AN INVESTIGATION OF FACTORS AFFECTING THE BINDING AND PATHOGENICITY OF HUMAN ANTI-DNA ANTIBODIES

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ABSTRACT

High affinity IgG anti-DNA antibodies are believed to be important in the pathogenesis of lupus nephritis. However, some anti-dsDNA antibodies appear relatively harmless. This thesis examines factors which determine the pathogenicity of anti-DNA antibodies and their ability to damage the kidney.

Chapter Three compares the pathological effect of implanting anti-DNA antibody RH-14 in 2 month old SCID mice, ‘leaky’ 8 month old SCID mice and ‘non-leaky’ Rag-1⁻/⁻ mice. RH-14 deposition resulted in hyaline thrombi associated with fibrin in 8 month old SCID mice but not in 2 month old mice. However, these thrombi were not associated with greater pathology than had been observed in 2-month old mice implanted with RH-14. Foot process effacement was not observed in the 8 month old mice.

Chapter Four describes how the CDRs were interchanged between the lambda chains of two human hybridoma derived anti-DNA antibodies (B3 & 33.H11) and a human antiphospholipid antibody (UK4). The chimeric light chains were paired with the heavy chain of B3 and whole IgG molecules were produced using a transient expression system. As predicted by computer modelling, arginine residues as positions 27a (B3 V₅CDR1) and 92 (33.H11 V₅CDR3) enhanced antibody binding to DNA, whilst an arginine at position 94 (UK4 V₅CDR3) blocked binding. The requirement of a co-factor, present in cell supernatant, was shown for the binding of affinity purified and DNase I treated recombinant anti-DNA antibodies to dsDNA.

Chapter Five describes stable CHO cell lines producing recombinant B3 and its mutants which were implanted into SCID mice to assess the pathogenicity of the different IgG molecules. This system had too much inherent background pathology to assess the pathogenicity of the anti-DNA IgG.

Chapter Six of this thesis demonstrates that two pathogenic human anti-dsDNA monoclonal antibodies bind to α-actinin, but not a non-pathogenic anti-dsDNA antibody. Patients with SLE had significantly higher binding to α–actinin than healthy controls. A greater proportion (6/10) of anti-DNA antibodies purified from the sera of lupus patients with renal disease bound to α-actinin than those purified from patients without renal disease (2/8).
This thesis is dedicated to Robert and my parents
for their constant support and encouragement
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DECLARATION

The work described in this thesis was carried out by the author unless otherwise stated.
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<td>Anti-nuclear antibodies</td>
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<tr>
<td>Anti-dsDNA</td>
<td>Antibodies to double stranded DNA</td>
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<tr>
<td>Anti-ssDNA</td>
<td>Antibodies to single stranded DNA</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APS</td>
<td>Antiphospholipid syndrome</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BIC</td>
<td>Bicarbonate buffer</td>
</tr>
<tr>
<td>BILAG</td>
<td>British Isles Lupus Assessment Group</td>
</tr>
<tr>
<td>BlyS</td>
<td>B lymphocyte stimulator protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs (of DNA)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BWF1</td>
<td>1st generation (F1) of New Zealand Black x New Zealand White mice</td>
</tr>
<tr>
<td>C</td>
<td>Complement component (as in C1q, C2 etc.)</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity-determining region</td>
</tr>
<tr>
<td>C_H</td>
<td>Constant domain of heavy chain</td>
</tr>
<tr>
<td>CHO cell</td>
<td>Chinese hamster ovary cell</td>
</tr>
<tr>
<td>C_L</td>
<td>Constant domain of light chain</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment of antibody</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fragment of antibody</td>
</tr>
<tr>
<td>Fcγ-R</td>
<td>Fc-gamma receptor</td>
</tr>
<tr>
<td>FR</td>
<td>Framework region</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
</tr>
<tr>
<td>H1</td>
<td>Histone 1 (also H2A, H2B, H3 and H4)</td>
</tr>
<tr>
<td>H</td>
<td>Heavy chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>-------------</td>
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<tr>
<td>SEC</td>
<td>Sample/enzyme/conjugate dilution buffer</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
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<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
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<tr>
<td>SLEDAI</td>
<td>SLE Disease Activity Index</td>
</tr>
<tr>
<td>Sm</td>
<td>smith antigen (spliceosome)</td>
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<td>SNF1</td>
<td>First generation (F1) of Swiss-Webster and New Zealand Black mice</td>
</tr>
<tr>
<td>SnRNP</td>
<td>small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
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<td>TE</td>
<td>Tris EDTA buffer</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TH</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VH</td>
<td>Variable domain of heavy chain</td>
</tr>
<tr>
<td>VK</td>
<td>Variable domain of kappa light chain</td>
</tr>
<tr>
<td>VL</td>
<td>Variable domain of lambda light chain</td>
</tr>
<tr>
<td>VL</td>
<td>Variable domain of light chain</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>MSB</td>
<td>martius yellow, brilliant crystal scarlet and soluble blue</td>
</tr>
<tr>
<td>PTAH</td>
<td>phosphotungstic acid haematoxylin</td>
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CHAPTER ONE

