visceral angiography in childhood PAN.

**Conclusion:** EMP may provide a "window" to the activated endothelium, and these data suggest that they may be useful diagnostically and for the monitoring of disease activity in SV of childhood.
5.2 Introduction

In Chapter 4 it was established that MHC class II\(^+\) HUVEC could operate as competent SAg-presenting cells resulting in T cell activation and adherence to HUVEC monolayers. HUVEC, in turn, become activated and upregulate cell adhesion molecules. Whether this endothelial activation correlates with injury, however, is unclear.

The studies outlined in this chapter examined in more detail the consequence to the endothelial cell of SAg-dependent T cell mediated endothelial cell activation by examining a novel surrogate marker of endothelial injury: the generation of endothelial microparticles (EMP). The \textit{in vitro} experiments and observations described in this chapter led to the \textit{in vivo} study of EMP in children with vasculitis subsequently described. Some further background information on the biology of cellular and platelet microparticle formation is first required.

5.2.1 Cellular membrane phospholipid asymmetry: pathophysiological implications

Membrane phospholipid asymmetry is a feature of all eukaryotic cells, and is tightly regulated by several energy dependent enzymatic processes (Zwaal and Schroit, 1997). The outer leaflet of eukaryotic cell membranes is formed predominantly by the cholinephospholipids (sphingomyelin [SM] and phosphatidylcholine [PC]), whereas the inner leaflet is largely comprised of the aminophospholipids (phosphatidylserine [PS] and phosphatidylethanolamine [PE]) (Zwaal and Schroit, 1997). It is now apparent that cells invest energy to maintain this phospholipid asymmetry, and the process is controlled by 3 enzymes- aminophospholipid translocase, floppase, and lipid scramblase. The first 2
enzymes are ATP-dependent, and the last enzyme is calcium dependent, but ATP independent (Zwaal and Schroit, 1997).

A general feature of all activated cells and cells undergoing apoptosis is a loss of normal cell membrane phospholipid asymmetry resulting in an increase in phosphatidylserine (PS) on the outer leaflet of the bi-lipid membrane layer (Martin, Reutelingsperger and others, 1995). Surface exposure of PS has important physiological and pathophysiological implications. For example, surface exposure of PS in platelet membranes ensures proficient propagation and control of the haemostatic process since PS on aggregated platelets can both restrict and promote thrombin formation at the site of injury by providing a catalytic membrane surface for procoagulant (prothrombinase and tenase) and anticoagulant (protein C) reactions (Zwaal and Schroit, 1997). The pathophysiological significance of this phenomenon is illustrated by the genetic disorder Scott’s syndrome - a bleeding disorder resulting from the loss of function of the scramblase enzyme, which normally serves to rapidly move PS from the inner membrane leaflet to the outer leaflet in response to injury (Toti, Satta and others, 1996).

Loss of cellular phospholipid asymmetry is often accompanied by blebbing and shedding of lipid-symmetric microvesicles from the cell surface (Comfurius, Senden and others, 1990; Sims, Wiedmer and others, 1989; Wiedmer, Shattil and others, 1990). It has been suggested that this microvesicle shedding is important to the cell to remove areas of localised symmetrical cell membrane phospholipid, thus allowing the cell to return to a resting (non-prothrombotic) state (Zwaal and Schroit, 1997). The process of blebbing
appears to be mediated by calcium-dependent calpain activation (Wiedmer, Shattil and others, 1990). Several agonists can induce platelet membrane asymmetry and microparticle formation: Calcium-ionophore is the most effective followed by complement membrane attack complex (C5b-9), collagen, and thrombin (Zwaal and Schroit, 1997).

The physiological significance of platelet microvesicles (hereafter referred to as microparticles [MP]) is unclear. Increased levels of circulating PMP have been demonstrated in patients with thrombotic disorders such as transient ischaemic attacks and myocardial infarction (Jy, Horstman and others, 1995; Lee, Jy and others, 1993). Since they are rich in surface exposed PS, it has been suggested that PMP may propagate prothrombotic processes. PMP can also bind to and activate neutrophils, providing an interesting link between haemostasis and inflammation (Jy, Mao and others, 1995).

5.2.2 Endothelial microparticles (EMP)

Whilst PMP have been extensively studied, until recently there has been very little interest in microparticles of endothelial cell origin (EMP). EMP were first described by Hamilton et al in 1990 (Hamilton, Hattori and others, 1990). In that study, the authors demonstrated that assembly of the complement membrane attack complex (C5b-9) on HUVEC resulted in the release of membrane-derived microparticles- particles less than 1 micron in diameter and expressing binding sites for activated factor V (factor Va). These microparticles were not merely the result of cellular lysis in response to the complement membrane attack complex. Subsequently, EMP release from HUVEC has been
demonstrated to occur \textit{in vitro} in response to TNF-\(\alpha\) (Combes, Simon and others, 1999), and in response to incubation of endothelial cells with serum of patients with the antiphospholipid syndrome (Combes, Simon and others, 1999), thrombotic thrombocytopenic purpura (TTP) (Jimenez, Jy and others, 2001), and multiple sclerosis (MS) (Minagar, Jy and others, 2001).

It is now becoming apparent that EMP may provide a window to the activated endothelium in a number of disease states where endothelial injury is central to the disease process including atherosclerosis (Mallat, Hugel and others, 1999), acute coronary syndromes (Mallat, Benamer and others, 2000), antiphospholipid syndrome (Combes, Simon and others, 1999), TTP (Jimenez, Jy and others, 2001), and MS (Minagar, Jy and others, 2001). The functional significance of endothelial cell and platelet PS externalisation and microparticle formation in these disease states has not been fully elucidated, but one important pathophysiological consequence may be a prothrombotic tendency mediated by activation of the extrinsic coagulation pathway (i.e. the tissue factor/factor VII-dependent pathway) (Berckmans, Neiuwland and others, 2001; Combes, Simon and others, 1999; Joop, Berckmans and others, 2001; Mallat, Hugel and others, 1999; Mallat, Benamer and others, 2000; Nieuwland, Berckmans and others, 2000). Recently, it has been suggested that EMP may be useful diagnostically for the detection of relapses of MS (Minagar, Jy and others, 2001), although others have suggested that EMP may increase in a number of autoimmune states other than MS (Larkin, 2001).
5.2.3 EMP: relevance to the vasculitides

Diagnosis and monitoring of disease activity of the systemic vasculitides is dependent on combinations of invasive tests such as tissue biopsy and visceral angiography, and clinical criteria with less than optimal sensitivity and specificity (Albert, Silverstein and others, 1988; Albert, Rimon, and Silverstein, 1988; Albert and Ekoe, 1983; Brogan, Davies and others, 2002; Brogan, Bose and others, 2002). Although serial measurement of anti-neutrophil cytoplasmic antibodies (ANCA) may play an important role in the diagnosis and monitoring of disease activity in adults with vasculitis (Hagen, Daha and others, 1998; Savage, 2001), so far there have been conflicting reports of their usefulness in this context (Kerr, Fleisher and others, 1993). Moreover, ANCA are present only in the minority of young patients with systemic vasculitis and are therefore poorly sensitive as a diagnostic marker for the majority of the vasculitides of childhood (Brogan and Dillon, 2000a; Brogan and Dillon, 2000b).

5.2.4 Study aims

1. To investigate the potential injurious response of the endothelial cell to SAg-dependent T cell mediated activation as determined by in vitro generation of endothelial microparticles.

2. Based on these in vitro observations, to examine the hypothesis that circulating EMP may be increased during active vasculitis, providing a non-invasive tool for assessing endothelial injury.
5.3 Methods

5.3.1 In vitro studies: analysis of endothelial microparticles in HUVEC-T cell coculture supernatants

Supernatants from the experiments described in section 4.3.4 (with plate plan in Appendix 7) were defrosted in a water bath at 37 ° C. Exact volumes of supernatant (400-800 microlitres) were then centrifuged at 17000 G for 60 minutes and the supernatant decanted to obtain the microparticle pellet. The microparticles were then reconstituted in 350 microlitres of annexin-V buffer (Bender Medsystems), and divided into six 35-microlitre aliquots plated onto the first 6 wells of a 96-well U-bottomed plate. The labelling and quantification of microparticles was then achieved as described in sections 2.5.3-2.5.5. Samples from each experiment were run 6 times and microparticle counts from individual experimental conditions were expressed as the mean number per ml of supernatant, with the intra-experiment standard error of the mean (SEM) based on 6 measurements. Each microparticle experiment was repeated in triplicate, with the final result reported as mean and SEM of these 3 experiments.

5.3.2 In vivo studies: Patients

A total of 39 children with primary systemic vasculitis were studied. The classification of the type of vasculitic illness was defined using the Chapel Hill criteria (Jennette, Falk and others, 1994).

29 children (14 males; mean age 7.24 years, range 0.7-15.8 years) had active systemic vasculitis as defined by a Birmingham vasculitis activity score (BVAS) (Luqmani, Bacon
and others, 1994) of greater than zero (mean BVAS score 10.6/63; range 3-24/63) in the absence of intercurrent infection. 10 had polyarteritis nodosa (PAN); 3 had microscopic polyangiitis (MPA- all with crescentic nephritis and perinuclear antineutrophil cytoplasmic antibodies, [pANCA]); 1 had biopsy-proven Wegener's granulomatosis (WG- with cytoplasmic antineutrophil cytoplasmic antibodies, [cANCA]); and 15 had complete Kawasaki disease (KD) as defined by the American Heart Association criteria (Dajani, Taubert and others, 1993). All KD patients were febrile (greater than 38 °C ), and in the second week of illness; 2 had coronary arterial aneurysms, and 1 had myocarditis with ventricular arrhythmia in the absence of coronary arterial abnormalities. In the KD group, all samples were obtained prior to treatment with intravenous immunoglobulin.

10 children with inactive vasculitis (4 males; mean age 9.0 years, range 1.6-15.8 years) were examined. In this group 4 had PAN, 3 had KD (post treatment, afebrile, and 4-7 weeks after the initial episode); 1 had WG (cANCA positive), and 2 had MPA (both pANCA positive). All had a BVAS score of 0/63.

Control samples were taken from 43 healthy subjects comprising 20 healthy children (10 males; mean age 10.3 years, range 2-15.1 years) and 23 young healthy adults (10 males; mean age 29.3 years, range 23-40 years).

The disease control group comprised 24 age-matched febrile children (11 males; mean age 7.16 years, range 0.6-15.2 years). 12 were attendees of their General Practitioner
with fever and (presumed) viral rash (N=9), or upper respiratory tract infection (N=3). 8 were hospital inpatients with viral or bacterial sepsis (inclusive of 2 patients with meningococcal sepsis). 4 were hospital out-patient attendees with inactive systemic lupus erythematosus (SLE) with fever secondary to upper respiratory tract infection, but in the absence of clinical vasculitis or anti-phospholipid syndrome (including negative anticardiolipin antibodies, and absence of lupus anticoagulant).

In addition, samples from 5 patients in the active vasculitis group were examined before and after induction of remission of vasculitis. The diagnoses in these 5 children were PAN (N=3), WG (N=1), and MPA (N=1).

Informed consent was obtained from the parents of all children involved in the study, and the study was approved by the local research ethics committee.

5.3.2 Preparation of platelet poor plasma

1.4-5 mls of whole blood was collected into bottles containing 3.2% trisodium citrate (Becton Dickinson). Platelet poor plasma was obtained by immediate centrifugation of the whole blood at 5000G for 5 minutes twice. Plasma was then stored at -70° Celcius until use.
5.3.3 Isolation of microparticles from platelet poor plasma

Platelet poor plasma was defrosted in a water bath at 37 °Celsius. Exact volumes of plasma (400-800 microlitres) were then centrifuged at 17000 G for 60 minutes and the supernatant decanted to obtain the microparticle pellet. The microparticles were then reconstituted in 350 microlitres of annexin V buffer (Bender Medsystems), and divided into ten 35-microlitre aliquots plated onto the first 10 wells of a 96 well U-bottomed plate. The labelling and quantification of microparticles (endothelial and platelet) was then achieved as described in sections 2.5.3-2.5.5.

5.3.4 Statistical analysis

Comparison of mean platelet and endothelial microparticle numbers between the different patient groups (independent analysis) was analysed using the Kruskal-Wallis test. Paired data obtained from children before and after induction of remission were compared using the Wilcoxon signed-rank test. Spearman rank correlation coefficients were calculated to investigate the relationship between microparticles, BVAS, and other conventional acute phase reactants. Non-parametric tests were used because the data were not easily transformed to normality (some upward skew, some downward skew, and some approaching “J-shaped” distributions). Receiver operator characteristic curves, sensitivity, specificity, positive and negative predictive values, and likelihood ratios were calculated to examine the diagnostic test characteristics of endothelial microparticles for active vasculitis.
5.4 Results

5.4.1 SAg-dependent T cell-mediated endothelial cell activation and injury: generation of endothelial microparticles (EMP)

To further assess endothelial cell injury in response to SAg-dependent T cell-mediated activation, endothelial microparticles (EMPs) released by HUVEC into the supernatant were analysed for the different experimental conditions (Appendix 7). TNF-α again served as a positive control in these experiments since this cytokine is known to stimulate microparticle formation from endothelial cell monolayers in vitro, and has been implicated as the main cytokine involved in SAg-mediated T cell-dependent endothelial cell activation. Supernatants from resting HUVEC monolayers, HUVEC stimulated with TNF-α, or activated by the SAg-mediated T cell-dependent process described in previous experiments, were sampled between 0 and 24 hours to investigate the kinetics of the formation of EMPs in response to the experimental conditions.

There was a progressive rise over 24 hours in the total number of EMPs released into the supernatant following stimulation with TNF-α as compared with resting HUVEC. Following TNF-α stimulation, the total EMP number rose from a baseline of zero at 0 hours to a mean of 1.6 million/ml (SEM 0.7 million/ml) at 24 hours (mean, SEM of 3 experiments) as shown in figure 5.1a. Similarly, TNF-α resulted in a rise in CD105 positive EMPs from 0 million/ml to 0.62 million/ml (SEM 0.4 millions/ml) at 24 hours.
(figure 5.1b), ICAM-1 positive EMPs from 0 million/ml to 0.73 millions/ml (SEM 0.6 million/ml) at 24 hours (figure 5.1c), and E-selectin positive EMPs from 0 million/ml to 0.63 million/ml (SEM 0.4 million/ml) at 24 hours (figure 5.1d). Although the observed rise in endothelial microparticle formation (for all the endothelial markers used) in response to TNF-α was consistent in all 3 experiments performed, the magnitude of the response in individual experiments varied considerably, as reflected in the wide error bars (figures 5.1a-d).

In contrast to the response to TNF-α, which was maximal at 24 hours, endothelial microparticle formation in response to SAg-mediated T cell-dependent stimulation peaked earlier (at around 2 to 6 hours), and was overall of a greater magnitude (figures 5.1a-d). Thus at 2 hours, there was a peak of E-selectin positive EMPs rising from a baseline of zero at 0 hours, to 1.76 million/ml (SEM 0.04 million/ml of 3 experiments), which subsequently dropped to 0.4 million/ml (SEM 0.07 million/ml) (figure 5.1d). For CD105-positive EMPs, there was a rise from a baseline of 0.004 million/ml (SEM 0.002 million/ml) at 0 hours, which peaked at 6 hours (0.33 million/ml, SEM 0.2 million/ml) and was sustained at 24 hours (0.33 million/ml, SEM 0.2 million/ml) (figure 5.1b). Although, similar results were obtained for the total number of annexin-V binding microparticles, and microparticles expressing ICAM-1 (figures 5.1a and 5.1c), it was not possible to attribute the origin of these microparticles entirely as endothelial since activated lymphocytes can express ICAM-1 (Barclay AN, Brown MH and others, 1997a), and were (necessarily) present in these co-culture experiments.
The peak of E-selectin positive EMPs at 2-4 hours in these co-culture experiments was SAg-dependent because no rise was observed in response to incubation with INF-γ alone, or with INF-γ and T cells in the absence of TSST-1 at 4 hours. There was, however, a slight rise in E-selectin EMP formation from MHC class II-negative HUVEC (i.e. HUVEC not pre-incubated INF-γ) which were incubated with T cells in the presence of TSST-1 for 24 hours, but not at 4 hours. This was presumably a consequence of the small number of MHC class II expressing monocytes (approximately 0.2%) contaminating the purified T cell population. Indeed, it was confirmed that the purified T cell population did become activated in a predominantly Vβ restricted manner when incubated with SAg (TSST-1 or SEB) at 24 hours, but not at 4 hours (figure 5.2), which could account for this observation. At 24 hours there was also some increased CD69 expression of CD4^ Vβ12 (figure 5.2a). This is not one of the classical responding Vβ families for this SAg suggesting that there could have been some bystander activation at 24 hours. This could also explain the increased E selectin EMP formation at 24 hours by MHC class II negative HUVEC in the presence of T cells and TSST-1.
Figure 5.1: Endothelial microparticle release from HUVEC in response to TNF-α, SAg stimulation (co-culture of MHC class II⁺ HUVEC with CD3 T lymphocytes in the presence of TSST-1 10 ng/ml) or control (resting) HUVEC; (Mean, SEM of 3 experiments)
Figure 5.2 Incubation of purified CD3⁺ lymphocytes with SAg after 24 hours

Incubation of purified CD3⁺ lymphocytes with SAg after 24 hours resulted in Vβ-restricted upregulation of CD69, presumably the consequence of contaminating MHC class II⁺ cells (approximately 0.2%).
Figure 5.2: Purified T lymphocyte incubation with SAg: V-beta specific upregulation of CD69 after 24 hours

Figure 5.2a: CD4 CD69 MFI

Figure 5.2b: CD8 CD69 MFI
5.4.2 Comparison of mean microparticle numbers between patient groups

The absolute numbers of microparticles (total, platelet, or endothelial origin) expressed in millions per ml of plasma from the different patient groups are summarised in figures 5.3-5.5.

The mean total microparticle number expressed as millions per ml of plasma for the active vasculitis, inactive vasculitis, controls, and disease controls were: 6.48 million/ml (SEM 1.07 million/ml), 1.09 million/ml (SEM 0.17 million/ml), 2.92 million/ml (SEM 0.63 million/ml), and 1.24 million/ml (SEM 0.25 million/ml) respectively (figure 5.3). Thus, the mean total number of annexin V binding microparticles in the children with active vasculitis was 6.0-fold higher than in the inactive vasculitis group (p=0.000), 2.2-fold higher than healthy controls (p=0.000), and 4.2 fold higher than in the disease controls (p=0.000).