Introduction
CHAPTER ONE

INTRODUCTION

1.1 Characterisation of Systemic Lupus Erythematosis and Lupus Nephritis

1.1.1 Autoimmunity

The adaptive immune response is a critical component of host defence against infection. Unfortunately, when the target of the immune response becomes self-antigen, as opposed to foreign antigen, the immune effector mechanisms may be unable to completely remove the antigen, resulting in a sustained immune response, chronic inflammation and self-tissue damage as seen in autoimmunity. Autoimmune diseases can be loosely classified as organ-specific, affecting a single organ, or systemic, affecting multiple organ systems.

1.1.2 What is systemic lupus erythematosis?

Systemic Lupus Erythematosis (SLE) is a systemic autoimmune rheumatic disease. Diagnosis of the disease is based on a set of criteria (Table 1.1) published by the American College of Rheumatology (Tan et al. 1982 and updated Hochberg 1997). Patients with SLE have a wide diversity of clinical problems. Musculoskeletal (arthralgia/arthritis and myalgia) and dermatological (butterfly rash, alopecia, purpura and vasculitis) manifestations are the most frequent, but there is often also involvement of kidney, heart, lungs and central nervous system. In patients with SLE, disease activity and the organ systems involved vary over time (i.e. cycles of relapse and remission). Flares of the disease may require drug treatment with non-steroidal anti-inflammatories, antimalarials, corticosteroids and other immunosuppressive agents. Survival rates have increased over recent decades. A recent multi-centre European study found a survival probability of 92% after 10 years; a lower survival probability was detected in those patients who had presented with nephropathy. The most frequent causes of death over a ten year period, were active SLE (26.5%, including renal 8.8%), thromboses (26.5%, always associated
Table 1.1 The American College of Rheumatology Criteria for the Diagnosis of Systemic Lupus Erythematosus

To be diagnosed as having SLE, a patient must either have serially, or simultaneously, satisfied four or more of these eleven criteria.

<table>
<thead>
<tr>
<th>CRITERION</th>
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<tr>
<td>1. Malar rash</td>
<td>Fixed malar erythema (Butterfly rash)</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches</td>
</tr>
<tr>
<td>3. Photosensitivity</td>
<td>Skin rash as an abnormal reaction to sunlight</td>
</tr>
<tr>
<td>4. Oral ulcers</td>
<td>Oral or nasopharyngeal ulcers, usually painless.</td>
</tr>
<tr>
<td>5. Arthritis</td>
<td>Non-erosive arthritis involving two or more peripheral joints</td>
</tr>
<tr>
<td>6. Serositis</td>
<td>Either pleuritis or pericarditis for which convincing clinical evidence exists</td>
</tr>
<tr>
<td>7. Renal disorder</td>
<td>Persistent proteinuria, greater than 0.5g/day or greater than 3+</td>
</tr>
<tr>
<td>8. Neurological disorder</td>
<td>Seizures or psychosis in the absence of offending drugs or other metabolic derangement</td>
</tr>
<tr>
<td>9. Haematological disorder</td>
<td>Haemolytic anaemia or leukopenia (&lt;4000/mm$^3$ total on two or more occasions) or lymphopenia (&lt;1500/mm$^3$ total on two or more occasions) or thrombocytopenia &lt;100 000/mm$^3$ total (non-drug induced)</td>
</tr>
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<td>10. Immunological disorder</td>
<td>Presence of anti-dsDNA or anti-Sm or anti-phospholipid antibodies, based on (i) an abnormal serum level of IgG or IgM anti-cardiolipin antibodies (ii) positive for lupus anticoagulant or (iii) a false positive serologic test for syphilis known to be positive for at least six months</td>
</tr>
<tr>
<td>11. Anti-nuclear antibody</td>
<td>Abnormal titre of ANA at any point in time and in the absence of drugs known to be associated with 'drug-induced' lupus</td>
</tr>
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</table>

Adapted from Tan et al. (1982) and updated Hochberg (1997)
with the presence of antiphospholipid syndrome), and infections (25%), probably secondary to immunosuppressive drug treatment (Cervera et al. 2003).