The number of endothelial microparticles as defined by E-selectin, and CD105 expression was higher in the active vasculitis group than in the other patient groups (figure 5.4a). The mean number of E-selectin positive microparticles in the active vasculitis was 8.5-fold higher than in the inactive vasculitis group (p=0.000), 5.6-fold higher than the healthy controls (p=0.000), and 10.4-fold greater than the disease controls (p=0.000). The number of CD105 positive microparticles in the active vasculitis group was 6.2-fold higher than the inactive vasculitis group (p=0.001), 4.1-fold higher than the healthy controls (p=0.000), and 11.4-fold higher than the disease controls (p=0.000)
(figure 5.4b). There was also a significant increase in platelet microparticles expressing CD42a in the active vasculitis group as compared to the other groups. The number of CD42a positive microparticles in the active vasculitis group was 8.4-fold higher than the inactive vasculitis group (p=0.006), 1.6-fold higher than the healthy controls (p=0.04), and 6.9-fold higher than the disease controls (p=0.000) (figure 5.5). There was no difference in the number of platelet microparticles expressing P-selectin comparing the active vasculitis, inactive vasculitis or healthy control groups, although the number of P-selectin microparticles was lower in the disease controls than the active vasculitis group (p=0.003), inactive vasculitis group (p=0.01), and healthy controls (p=0.001) (figure 5.5).
FIGURE 5.3: TOTAL MICROPARTICLE NUMBERS

Column scatter plot of the total microparticle number taken from patients with active vasculitis (n=29), inactive vasculitis (n=10), healthy controls (n=43), and disease controls (n=24).
Figure 5.3: Total Microparticle Number

- Active Vasculitis
- Inactive Vasculitis
- Healthy Controls
- Disease Controls

p = 0.000
Figure 5.4 E selectin and CD105 microparticle numbers

Column scatter plots of E-selectin and CD105 EMPs taken from patients with active vasculitis (n=29), inactive vasculitis (n=10), healthy controls (n=43), and disease controls (n=24).
Figure 5.4a: E Selectin Microparticle Number

- Active Vasculitis
- Inactive Vasculitis
- Healthy Controls
- Disease Controls

Figure 5.4b: CD105 Microparticle Number
FIGURE 5.5 COMPLETE MICROPARTICLE PROFILE

Bar chart of the complete microparticle profile from patients with active vasculitis (n=29), inactive vasculitis (n=10), healthy controls (n=43), and disease controls (n=24). * = microparticle population significantly higher in the active vasculitis group; ‡ = microparticle population significantly lower in the disease control group.
Figure 5.5: Complete microparticle profile

- **Figure 5.5** provides a histological profile of complete microparticles, showing different biomarkers and their significance in active vasculitis, inactive vasculitis, controls, and disease controls.

- The graph illustrates the levels of biomarkers such as CD42a, Psel, ICAM-1, CD105, VCAM-1, and Esel, with markers indicating statistical significance (e.g., *).
There was no difference in the numbers of microparticles expressing the adhesion molecules ICAM-1 or VCAM-1 between the groups (figure 5.5).

5.4.2 Longitudinal analysis of endothelial and platelet microparticle profiles before and after induction of remission of vasculitis

Paired samples obtained from 5 children before and after induction of remission of vasculitis were analysed for changes in endothelial and platelet microparticle profiles. All 5 patients with active vasculitis demonstrated high levels of endothelial MPs which fell to normal following induction of remission (a 10.5-fold decrease for CD105 MPs (p=0.04) and an 8.7-fold decrease for E-selectin MPs (p=0.04). There was also a significant fall in platelet microparticles expressing CD42a after induction of remission (a 3.9-fold decrease, p=0.04), but no significant change in microparticles expressing P-selectin. There was no significant difference in microparticles expressing ICAM-1 or VCAM-1 before and after induction of remission of vasculitis. These data are summarised in figure 5.6.

5.4.3 Correlation of EMPs with BVAS and conventional acute phase reactants

Correlation coefficients between microparticles and BVAS and conventional acute phase markers are shown in table 5.1. There was a significant correlation between E-selectin microparticles and the BVAS in the vasculitis patients as a whole (R=0.65, p=0.000; active and inactive vasculitis patients combined); CD105 microparticles (R=0.51, p=0.001); and total microparticle number (R=0.65,
Figure 5.6 Changes in MP profile before and after induction of remission of vasculitis

Changes in the endothelial (E-selectin and CD105) and platelet (CD42a) microparticle profile before and after induction of remission in 5 children with vasculitis (PAN=3; MPA=1; WG=1).
Figure 5.6a: E-selectin microparticles pre and post induction of remission

Figure 5.6b: CD105 microparticles pre and post induction of remission

Figure 5.6c: CD42a microparticles pre and post induction of remission

P=0.04
There was a weaker (but significant) correlation between CD42a microparticles and the BVAS (R=0.42, p=0.01).

For the conventional acute phase laboratory markers there was a significant correlation between the BVAS and the haemoglobin (R= -0.61, p= 0.000); total white cell count (R=0.35, p= 0.038); ESR (R=0.72, p= 0.000); and plasma albumin (R= -0.50, p= 0.02).

In the patients with vasculitis (active or inactive) there was a correlation between E-selectin and CD105 positive microparticles and other conventional acute phase reactants (table 5.1). In contrast, in the disease control group the strength of the relationship between microparticles (total, endothelial, or platelet) and conventional acute phase reactants was overall weaker (table 5.1), with the notable exception of P-selectin platelet microparticles which were negatively correlated with plasma albumin levels (R= -0.69, p=0.002). Notably, there was no significant correlation between the platelet count and CD42a microparticles or P-selectin microparticles in either the vasculitis patients or the disease controls (table 5.1).

### 5.4.4 Test characteristics of EMPs for the diagnosis of active vasculitis

The receiver operator characteristic curves (ROC curves) for EMPs expressing E-selectin or CD105 at varying definitions of positivity are shown in figure 5.7. For comparison the ROC curve for renal angiography in childhood PAN based on a previously published series of 25 children is also shown (Brogan, Davies and others, 2002). Table 5.2 summarises the test characteristics of EMPs for the diagnosis of active vasculitis at
Table 5.1 Correlation between EMP and clinical indices of vasculitic disease activity

Correlation between EMPs and conventional acute phase reactants in the vasculitis and disease control patient groups. Hb= Haemoglobin; ESR= Erythrocyte sedimentation rate; CRP= C-reactive protein; BVAS= Birmingham Vasculitis Activity Score.
Table 5.1: Correlation between EMP and clinical indices of vasculitic disease activity

<table>
<thead>
<tr>
<th></th>
<th>Hb</th>
<th>Total WCC</th>
<th>Platelets</th>
<th>ESR</th>
<th>CRP</th>
<th>Albumin</th>
<th>BVAS</th>
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<tr>
<td><strong>Total MP count</strong></td>
<td>R = -0.52</td>
<td>R = 0.23</td>
<td>R = 0.41</td>
<td>R = 0.50</td>
<td>R = 0.25</td>
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<td></td>
<td>(p = 0.001)</td>
<td>(p = NS)</td>
<td>(p = 0.01)</td>
<td>(p = 0.007)</td>
<td>(p = NS)</td>
<td>(p = 0.009)</td>
<td>(p = 0.000)</td>
</tr>
<tr>
<td><strong>E-selectin MP</strong></td>
<td>R = -0.50</td>
<td>R = 0.05</td>
<td>R = 0.30</td>
<td>R = 0.40</td>
<td>R = 0.06</td>
<td>R = -0.40</td>
<td>R = 0.65</td>
</tr>
<tr>
<td><strong>MPs</strong></td>
<td>(p = 0.005)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = 0.02)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = 0.000)</td>
</tr>
<tr>
<td><strong>CD105 MP</strong></td>
<td>R = -0.4</td>
<td>R = 0.10</td>
<td>R = 0.3</td>
<td>R = 0.6</td>
<td>R = 0.3</td>
<td>R = -0.6</td>
<td>R = 0.51</td>
</tr>
<tr>
<td><strong>E-selectin</strong></td>
<td>(p = 0.01)</td>
<td>(p = NS)</td>
<td>(p = 0.002)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = 0.007)</td>
<td>(p = 0.001)</td>
</tr>
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<td><strong>CD42a MP</strong></td>
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<td>R = 0.03</td>
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<td>(p = NS)</td>
<td>(p = NS)</td>
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<td>(p = 0.01)</td>
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<td>(p = NS)</td>
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<td>(p = NS)</td>
<td>(p = NS)</td>
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<tr>
<td><strong>VCAM-1 MP</strong></td>
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<td>(p = NS)</td>
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<td>(p = NS)</td>
<td>(p = 0.04)</td>
<td>NA</td>
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<tr>
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<td>R = 0.04</td>
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<td>R = -0.03</td>
<td>R = 0.00</td>
<td>R = -0.03</td>
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</tr>
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<td><strong>MPs</strong></td>
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<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
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</tr>
<tr>
<td><strong>P-selectin MP</strong></td>
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<td>R = 0.05</td>
<td>R = 0.08</td>
<td>R = 0.35</td>
<td>R = 0.23</td>
<td>R = -0.41</td>
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</tr>
<tr>
<td><strong>ICAM-1 MP</strong></td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>VCAM-1 MP</strong></td>
<td>R = -0.12</td>
<td>R = 0.08</td>
<td>R = -0.22</td>
<td>R = -0.12</td>
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<td>R = 0.063</td>
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</tr>
<tr>
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<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>NA</td>
</tr>
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<td>R = 0.00</td>
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</tr>
<tr>
<td></td>
<td>(p = NS)</td>
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<td>(p = NS)</td>
<td>(p = NS)</td>
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Table 5.2 Test characteristics of EMPs for the diagnosis of active vasculitis

PPV = positive predictive value; NPV = negative predictive value; LR+ = Likelihood ratio for a positive test result; LR- = Likelihood ratio for a negative test result.
Table 5.2: Test characteristics of EMP for the diagnosis of active vasculitis

<table>
<thead>
<tr>
<th>Cut-off for test positivity</th>
<th>Sensitivity % (95%CI)</th>
<th>Specificity % (95%CI)</th>
<th>PPV % (95%CI)</th>
<th>NPV % (95%CI)</th>
<th>LR+</th>
<th>LR-</th>
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<tbody>
<tr>
<td>E-selectin microparticles (millions/ml of plasma)</td>
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<tr>
<td>0.1</td>
<td>97 (82-100)</td>
<td>43 (32-55)</td>
<td>39 (30-48)</td>
<td>97 (94-100)</td>
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<td>0.08</td>
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<td>65 (53-75)</td>
<td>49 (40-59)</td>
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<tr>
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<td>79 (72-87)</td>
<td>92 (87-97)</td>
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<td>66 (46-82)</td>
<td>92 (84-97)</td>
<td>76 (68-84)</td>
<td>88 (81-94)</td>
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<td>0.37</td>
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</tr>
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<td>CD-105 microparticles (millions/ml of plasma)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>46 (31-50)</td>
<td>92 (81-98)</td>
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Figure 5.7 Receiver operator characteristic (ROC) curves for E-selectin and CD105 EMPs for the diagnosis of active vasculitis.

For comparison is the ROC curve for the diagnosis of polyarteritis nodosa in a previously published cohort of children (Brogan, Davies, and others. 2002).
Figure 5.7: Receiver operator characteristic curves for endothelial microparticles (E selectin and CD105) for the diagnosis of active vasculitis.
varying definitions of test positivity. The cut-off values for test positivity in table 5.2 correspond to individual points on the ROC curves.