1.1.3 Assessment of anti-dsDNA antibodies and disease activity in patients with SLE

The presence of anti-nuclear antibodies (ANAs), and anti-double-stranded deoxyribonucleic acid (anti-dsDNA) antibodies are diagnostic criteria for SLE. Rapid rises in anti-dsDNA antibodies can predict exacerbations (flares) of disease activity. The most commonly used assays to detect anti-dsDNA antibodies are the Enzyme-linked immunosorbent assay (ELISA), the Farr assay and binding to Crithidia luciliae. The properties of these assays are summarised in Table 1.2. Large cohort studies of patients with SLE have generally demonstrated an increase in levels of anti-dsDNA IgG antibodies, with increasing activity of lupus nephritis (Lloyd and Schur 1981; ter Borg et al. 1990; Okamura et al. 1993). One study demonstrated that a sharp drop in anti-DNA levels preceded by a rise in levels, was shown to predict a serious exacerbation of renal disease, presumably due to deposition of the antibodies in the kidney (Swaak et al. 1979).

However, many of the studies carried out prior to the mid-1980s attempted to correlate anti-DNA levels with unreliable and unvalidated disease activity indices. Several validated indices are now in use by clinicians, including the SLE disease activity index, SLEDAI (Bombardier et al. 1992); the systemic lupus activity measure, SLAM (Liang et al. 1988); and the European Community lupus activity measure, ECLAM (Vitali et al. 1992). However, these global points scores may not distinguish patients who may have a life threatening disease in one organ or system from those who have mild disease in several organ systems. A more detailed if more complex index, was developed by the British Isles Lupus Assessment Group (BILAG) (Hay et al. 1993). This index, based on the ‘physician’s intention to treat’ principle, distinguishes activity in eight organs or systems. For example, the renal assessment system of BILAG (which was used in the selection of patient sera for the experiments in Chapter six), is shown in Table 1.3. Using the BILAG score, antibodies to dsDNA were shown to correlate with renal disease (P=0.0006),
<table>
<thead>
<tr>
<th>ASSAY</th>
<th>PRINCIPLE IN BRIEF</th>
<th>ADVANTAGES</th>
<th>LIMITATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farr Assay</td>
<td>Radiolabelled ssDNA or dsDNA is added to sample to be tested for anti-DNA antibodies. Resulting radioactive immune complexes are precipitated by ammonium sulphate and results expressed as % of radioactivity in precipitate.</td>
<td>Distinguishes between high and low-affinity anti-DNA antibodies.</td>
<td>Does not distinguish IgG &amp; IgM isotypes.</td>
</tr>
<tr>
<td>ELISA</td>
<td>ssDNA or dsDNA is applied to the wells of polystyrene plates and the amount of anti-DNA antibodies in the serum is measured colorimetrically by adding an enzyme-labelled, affinity-purified anti-immunoglobulin secondary and appropriate substrate.</td>
<td>Rapid, simple, quantitative and reproducible assay. Can determine antibody isotype and light chain usage depending on the specificity of the secondary antibody.</td>
<td>May detect anti-nucleosome antibodies, if DNA coating the plate or sample contains histones.</td>
</tr>
<tr>
<td>Crithidia luciliae</td>
<td>Anti-dsDNA antibodies are detected by their ability to bind the kinetoplast of the protozoan, <em>Crithidia luciliae</em>. The kinetoplast is pure circular dsDNA. Anti-dsDNA antibodies in the test sample, are detected by fluoresceinated secondary antibody.</td>
<td>The most specific test for anti-DNA antibodies. Can determine anti-dsDNA subsets, depending on the specificity of the secondary antibody.</td>
<td>Not as sensitive as the ELISA.</td>
</tr>
</tbody>
</table>

Table 1.2  Assays for the detection of anti-DNA antibodies
Adapted from Hahn (1998), and Isenberg and Smeenk (2002)
Table 1.3 British Isles Lupus Assessment Group (BILAG) renal system assessment

**Category A**
Two or more of the following providing 1, 4, or 5 is included:

1. Proteinuria, defined as (a) urinary dipstick increased by 2 or more levels; or (b) 24 h urinary protein rising from < (but not >) 0.2g to > 1g; or (c) 24 h urinary protein rising from >1g by 100%; or (d) newly documented proteinuria of > 1g.
2. Accelerated hypertension.
3. Deteriorating renal function, defined as (a) plasma creatinine > 130μM/L and having risen to > 130% of previous value; or (b) creatinine clearance having fallen to <67% of previous value; or (c) creatinine clearance < 50ml/min, and last time was > 50ml/min or was not measured.
4. Active urinary sediment (on uncentrifuged specimen): pyuria (>5wc/hpf), haematuria (>5rbc/hpf) or red cell casts in the absence of infection or other cause.
5. Histological evidence of active nephritis by WHO criteria within the last 3 months (or since previous assessment if seen less than 3 months ago: Sclerosis without inflammation is not counted).

**Category B**
One of the following:

1. One of the category A criteria.
2. (a) Urinary dipstick which has risen by 1+ or more to at least 2+, or (b) 24 h urinary protein rising by > 1g by > 50% but <100%.
3. Plasma creatinine > 130μM/L and having risen to 115% of previous value.

**Category C**
One of the following:

1. 24 h urinary protein > 0.25g.
2. Urinary dipstick 1+ or more.
3. Rising blood pressure, defined as (a) systolic rise of ≥ 30mm and (b) diastolic rise of ≥ 15mm (providing the recorded values are > 140/90).

**Category D**
Previous renal disease.

**Category E**
No previous renal disease.

*Adapted from Isenberg and Gordon (2000)*
cardiopulmonary disease (P=0.0004) and global score (P=0.002), but not with musculoskeletal, central nervous system or haematological involvement. This study was carried out on 14 Afro-Caribbean patients with SLE, over 3 to 15 years of follow-up (Isenberg et al. 1997a). A further study of 33 patients with SLE (11 with nephritis, 22 without nephritis and 21 healthy controls) reported higher levels of anti-dsDNA and anti-heparan sulphate (HS) antibodies in patients with lupus nephritis. The level of anti-HS antibodies correlated with the BILAG renal score (Ravirajan et al. 2001).

The predictive value of tests for anti-dsDNA antibodies in SLE patients is controversial. Not all patients show a correlation between high anti-dsDNA levels and active SLE. Some patients have high anti-dsDNA levels but inactive disease (Swaak et al. 1979; Schur and Sandson 1968), whilst others have active SLE disease but no anti-dsDNA antibodies (McCarty et al. 1993). There are several reasons for the discrepancies in these studies. Firstly, there is variation in the specificity and sensitivity of the assays that are used to detect the anti-dsDNA antibodies. Secondly, the anti-DNA levels may not have been correlated with validated clinical or histological indices. Thirdly, an additional factor affecting all clinical research is interpretation of results in patients who have received widely varying drug treatments, especially immunosuppressives which can affect anti-dsDNA levels and renal histological features. However, the most important reason why some studies may show a lack of correlation between the level of anti-dsDNA antibodies and disease activity, is that not all anti-dsDNA antibodies are pathogenic. Factors that may affect the pathogenicity of anti-dsDNA antibodies in SLE, and in particular in lupus nephritis, are the major focus of this thesis and will be discussed further in later sections.

1.1.4 Glomerulonephritis in SLE

Glomerulonephritis in the form of lupus nephritis becomes clinically apparent in 50% to 80% of patients with SLE, resulting in end-stage renal failure in 10% to 15% of patients (Cameron 1999). In 1982, the World Health Organisation (WHO) classification divided renal lupus into six categories, based upon histological analysis of kidney biopsies (Churg and Sobin 1982). The groups are broadly defined as minimal or mesangial change; mild or focal proliferative; severe or diffuse
proliferative, membranous, and end stage sclerosing nephritis. There are also two distinct manifestations of glomerular nephritis not included within the WHO score, tubulointerstitial disease and renal vascular thrombosis. A modification to the classification was recently suggested to include these types of nephritis (Weening et al. 2004). From the point of view of pathogenesis, lupus nephritis is more usefully divided into four categories: proliferative, membranous, or tubulointerstitial glomerulonephritis, and renal thrombosis.
1.2 Immunopathogenesis of Systemic Lupus Erythematosus

Despite the diverse nature of SLE, all clinical presentations of the disease are usually accompanied by the presence of circulating autoantibodies against a multiplicity of cellular antigens. Although in individual patients the antibody response is usually restricted to one or relatively few antigen specificities. The initial stimulus for the disease is likely to be one or more environmental factors combined with the occurrence of susceptibility genes in predisposed individuals. Once the critical threshold is breached, there is also a failure of the immune system to down regulate the ensuing abnormal immune response (Figure 1.1). Key questions include, what are the disease mechanisms that allow the availability of autoantigens and the breakdown of tolerance, to give rise to pathogenic autoantibodies?