5.5 Discussion

In Chapter 4 it was established that SA g-dependent T cell Vβ-restricted adherence and activation on the surface of HUVEC monolayers results in activation of the HUVEC. The in vitro studies outlined in this chapter now demonstrate that a further consequence of this interaction is EMP generation, (both increased E-selectin and CD105 EMP formation). This effect occurred earlier, and was comparable (and on the whole greater) than the HUVEC response to stimulation with TNF-α, emphasising the potential for vascular injury in response to SA g s.

It is currently still unclear in this in vitro model whether this microparticle formation occurs as a result of apoptosis alone, or is the result of cellular activation in the absence of apoptosis. Moreover, the functional significance of endothelial microparticle formation in response to SA g dependent T cell activation remains to be elucidated, although it is known that microparticles rich in PS can participate in the dissemination of pro-adhesive and pro-coagulant activities in thrombotic disorders (Boulanger, Scoazec and others, 2001; Combes, Simon and others, 1999; Jimenez, Jy and others, 2001; Joop, Berckmans and others, 2001; Mallat, Hugel and others, 1999; Mallat, Benamer and others, 2000; Nieuwland, Berckmans and others, 2000), thus potentially amplifying the vascular injury.
It was the opinion of the author that the generation of endothelial microparticles in response to many injurious stimuli including TNF-α and, (as described for the first time in this chapter) in response to SAg-dependent T cell mediated endothelial injury, constituted a fascinating and possibly important observation, with potential direct relevance to the study of patients with vasculitis. This result therefore prompted the studies of this phenomenon in children with vasculitis syndromes described in this chapter.

There is an emerging concept that endothelial cell dysfunction is central to the pathogenesis of many diseases including atherosclerosis (Mallat and Tedgui, 2001; Tedgui and Mallat, 2001), multiple sclerosis (Minagar, Jy and others, 2001b), and autoimmune diseases which are typified by thrombosis and vascular injury (TTP (Jimenez, Jy and others, 2001) and SLE with antiphospholipid syndrome (Combes, Simon and others, 1999)). In support of this concept, previous studies have been able to demonstrate an elevated number of microparticles of endothelial origin in these disorders, as a consequence of increased endothelial cell activation or apoptosis. The data presented in this study would now suggest that the same is true of the systemic vasculitides of childhood, and it was possible to demonstrate increased levels of circulating endothelial microparticles expressing E-selectin and CD105 in the active stage of the disease.

It could be argued that childhood vasculitides would be the “prototypic” disease states in which to study this phenomenon since endothelial dysfunction occurs in most vasculitis
syndromes, and children do not have atherosclerosis, which may be a confounding factor when studying endothelial microparticles in adult disease states. Thus it is now apparent that increased endothelial microparticle formation is not specific to any single disease entity, but rather is the endpoint of a variety of immunological and inflammatory pathways, which culminate in endothelial injury.

A number of endothelial surface markers of varying specificity were utilised to characterise the microparticles as being of endothelial or platelet origin. CD105 (endoglin) is constitutively expressed by endothelial cells, but is also expressed on some activated monocytes and leukaemic cells (Barclay AN, Brown MH and others, 1997b). As such, it is relatively specific for the endothelium. E-selectin is only expressed on activated endothelial cells \textit{in vivo} (Barclay AN, Brown MH and others, 1997c), although very recently has been demonstrated to be upregulated on CD4 T cells \textit{in vitro} stimulated by a novel interaction between membrane-bound TNF-\(\alpha\) and rabbit anti-human TNF-\(\alpha\) antibody (Harashima, Horiuchi and others, 2001). Thus, at least \textit{in vivo}, E-selectin is an entirely specific marker for endothelial cells. ICAM-1 is constitutively expressed on endothelial cells, although it is upregulated during endothelial cell activation (Barclay AN, Brown MH and others, 1997a). It has a much wider tissue expression, however, and is also expressed on activated leucocytes (Barclay AN, Brown MH and others, 1997a). As such, it is a less specific endothelial cell marker. Similarly, although VCAM-1 is predominantly expressed on vascular endothelium, it also has been identified on dendritic cells, macrophages, and non-vascular cell populations within several organs including the joints and kidney (Barclay AN, Brown MH and others, 1997d). CD42a is restricted to
and constitutively expressed on platelets and megakaryocytes and is therefore a relatively specific platelet marker (Barclay AN, Brown MH and others, 1997e). P-selectin is present on activated platelets and activated endothelial cells, and is therefore a poor discriminator between activated platelets and endothelial cells (Barclay AN, Brown MH and others, 1997f).

The main finding in the in vivo study was an increased level of circulating microparticles expressing E-selectin and CD105 in the children with active vasculitis. It is suggested that these microparticles are truly of endothelial cell origin, although one potential confounder which cannot be excluded is that soluble E-selectin (known to be increased in many vasculitis syndromes) (Nash, Shah, and Dillon, 1995) could bind phosphatidylserine-rich microparticles released from platelets. We suggest that this is unlikely however, because although it is known that L-selectin can bind to negatively charged phospholipids such as phosphatidylserine and cardiolipin, E-selectin has been shown unequivocally not to have any binding capacity to phospholipid (Malhotra, Taylor, and Bird, 1996). Moreover, the fact that both E-selectin and CD105 microparticles were observed to correlate with vasculitic disease activity, whereas microparticles expressing less specific endothelial markers failed to do so indicates that the E-selectin and CD105 microparticles are truly of endothelial origin. It should be stated, however, that 3-colour flow cytometry was not performed (because of time constraints) to examine for CD42a and E-selectin “double-positives” to unequivocally exclude the possibility of soluble E-selectin (which is known to be increased in vasculitis syndromes, including KD) binding.
to platelet-derived MP and thus potentially confounding the observations described in this study.

An increased number of microparticles of platelet origin expressing CD42a in the active vasculitis group was also observed, although the size of this difference between the groups was less dramatic than that observed for EMP. This finding is not entirely surprising since most vasculitis syndromes are characterised by high platelet counts and by micro and macroscopic thrombus formation; indeed anti-platelet therapy is recommended for most forms of childhood vasculitic illness (Brogan and Dillon, 2000b). Interestingly, however, there was no correlation between platelet microparticles and absolute platelet count in the vasculitis patients, or the disease controls.

Importantly, this study did not address the functional significance of increased microparticle numbers (of all types) in active vasculitis, although it is possible that microparticles could contribute to the pathogenesis of vasculitis via several mechanisms. Firstly, the prothrombotic potential of cellular and platelet microparticles is well-established, and has been demonstrated in acute coronary syndromes, atherosclerosis, anti-phospholipid syndrome, and meningococcal sepsis. This occurs mainly as a result of the rich PS content of the microparticles, which acts as the one of the essential lipid co-factors for clotting and supports thrombin generation via the tissue factor-factor VII-mediated pathway. Since a prothrombotic tendency occurs in vasculitis (Kiraz, Ertenli and others, 2002; Savage and Cooke, 1993), it is entirely conceivable that the increased circulating microparticles observed during active vasculitis may contribute to this.
Secondly, it has been demonstrated *in vitro* that microparticles of neutrophil origin can activate endothelial cells in tissue culture (Mesri and Altieri, 1998). This is interesting since an important sub-group of small vessel vasculitides (Wegener's granulomatosis, microscopic polyangiitis, and Churg-Strauss syndrome) are associated with the presence of anti-neutrophil cytoplasmic antibodies (ANCA) which are thought to activate neutrophils and endothelial cells, causing vascular injury. It is not yet known if neutrophil microparticles are released from neutrophils following activation by ANCA, or indeed whether microparticles derived from other leucocytes could further contribute to endothelial activation in vasculitis, but clearly this is an important area worthy of further study.

The test characteristics of endothelial microparticles for the diagnosis of active vasculitis based on a limited number of children with active vasculitis are encouraging, and certainly compare favourably with invasive tests such as visceral angiography (Brogan, Davies and others, 2002), and tissue biopsy (Albert, Rimon, and Silverstein, 1988). It must be emphasised, however, that increased circulating EMP are not specific to the vasculitides, but rather are a feature of a number of diseases characterised by endothelial cell dysfunction. Used in the right clinical context, however, they may provide important diagnostic information. Furthermore, we have shown that they correlate with clinical parameters of vasculitic disease activity such as the BVAS, and may be useful when measured longitudinally in individual patients to monitor disease activity.
Lastly, the observation that endothelial microparticles correlated with conventional acute phase reactants in the context of vasculitic disease activity, but less so in the disease control group suggests that they are not behaving like a conventional acute phase reactant, and may be able to discriminate between sepsis and the inflammatory response associated with active vasculitis. In accordance with this result, a study of children with meningococcal sepsis failed to show any difference in the levels of circulating EMPs in this disease compared with controls, although patient numbers were limited (Nieuwland, Berckmans and others, 2000).

In conclusion, the consequence of SAg-dependent T cell mediated HUVEC interactions is HUVEC activation and EMP formation, potentially a marker of endothelial injury. In addition, this study has demonstrated increased levels of circulating EMP in children with active vasculitis, and it is proposed that this novel finding may provide a "window" to the activated endothelium. The functional significance of this observation, however, remains to be elucidated.
Chapter 6: General discussion and future directions
6.1 General discussion

This thesis has attempted to examine one aspect of the relationship between infections and vasculitis in the young and specifically asks the question whether or not S Ags could be involved in the aetiopathogenesis of vascular injury associated with vasculitis syndromes in the paediatric population. Throughout this thesis a general theme has been the role that the endothelium plays in vasculitis- both as a target for injury and as a potential amplifier of the inflammatory response. Chapter 5 expands on this concept, and for the first time demonstrates increased endothelial microparticles in children with active vasculitis.

There are important limitations to the data presented in this thesis many of which have been discussed in the relevant chapters, and in addition many unanswered questions. This chapter will highlight these limitations and proposes areas of further research that may address some of the unresolved issues. In addition, limited preliminary data will be presented to illustrate the sort of approaches that may be used in the future to further the data presented in this thesis.

6.1.1 What is true skewing of the Vβ repertoire?

In chapter 1, evidence for peripheral blood T cell activation (CD69 upregulation) in patients with active vasculitis was found, and there was some evidence of T cell Vβ repertoire skewing in the vasculitis patients over and above that observed in the disease control children despite comparable degrees of T cell activation. As discussed, this
provides evidence for a role of T cells in vasculitis but does not provide definitive evidence that SAgs are involved in all forms of vasculitis. Despite attempts to control for potential confounding factors, this observation may still be artefactual.

It is useful in this context to consider what is actually meant by “skewing” of the Vβ repertoire to highlight the complexity of this concept, particularly when considering diseases with a chronicity that can span months to years (such as PAN or WG). The typical response of a T cell (CD4+ or CD8+) following SAg activation has been described: activation, followed by proliferation (expansion), and finally deletion. Thus, depending on the timing of the blood sampling in relation to disease onset, particular peripheral blood Vβ family percentages may be normal, increased, or decreased. Even when considering Kawasaki disease, which in contrast to other vasculitic syndromes such as PAN has a well-defined and relatively acute onset, previous studies have suggested that the timing of the blood sample is crucial to detect expansion of Vβ2 T cells (Curtis, Zheng and others, 1995). This point is summarised in figure 6.1 which considers a hypothetical study examining the percentage of Vβ2 T cells in the peripheral blood of healthy controls, KD at the acute (day 10) and convalescent (week 5) phases of the disease, and PAN with disease onset anywhere between 3 and 12 months from the time of sampling. As illustrated in this figure, studies which only examined changes in the mean percentage of Vβ families and ignored changes in the variance (spread) may miss true “skewing”. This could explain some of the negative studies relating to KD, and illustrates the importance of considering both expansions and deletions when looking for evidence within the Vβ repertoire of an effect of SAgs.
Figure 6.1: What is T cell Vβ skewing? A hypothetical study examining controls, KD and PAN (n=3 in each group)

- Higher mean same variance
- Same mean bigger variance
- Lower mean same variance

- ● controls
- ■ KD day 10
- ▲ KD week 5
- ○ PAN 3-12 months?

vβ2
In fact the concept of skewing is even more complex than this, purely as a result of simple arithmetic: when an individual responds to a SAg (e.g. TSST-1) and increases the percentage of circulating Vβ2 T cells, by definition there must be a concomitant decrease in the percentage of other Vβ families. Conversely, if an individual has a deletion of a particular Vβ family in response to a SAg, there will always be a concomitant increase in the percentage of other Vβ families. In other words, true skewing also dictates that SAg's will cause specific changes in the responding Vβ family, and non-specific changes shared over the whole T cell Vβ repertoire. Again, this could account for some of the inconsistencies regarding changes in the Vβ repertoire observed in previous studies relating to KD.

6.1.2 Vβ-specific activation in the absence of Vβ skewing

Another issue relating to the previously described model of the response of a T cell to a SAg which may explain some of the negative data relating to KD is that Vβ-specific activation may occur in the absence of proliferation early on in the response. This concept is highlighted by the *in vitro* experiments described in chapter 4, which showed Vβ-specific CD69 upregulation on T cells incubated for 4 hours with SAg, but with relatively little change in Vβ percentages. Theoretically if a patient with vasculitis which was triggered by a SAg was sampled early in the disease process, there may be Vβ-specific activation without Vβ expansion. The 3-colour flow cytometry protocol for examining specific Vβ family CD69 upregulation within the CD4 or CD8 T cell subsets
(which effectively provides data relating to both Vβ-specific activation and expansion/deletion) would be the obvious way to address this potential confounding factor, and it is with some regret that this approach was not utilised from the outset in the studies leading to this thesis.

Preliminary results using this approach were obtained from one infant aged 3-months with KD who was included in the study described in chapter 3. This infant was of particular interest since he was the identical twin brother of a case of infantile KD (complicated by coronary and axillary arterial aneurysm formation) who had presented 10 days previously (again included in chapter 3). Since the parents had been warned regarding an increased risk of KD in siblings of index cases they presented relatively early with the second twin, allowing blood sampling on day 6 of the illness. Analysis of the peripheral blood CD4 and CD8 Vβ repertoire (by percentages) did not reveal any major expansions or deletions. Specific Vβ expression of CD69 was measured for Vβ2 and Vβ14 (limited blood volume was obtained from this small infant thus precluding analysis of CD69 expression on other Vβ families). These preliminary results are shown in figure 6.2. As illustrated, although only two Vβ families were analysed within the CD4 and CD8 populations and cell numbers were limited, there was CD69 upregulation within the Vβ2 subset (true for both CD4 and CD8), which was not observed for Vβ14, and with comparatively little perturbation of the percentage of cells expressing Vβ2 as compared with healthy control children. Polymerase chain reaction for toxin genes from nasal isolates of Staphylococcus aureus from this infant revealed genes encoding for the toxins SEA, SEG, SEI and TSST-1 (analysed by the Central Public Health Laboratory,
Colindale). The presence of TSST-1 and the observed specific activation of CD4 and CD8 T cells expressing Vβ2 is of considerable interest in this context, and emphasises that analysis of Vβ repertoire skewing by consideration of percentages is a relatively insensitive means of studying this phenomenon.
Figure 6.2: Vβ-specific expression of CD69 on peripheral blood CD4 and CD8 T cells in a 3-month old infant with KD (day 6 of illness).
A further extension of this argument would be to correlate such Vβ-specific activation profiles in patients with KD (or other vasculitides) with toxins isolated from individual patients. For example, bacterial culture supernatants could be incubated with PBMCs from a healthy adult donor, and the specific Vβ activation profile obtained (CD69 upregulation across the Vβ repertoire) could be compared with that actually observed in the patient. Such an approach has been proposed in KD and sample collection is underway as part of a collaborative study undertaken by the London Kawasaki Disease Research Group (KDRG) (Brogan, Bose and others, 2002).

6.1.3 Vβ-restricted binding of the T cells to the HUVEC monolayer: compartmentalisation of the immune response in vasculitis

In chapter 4 it was demonstrated that in vitro MHC class II-expressing HUVEC can present SAg to T cells, and one consequence of this is Vβ-restricted binding of the T cells to the HUVEC monolayer. This observation may provide insight into the compartmentalisation of the immune response in vasculitis, and could also explain some of the conflicting data relating to studies of peripheral blood T cell Vβ repertoire skewing in KD. For example, if this mechanism was operational in vasculitic syndromes such as KD it could be anticipated that sampling the peripheral blood compartment may not be revealing in terms of T cell activation since the majority of the activated T cells would be adherent to the endothelium of lesional tissue. As discussed in chapter 1 there are data suggesting that lesional sequestration of T cells expressing Vβ2 occurs in KD which would be compatible with this theory (Leung, Giorno and others, 1995; Yamashiro Y,
Nagata S and others, 1996). However it should be acknowledged that the mechanism proposed in chapter 4 is not the only possible way that T cells expressing Vβ2 may be sequestered into lesional tissue in KD. For example, T cells may become activated at sites away from the endothelial surface (for example, within the respiratory tract, or gut-associated lymphoid tissue) and subsequently migrate to cardiovascular tissue under the influence of endothelial cell adhesion molecules. This latter mechanism may also utilize homing receptors and IL-12 (Leung, Gately and others, 1995). The contribution of homing receptors is currently being examined in KD as part of the London KDRG collaborative study (Brogan, Bose and others, 2002).

It was anticipated that a study of lesional T cell Vβ expression in childhood PAN and MPA would have been undertaken as part of this thesis. Indeed, ethical committee approval for this was obtained, and preliminary work in establishing antibody binding using T cells embedded in wax blocks was undertaken. The idea had been to examine tissue from patients in whom peripheral blood T cell Vβ profiles were known, and calculate in vivo “Vβ ratios” (in other words, the ratio of the percentage of particular Vβ families of T cells in lesional tissue to peripheral blood) in order to further examine the hypothesis that there may be specific Vβ T cell sequestration in vasculitic lesional tissue in PAN. Time constraints precluded such a study, however.

6.1.4 Clonal versus polyclonal Vβ restricted expansion

Another limitation of the T cell study presented in chapter 3 was that it did not address the issue of clonal versus polyclonal expansion. Generally speaking SAg stimulation
produces a Vβ restricted expansion of both CD4 and CD8 T cells that is polyclonal since stimulation is induced independent of the TCR junctional regions. Conventional antigen stimulation typically leads to a clonal expansion of T cells (with consequently limited TCR junctional diversity) that is not Vβ restricted. However, there have been reports suggesting that conventional antigen exposure can lead to a clonal expansion of cells with restricted Vβ usage (Deckhut, Allan and others, 1993; Wedderburn, O'Hehir and others, 1993). In addition, the expansion of a CD8+ Vβ4+ clone was reported in an individual following hepatitis B vaccination (Abbott, Geursen and others, 1996). Therefore, to be certain that an expansion of T cells belonging to a particular Vβ family is caused by SAg exposure, it is preferable to confirm polyclonality by analysis of the CDR3 region. That said, this paradigm can arguably now be challenged since it has been established the Mycoplasma SAg MAM binds both to human Vβ17 segments and the CDR3 region (see chapter 1, section 1.3.2.1). Whether this is true of other SAGs remains to be established, but from a mechanistic point of view it is no longer true to say that SAGs always induce polyclonal CDR3 Vβ restricted expansion, or conversely that clonal expansion as determined by CDR3 analysis precludes a SAg-mediated response.

6.1.5 Flow cytometry versus polymerase chain reaction for the study of T cell Vβ repertoires

Flow cytometry was used in the studies described in this thesis to analyse changes in the peripheral blood T cell Vβ repertoire although some comment regarding semi-quantitative polymerase chain reaction (PCR), and the advantages and disadvantages of
these two alternative means of examining the Vβ repertoire is required. One of the principal advantages of the use of monoclonal antibodies and flow cytometry is that this technique detects TCR proteins rather than measuring RNA levels. PCR analysis may detect non-functioning transcripts including pseudogene transcripts, transcripts of non-functional rearrangements, and transcripts that are not expressed as functional TCR proteins due to poor pairing with the TCR alpha chain. Secondly, the use of monoclonal antibodies and flow cytometry allows accurate measurement of the number of cells expressing a particular Vβ gene product. Direct comparison of the results of analysing specific T cell Vβ families with well-characterised monoclonal antibodies with that performed by semi-quantitative PCR has demonstrated how PCR analysis can give misleading quantitative results (Diu, Romagne and others, 1993). This is because RNA levels are not necessarily equivalent to actual cell numbers or percentages since the amount of TCR β chain mRNA expressed by individual T cell clones may differ up to 100 fold. Thirdly, the use of multicolour staining with monoclonal antibodies allows the T cell Vβ repertoire to be analysed with respect to other phenotypic characteristics such as CD4 or CD8 positivity, or expression of activation markers such as CD69. Such analysis is more difficult using PCR and requires the T cell population to be separated, for example by magnetic beads (see chapter 2), before mRNA isolation.

PCR analysis has two main advantages over FACS analysis. Firstly, oligonucleotide primers can be designed to probe the complete T cell Vβ repertoire. In contrast the availability of monoclonal antibodies against many of the TCR Vβ families has historically been limited, although recently much improved. The second advantage of
PCR techniques is that less sample is required, a feature which is of particular relevance to the study of children, where the volume of blood available for study is usually limited. Lastly, PCR techniques allow Vβ expansions to be classified as polyclonal or clonal by probing the CDR3 region (see table 1.3.2, and text above).

Taking into account these considerations flow cytometry was overall considered to be a suitable technique to answer the main question being asked regarding T cell activation and T cell Vβ-repertoire skewing in the studies described in this thesis, although the limitations of this technique need to be acknowledged.

6.1.6 Colonisation of study subjects with SAg-producing organisms

A final limitation of the study described in chapter 3 is that it did not provide data relating to colonisation of vasculitis patients with SAg-producing organisms. Clearly this would have been a useful adjunct to the argument and indeed it was anticipated that swabs (nasal, pharyngeal, and rectal or stool) would have been obtained from all the vasculitis patients, controls, and disease control children described in chapter 3. This would have allowed comparison of the toxin profiles (analysed using PCR for known common staphylococcal and streptococcal SAgS) and the peripheral blood T cell Vβ repertoire. In fact, none of the children (or their parents) included in the studies described consented to rectal swabs, and only a minority of vasculitis patients consented to nasal swabs (it was of some surprise to this author that children would rather have a blood test than a nasal swab!). That said, nasal colonisation with staphylococcus and/or β-haemolytic streptococci was detected in 5/16 children with PAN, a rate comparable to
previously published nasal carriage rates within the healthy paediatric population as a whole (Burgner, Curtis and others, 2001). Again, because of time constraints, these isolates were not analysed for toxin production. In any case such an approach would only have identified known or common staphylococcal or streptococcal SAgS, and novel SAgS would not have been detected. Moreover, the chronicity of the vasculitides studied (mainly PAN) and the fact that many of these children were hospitalised for considerable periods of time and received several courses of antibiotics would make such data very difficult to interpret, and indeed control for.

6.1.7 HLA-mismatch between T cells and HUVEC in co-culture experiments

In chapter 4 data were presented suggesting that endothelial cells can function as competent SAg presenting cells for T cells. It was previously discussed that the major limitation of those data was that no attempt was made to control for HLA-mismatch, and any observations made are occurring on the background of a mixed lymphocyte-endothelial culture. There are two potential ways to overcome this problem. Firstly, the experiments could be repeated using an endothelial cell line with known tissue type, and T cells donated from an adult with a compatible match. The practical limitation of this approach is that it would place a considerable burden on that individual donor (throughout the course of 2 years, over 1000 mls of blood was collected from healthy adult donors in order to obtain T cells or PBMC). In addition, the use of immortalised cell lines as opposed to first or second passage HUVEC may introduce another confounding factor relating to cell adhesion molecule expression, or response to cytokine stimulation (Haraldsen, Kvale and others, 1996). The second way of overcoming the HLA mismatch
problem would be to use HUVEC from a single umbilical cord and T cells extracted from cord blood from the same individual. From a practical point of view this is simpler, but the main disadvantage is that naive T cells from the foetus may not respond to SAgS in the same way as T cells from more mature donors. That said, KD has been described in neonates (Stanley and Grimwood, 2002), and such studies may therefore be valid in their own right.

6.1.8 EMP generation from SAg-dependent T cell mediated activation of HUVEC

Another intriguing result presented in chapter 5 related to the generation of EMP in response to SAg-dependent T cell mediated activation. As shown in figure 5.1, there was an initial increase in EMP in the co-culture supernatant maximal by 2-4 hours, followed by a return to just above normal by 24 hours. This was most obvious for E selectin EMP (figure 5.1d). The obvious question relating to this observation is: where are the EMP going? One clue may be provided by the study by Brezinschek et al (Brezinschek, Oppenheimer-Marks, and Lipsky, 1999). Using an in vitro model in which human peripheral blood CD4+ T cells migrated through confluent monolayers of HUVEC, the authors demonstrated that activated T cells acquired a variety of endothelial cell surface determinants including CD31, CD49d, CD54, CD61 and CD62E (E selectin). Importantly, the acquisition of these endothelial markers occurred as a result of endothelial cell membrane transfer as documented using endothelial cells whose cell membrane had been pre-labelled with the lipophilic dye, DiOC-16. Another relevant observation that the authors made was that membrane blebs occurred on the surface of the endothelial cells during their interaction with T cells. It is intriguing to speculate that
this endothelial cell membrane transfer to T cells occurred as a result of endothelial microparticle formation, and certainly this observation would explain the results illustrated in figure 5.1. To examine the hypothesis that the EMP were binding to the T cells present in the co-culture (and thus were effectively being removed from the supernatant), preliminary experiments were performed examining surface E selectin expression on T cells in the co-culture model described in chapter 4, and the results of a single experiment are shown in figure 6.3. At 2 hours, there was increased surface expression of E selectin on CD3+ T cells co-cultured with MHC class II+ HUVEC and TSST-1 (10 ng/ml) which would be compatible with the hypothesis that E selectin EMP released from activated HUVEC are binding to the T cells present in the co-culture. To confirm this, however, it would be important to examine mRNA expression for E selectin in these T cells to ensure that the T cells are not synthesising E selectin de novo (Harashima, Horiuchi and others, 2001). Additionally, it would be important to examine this phenomenon over a longer time frame (0-24 hours). Nonetheless, these preliminary data do suggest that uptake of EMP by T cells may be a valid hypothesis to account for the decrease in EMP in the supernatant of the co-culture experiments described in chapter 5. Moreover, transfer of E selectin to the T cell may have functional implications, for example by conferring an increased tendency for T cells to adhere to other leukocytes or endothelial cells.
Figure 6.3: Preliminary experiment demonstrating surface expression of E selectin (CD62E) on T cells (CD3+) from a healthy adult donor following co-culture with MHC class II+ HUVEC and TSST-1 (10 ng/ml).
6.1.9 A proposed model for SAg-induced vascular injury

The exact mechanism by which SAg induces disease and in particular vascular injury remains unclear. Intense cytokine release, especially TNF-α, is believed to be particularly important in the pathogenesis of the capillary leak underlying the toxic shock syndrome. Based on the data described in chapter 4 it is possible to propose a model whereby SAg may cause vascular injury (figure 6.4). Central to this model is the concept that the endothelial cell can operate as a SAg presenting cell. SAg may initially meet and be presented to T cells at sites such as the upper respiratory tract or gut-associated lymphoid tissue. It is well established that such an interaction results in the production of a plethora of pro-inflammatory cytokines including IFN-γ, TNF-α, IL-1, and IL-2. This in itself could upregulate MHC class II and cell adhesion molecule expression on endothelial cells locally and/or systemically. Systemic absorption of SAg on the background of this endothelial activation could result in further Vβ-restricted adherence to the endothelium, with further massive T cell activation directly on the luminal endothelial surface of blood vessels. This could result in further endothelial activation and injury mediated by several potential downstream events including recruitment of other inflammatory cells. In some vasculitic syndromes (e.g. KD) this may be the initial and predominant triggering event, whereas in others (such as PAN or WG) such SAg-mediated endothelial injury may be operating as an additional risk factor on the background of several other genetic (e.g. FcγR polymorphisms) and environmental (e.g. silica exposure) predisposing factors. This
Figure 6.4: Proposed model for SAg-induced vascular injury

**KEY**

- **APC** = antigen presenting cell
- **T Ly** = T lymphocyte
- **Vα/Vβ**
- **INF-γ**
- **TNF-α**

**Vascular injury**

**APC**

- **MHC II**

**T lymphocyte** (CD4 or CD8)

Activated endothelial cell

MHC class II

SAg

APC = antigen presenting cell

T Ly = T lymphocyte
proposed model does not, however, preclude other mechanisms of vascular injury and may operate in conjunction with endothelial and vascular injury mediated by conventional antigens, autoantibodies, complement, or immune complexes.

6.1.10 Future work: SAg peptide antagonists

The fascinating experiments relating to SAg peptide antagonists published by Arad et al (Arad G, Levy R and others, 2000) open up another line of future experimental work. It would be interesting to see whether the p12 SAg antagonist could block Vβ restricted T cell endothelial adherence and activation. In September 2000 contact was made with the senior author of this paper who unfortunately declined any collaboration. This may have been because their group is developing this technology as protection against potential germ warfare with superantigenic toxins (Kaempfer, Arad and others, 2002). SAg antagonists may prove to be important blockers of SAg-dependent T cell mediated endothelial injury, however, and this would be an area worthy of considerable future research.

6.1.11 Cellular microparticles: Potential novel mechanisms of vascular injury

The observations made in chapter 5 relating to EMP generation from stimulated HUVEC in vitro led to the in vivo study of EMP in children with vasculitis. In some respects this was a “sideways” step, but (in the opinion of the author) an important step to take since the results provide important insight into the endothelial injury associated with vasculitis in the young. It should be emphasised that the observation of increased EMP in children
with active vasculitis in no way implies that SAgs are necessarily involved in the pathogenesis of these vasculitic syndromes (although SAg-dependent T cell mediated endothelial injury clearly is associated with increased EMP formation \textit{in vitro}), and it is likely that EMP formation is the endpoint of a number of different immunological pathways culminating in endothelial injury.

It is conceivable that microparticles (endothelial, platelet, or other) could be functionally important in the vascular injury and prothrombotic state associated with vasculitis, and future work examining this area may provide insight into novel pathogenetic mechanisms. For example, the prothrombotic potential of PS-rich cellular and platelet microparticles is likely to be relevant to the vasculitides. An obvious future experiment would be to compare the fibrin-generating potential of microparticles derived from either activated or resting endothelial cells \textit{in vitro}. Furthermore, it is interesting that microparticles of neutrophil origin can activate endothelial cells \textit{in vitro} (Mesri and Altieri, 1998). Another set of experiments could examine the potential for neutrophil microparticle generation in response to ANCA stimulation (following TNF-\(\alpha\) priming).

In terms of SAgs, it might also be interesting to study the generation of V\(\beta\) specific microparticles from T cells in vasculitis patients. Given the inherent problems in considering T cell V\(\beta\) repertoire skewing such an approach may cast further light on this complex issue by providing evidence of V\(\beta\)-specific lymphocyte activation and/or apoptosis, which would be compatible with an effect of SAgs.
It would also be fascinating to investigate if MHC class II positive endothelial cells can generate MHC class II positive microparticles. One implication of this hypothesis would be that such MHC class II positive microparticles might be able to present SAg to T cells raising the possibility of a mechanism which could massively amplify the immune response to SAGs and profoundly contribute to the systemic inflammatory response associated with SAg-mediated diseases.

6.1.12 Organ-specific endothelial microparticles?

Another intriguing question relating to EMP would be to ask whether it was possible to identify different phenotypic subclasses of EMP that correlated with either the size of the blood vessel involved or the organs involved in the vasculitic process. For example, identification of a specific glomerular endothelial marker may allow the study of specific glomerular EMP profiles, which may then cast light on the extent of specific renal injury associated with vasculitis. Another obvious example would be in KD where the identification of a coronary arterial endothelial marker expressed on EMP may have prognostic implications in terms of the risk of developing coronary arterial aneurysms. At the time of writing this thesis the author is not aware of any organ-specific endothelial markers that would allow such a study, although clearly this approach would be of considerable interest.
6.1.13 Concluding remarks

In conclusion the data presented in this thesis would suggest that T cells are involved in the aetiopathogenesis of vasculitis syndromes of the young. Moreover, it was possible to demonstrate peripheral blood T cell Vβ skewing over and above that observed in disease controls. Whilst these observations would be compatible with a superantigenic effect, these data do not unequivocally resolve this contentious issue. One way that SAgs could contribute to vascular injury may involve the endothelial cell as a SAg presenting cell, resulting in Vβ specific CD4 and CD8 T cell adherence and activation directly on the luminal endothelial surface. The consequence of such an interaction is upregulation of endothelial cell adhesion molecules and the generation of endothelial microparticles. Lastly, endothelial microparticles are increased in the circulation of children with active vasculitis, and correlate with clinical and laboratory parameters of vasculitic disease activity. This observation provides further evidence of endothelial injury in vasculitis, and it is anticipated that it may provide insight into novel pathogenetic mechanisms of vascular injury.
### Appendix 1: Classification of vasculitic syndromes

#### 1. Chapel Hill Consensus (Jennette, Falk and others, 1994)

<table>
<thead>
<tr>
<th>Large vessel vasculitis</th>
<th>Medium-sized vessel vasculitis</th>
<th>Small vessel vasculitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giant cell (temporal) arteritis</td>
<td>Polyarteritis nodosa* (classic polyarteritis nodosa)</td>
<td>Wegener’s granulomatosis†</td>
</tr>
<tr>
<td>Takayasu arteritis</td>
<td>Kawasaki disease</td>
<td>Churg-Strauss syndrome‡</td>
</tr>
<tr>
<td></td>
<td>Medium-sized vessel vasculitis</td>
<td>Microscopic polyangiitis† (microscopic polyarteritis)‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Henoch-Schönlein purpura</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Essential cryoglobulinemic vasculitis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cutaneous leukocytoclastic angiitis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Large vessel vasculitis</th>
<th>Medium-sized vessel vasculitis</th>
<th>Small vessel vasculitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulomatous arteritis of the aorta and its major branches, with a predilection for the extracranial branches of the carotid artery. Often involves the temporal artery. Usually occurs in patients older than 50 and often is associated with polymyalgia rheumatica.</td>
<td>Necrotizing inflammation of medium-sized or small arteries without glomerulonephritis or vasculitis in arterioles, capillaries, or venules.</td>
<td>Necrotizing inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium-sized vessels (e.g., capillaries, venules, arterioles, and arteries). Necrotizing glomerulonephritis is common.</td>
</tr>
<tr>
<td>Takayasu arteritis</td>
<td>Kawasaki disease</td>
<td>Churg-Strauss syndrome‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mississippi polyangiitis† (microscopic polyarteritis)‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Henoch-Schönlein purpura</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Essential cryoglobulinemic vasculitis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cutaneous leukocytoclastic angiitis</td>
</tr>
</tbody>
</table>

*Large vessel refers to the aorta and the largest branches directed toward major body regions (e.g., to the extremities and the head and neck); medium-sized vessel refers to the main visceral arteries (e.g., renal, hepatic, coronary, and mesenteric arteries); small vessel refers to venules, capillaries, arterioles, and the intraparenchymal distal arterial radicals that connect with arterioles. Some small and large vessel vasculitides may involve medium-sized arteries, but large and medium-sized vessel vasculitides do not involve vessels smaller than arteries. Essential components are represented by normal type, italicised type represents usual, but not essential, components.

†Preferred term. ‡Strongly associated with antineutrophil cytoplasmic autoantibodies (ANCA).
2. American College of Rheumatology (ACR) Criteria (for polyarteritis nodosa (Lightfoot, Jr., Michel and others, 1990) modified for children (Brogan, Davies and others, 2002).

<table>
<thead>
<tr>
<th>CRITERION</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Weight loss of 5% or more, OR failure to thrive</td>
<td>Loss of 5% or more of body weight since illness began, not caused by dieting or other factors, OR fall off in weight from the child's normal centile</td>
</tr>
<tr>
<td>2. Livedo reticularis</td>
<td>Mottled reticular pattern over the skin of portions of the extremities or torso</td>
</tr>
<tr>
<td>3. Testicular pain or tenderness</td>
<td>Pain or tenderness of the testicles, not caused by infection, trauma, or other causes</td>
</tr>
<tr>
<td>4. Myalgias, weakness, or leg tenderness</td>
<td>Diffuse myalgias (excluding shoulder and hip girdle) or weakness of muscles or tenderness of leg muscles</td>
</tr>
<tr>
<td>5. Mononeuropathy or polyneuropathy</td>
<td>Development of mononeuropathy, multiple mononeuropathies, or polyneuropathy</td>
</tr>
<tr>
<td>6. Systemic hypertension</td>
<td>Systolic or diastolic BP greater than age-related reference range</td>
</tr>
<tr>
<td>7. Elevated blood urea nitrogen or creatinine</td>
<td>Elevation of BUN or creatinine above age related reference range, not due to dehydration or obstruction</td>
</tr>
<tr>
<td>8. Hepatitis B virus</td>
<td>Presence of hepatitis B surface antigen or antibody in serum</td>
</tr>
<tr>
<td>9. Arteriographic abnormality</td>
<td>Arteriogram showing aneurysms or occlusions of the visceral arteries, not caused by arteriosclerosis, fibromuscular dysplasia, or other non-inflammatory causes</td>
</tr>
<tr>
<td>10. Biopsy of small- or medium-sized artery containing polymorphs</td>
<td>Histological changes showing the presence of granulocytes or granulocytes and mononuclear leukocytes in the artery wall</td>
</tr>
</tbody>
</table>

The presence of 3 or more of the criteria will define a patient as having PAN.

3. ACR criteria for classification of Henoch-Schönlein purpura (Mills, Michel and others, 1990)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palpable purpura</td>
<td>Slightly raised “palpable” hemorrhagic skin lesions not related to thrombocytopenia</td>
</tr>
<tr>
<td>Age &lt;=20 yr at onset</td>
<td>Patient &lt;=20 yr old at onset of first symptoms</td>
</tr>
<tr>
<td>Bowel angina</td>
<td>Diffuse abdominal pain, worse after meals, or the diagnosis of bowel ischemia, usually including bloody diarrhea</td>
</tr>
<tr>
<td>Wall granulocytes on biopsy</td>
<td>Histologic changes showing granulocytes in he walls of arterioles or venules</td>
</tr>
</tbody>
</table>

For purposes of classification, a patient shall be said to have Henoch-Schönlein purpura if at least two of these criteria are present. The presence of any two or more criteria yields a sensitivity of 87.1% and specificity of 87.7%.
4. ACR criteria for the classification of hypersensitivity vasculitis (Calabrese, Michel and others, 1990)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset &gt;16 yr</td>
<td>Development of symptoms after age 16 yr</td>
</tr>
<tr>
<td>Medication at disease onset</td>
<td>Medication that may have been a precipitating factor was taken at the onset of symptoms</td>
</tr>
<tr>
<td>Palpable purpura</td>
<td>Slightly elevated purpuric rash over one or more areas of skin; does not blanch with pressure and is not related to thrombocytopenia</td>
</tr>
<tr>
<td>Maculopapular rash</td>
<td>Flat and raised lesions of various sizes over one or more areas of the skin</td>
</tr>
<tr>
<td>Biopsy (including arteriole and venule)</td>
<td>Histologic changes showing granulocytes in a perivascular or extravascular location</td>
</tr>
</tbody>
</table>

For purposes of classification, a patient shall be said to have hypersensitivity vasculitis if at least three of these criteria are present. The presence of any three or more criteria yields a sensitivity of 71.0% and specificity of 83.9%. The age criterion is not applicable for children.

5. ACR criteria for the classification of Wegener’s Granulomatosis (Leavitt, Fauci and others, 1990)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal or oral inflammation</td>
<td>Painful or painless oral ulcers or purulent or bloody nasal discharge</td>
</tr>
<tr>
<td>Abnormal-appearing chest radiograph</td>
<td>Nodules, fixed infiltrates or cavities</td>
</tr>
<tr>
<td>Abnormal urinary sediment</td>
<td>Microhematuria (&gt;5 RBC/hpf) or RBC casts</td>
</tr>
<tr>
<td>Granulomatous inflammation</td>
<td>Granulomatous inflammation within the wall of an artery or in the perivascular or extravascular area of an artery or arteriole</td>
</tr>
</tbody>
</table>

Diagnosis of Wegener’s granulomatosis requires the presence of two of the four criteria. The presence of any two or more criteria has a sensitivity of 88.2% and a specificity of 92.0%. RBC, red blood cells.
Appendix 2: The Birmingham Vasculitis Activity Score (BVAS) (Luqmani, Bacon and others, 1994)

Tick box only if abnormality is newly present or worsening within the previous 4 weeks and ascribable to vasculitis

<table>
<thead>
<tr>
<th>SYSTEMIC</th>
<th>3 (maximum total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>malaise</td>
<td>1</td>
</tr>
<tr>
<td>myalgia</td>
<td>1</td>
</tr>
<tr>
<td>arthralgia/arthritis</td>
<td>1</td>
</tr>
<tr>
<td>fever (&lt;38.5°C)</td>
<td>1</td>
</tr>
<tr>
<td>fever (&gt;38.5°C)</td>
<td>2</td>
</tr>
<tr>
<td>wt loss (1-2 kg) within past month</td>
<td>2</td>
</tr>
<tr>
<td>wt loss (&gt;2 kg) within past month</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CUTANEOUS</th>
<th>6 (maximum total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>infarct</td>
<td>2</td>
</tr>
<tr>
<td>purpura</td>
<td>2</td>
</tr>
<tr>
<td>other skin vasculitis</td>
<td>2</td>
</tr>
<tr>
<td>ulcer</td>
<td>4</td>
</tr>
<tr>
<td>gangrene</td>
<td>6</td>
</tr>
<tr>
<td>multiple digit gangrene</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MUCOUS MEMBRANES/EYES</th>
<th>6 (maximum total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>mouth ulcers</td>
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<td>genital ulcers</td>
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<td>episcleritis</td>
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<td>uveitis</td>
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<tr>
<td>retinal exudates</td>
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<td>retinal haemorrhage</td>
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<td>epistaxis</td>
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<tr>
<td>crusting</td>
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<tr>
<td>aural discharge</td>
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<td>otitis media</td>
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<tr>
<td>new deafness</td>
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<tr>
<td>hoarseness/laryngitis</td>
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<td>subglottic involvement</td>
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<td>nodules or fibrosis</td>
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</tr>
<tr>
<td>Condition</td>
<td>Score</td>
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<tr>
<td>-----------------------------------</td>
<td>-------</td>
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<td>pleural effusion/pleurisy</td>
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<tr>
<td>infiltrate</td>
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<td>haemoptysis/haemorrhage</td>
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<td>new loss of pulses</td>
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<td>aortic incompetence</td>
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<td>CCF/cardiomyopathy</td>
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<td>gut infarction</td>
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<td>pancreatitis</td>
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<td>haematuria (&gt;1 + or &gt;10 rbc/ml)</td>
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<td>creatinine &gt;500 µmol/l</td>
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<td>rise in creatinine &gt;10%</td>
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<td><strong>9. NERVOUS SYSTEM</strong></td>
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<td>organic confusion/dementia</td>
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<td>cord lesion</td>
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<td>peripheral neuropathy</td>
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<td>motor mononeuritis multiplex</td>
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<td><strong>MAXIMUM SCORE</strong></td>
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Appendix 3: 96-well Antibody plate plan for determination of
the T cell Vβ repertoire

<table>
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<tr>
<th>Cells alone</th>
<th>CD3F</th>
<th>Vβ3F Isotype control</th>
<th>Vβ8F</th>
<th>CD3PE</th>
<th>CD69PE Isotype control</th>
<th>CD69 PE</th>
<th>CD25PE Isotype control</th>
<th>CD25 PE</th>
<th>CD4 QR</th>
<th>CD8QR</th>
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<tr>
<td>VP1F</td>
<td>VP1F</td>
<td>VP2F</td>
<td>VP3F</td>
<td>VP3F</td>
<td>VP4F</td>
<td>VP4F</td>
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<td>VP5F</td>
<td>VP13.1F</td>
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<td>VP16F</td>
<td>VP16F</td>
<td>VP17F</td>
<td>VP18F</td>
<td>VP18F</td>
<td>VP19F</td>
<td>VP19F</td>
<td>VP20F</td>
<td>VP20F</td>
<td>VP13.6F</td>
<td>VP14F</td>
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<td>CD3PE CD8QR</td>
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<td>VP1F</td>
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<td>VP5F</td>
<td>VP13.1F</td>
<td>VP14F</td>
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Appendix 4: 96-well antibody plate plan for determination of T cell Vβ-specific CD69 expression

<table>
<thead>
<tr>
<th>Cells alone</th>
<th>Vβ8.2F Isotype control</th>
<th>Vβ8.2F</th>
<th>CD3PE</th>
<th>CD69PE Isotype control</th>
<th>CD69PE</th>
<th>CD4 QR</th>
<th>CD3QR</th>
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<tbody>
<tr>
<td>CD69PE CD4QR Vβ1F</td>
<td>CD69PE CD4QR Vβ1F</td>
<td>CD69PE CD4QR Vβ5.1F</td>
<td>CD69PE CD4QR Vβ8F</td>
<td>CD69PE CD4QR Vβ12F</td>
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<td>CD69PE CD8QR Vβ1F</td>
<td>CD69PE CD8QR Vβ1F</td>
<td>CD69PE CD8QR Vβ5.1F</td>
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<td>CD69PE CD8QR Vβ12F</td>
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Appendix 5: Flow cytometry instrument settings for PBMC and T cells, HUVEC and microparticles

<table>
<thead>
<tr>
<th>PBMC/T cells</th>
<th>Detector</th>
<th>Voltage</th>
<th>Amp gain</th>
<th>Mode</th>
<th>Threshold</th>
<th>Compensation</th>
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<tr>
<td></td>
<td>Forward</td>
<td>E00</td>
<td>1.00</td>
<td>Linear</td>
<td>105</td>
<td>FL1-0.5%FL2</td>
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<tr>
<td></td>
<td>scatter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FL2-36% FL1</td>
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<tr>
<td></td>
<td>Side</td>
<td>440</td>
<td>2.3</td>
<td>Linear</td>
<td>52</td>
<td>FL2-37%FL3</td>
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<td>scatter</td>
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<td></td>
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<td></td>
<td>(for CD4)</td>
</tr>
<tr>
<td></td>
<td>FL1 (FITC)</td>
<td>582</td>
<td>-</td>
<td>Logarithmic</td>
<td>52</td>
<td>FL2-3.7% FL3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(for CD8)</td>
</tr>
<tr>
<td></td>
<td>FL2 (PE)</td>
<td>645</td>
<td>-</td>
<td>Logarithmic</td>
<td>52</td>
<td>FL3-22.4%FL2</td>
</tr>
<tr>
<td></td>
<td>FL3 (QR)</td>
<td>715</td>
<td>-</td>
<td>Logarithmic</td>
<td>52</td>
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</table>

| HUVEC                 | Forward   | E1      | -        | Logarithmic| 80        | Non-applicable                  |
|                       | scatter   |         |          |           |           | since only single colour FACS |
|                       | Side      | 359     | 1.00     | Linear    | 52        | analysis performed              |
|                       | scatter   |         |          |           |           |                                |
|                       | FL1 (FITC)| 401     | -        | Logarithmic| 52        |                                |
|                       | FL2 (PE)  | 408     | -        | Logarithmic| 52        |                                |
|                       | FL3 (CYC) | 159     | -        | Logarithmic| 52        |                                |

| Microparticles        | Forward   | E01     | -        | Logarithmic| 80        | FL1-4.8%FL2                     |
|                       | scatter   |         |          |           |           | FL2-13.7% FL1                   |
|                       | Side      | 377     | -        | Logarithmic| 52        | FL2-0%FL3                       |
|                       | scatter   |         |          |           |           | FL3-18.1%FL2                    |
|                       | FL1 (FITC)| 782     | -        | Logarithmic| 136       |                                |
|                       | FL2 (PE)  | 749     | -        | Logarithmic| 52        |                                |
|                       | FL3 (CYC) | 803     | -        | Logarithmic| 52        |                                |
Appendix 6: Equation used to derive the number of 3 μm latex beads added for the analysis of microparticles (Sigma product information)

\[
N = \frac{(6 \times 10^{10}) \times S \times P_l}{\pi \times P_s \times d^3} \quad \text{OR} \quad N = 1.828 \times 10^{11} d^3
\]

Where:
- \( N \) = number of beads per ml
- \( S \) = % solids (w/w)
- \( D \) = diameter (μM)
- \( P_s \) = density of bulk polymer (g/ml)
- \( P_l \) = density of latex (g/ml)

When:
- \( S \) = 10% solids
- \( S = 1.05 \text{ g/ml (polystyrene)} \)
- \( P_l = 1.005 \text{ g/ml} \)
Appendix 7: Plate plan for T cell and HUVEC co-culture experiments
Appendix 8: Plate plan for integrin blockade of SAg-mediated T cell adhesion to HUVEC
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Publications from this thesis:


Papers submitted and pending peer review:


Brogan PA. Recent advances in Kawasaki disease: In: Recent advances in paediatrics (David TJ, Ed; book chapter submitted April 2003).