1.2.1 Environmental factors

1.2.1.1 Infectious agents

A common but unproven hypothesis is that SLE is triggered by infectious agents, including viruses (reviewed James et al. 2001). Co-immunisation with virus-self complexes has been shown to be a possible mechanism, capable of breaking tolerance and generating autoimmunity. When apoptosis is induced by sindbis virus infection, viral antigens and self-antigens are co-clustered in surface blebs, which may allow breakdown of self-tolerance (Rosen et al. 1995). Many viruses have been shown to cause apoptosis of infected cells in vitro, but this mechanism would probably only play a role in disease pathogenesis in susceptible individuals with defective clearance of apoptotic cells. A role for human polyoma virus BK in the development of anti-dsDNA autoantibodies (discussed 1.3.3.3) has been supported by animal experiments (Rekvig et al. 1997), but their role in human SLE is still unknown.

Epitopes of Sm and nRNP (components of the spliceosome [Sm, Smith antigen; nRNP, nuclear ribonucleoprotein]) autoantigens have been identified, which resemble antigenic peptides derived from Epstein-Barr virus nuclear antigen (EBNA-1), suggesting a possible role for these agents in the etiology of lupus (discussed in section 1.3.3.2, and reviewed James and Harley 1998). A significant increase in the prevalence of Epstein-Barr virus (EBV) infection was found in paediatric patients with lupus compared to controls (James et al. 1997). These patients had not been
Figure 1.1 Hypothesis for the induction and amplification of lupus autoimmunity

(A) T cells exposed to autoantigen in the periphery are normally tolerised. However, in SLE a number of mechanisms appear to cause breakdown of tolerance. Defects in apoptosis and clearance may allow increased presentation of altered self-peptides or foreign antigens may act as molecular mimics. In addition signalling defects may lead to lupus T cells having a lowered threshold of activation. (B) Once activated the T cells can provide help to B cells, which undergo somatic hypermutation and affinity maturation (C) to produce high affinity autoantibodies. Deposition of the autoantibodies in the kidney (D) triggers an inflammatory reaction which results in tissue damage and the release of more self-antigens. These self-antigens can be presented by specific antigen presenting B cells in a second round of T cell activation, resulting in a positive feed back cycle (E,F,G). Processing of the self-antigens allows presentation of novel self-epitopes, resulting in a process of epitope spreading where autoimmune T and B cell responses become diversified and amplified (H). Activated T cells can also cause direct pathology by migration to target tissues and releasing cytokines and mediating direct cytotoxicity (I). Key: T cells are shown in orange and B cells are red. APC, antigen presenting cell (eg dendritic cell).

Figure adapted from Shlomchik et al. (2001).
recently infected with EBV, for example due to immunosupression. Since approximately 95% of adults have been infected with EBV, EBV infection cannot be sufficient to initiate autoimmunity, except in susceptible individuals.

Patients with SLE produce high titre antibodies to various retroviral proteins, including Gag, Env, and Nef of human immunodeficiency virus (HIV) and human T-cell leukaemia virus (HTLV), in the absence of overt retroviral infection. At least 1% of the human genome is encoded by human endogenous retroviral (HERV) sequences, which are thought to have integrated into the human genome during the time of the Old World primates (Lower et al. 1996). In a study of 39 patients with SLE, 86 patients with other rheumatic diseases and 39 healthy controls, antibodies which bound an immunodominant epitope of HERV-K10 Env sequence were significantly elevated in the patients with SLE, as compared to the healthy or disease controls (Herve et al. 2002). However, it is not clear whether these antibodies are driven by expression of endogenous retrovirus protein, by encounter with an exogenous retrovirus or are cross-reactive and driven by an autoantigen. A recent article reviews the role of HERVs in SLE; and proposes that molecular mimicry, between HTLV-1-related endogenous sequence (HRES-1) and the small nuclear ribonucleoproteins (snRNP) complex, initiates autoantibody formation (Adelman and Marchalonis 2002). However, the relationship between either viral infection or HERVs and the initiation of SLE remains controversial.

1.2.1.2 Ultraviolet radiation

Ultraviolet (UV) radiation, particularly UVB, has been shown to trigger and exacerbate the photosensitive lupus rash. There is also evidence that UV light may cause damage to DNA by catalase induction of reactive oxygen species (ROS), which could provide novel self-antigens (Heck et al. 2003). UV light has been shown to induce apoptosis in human keratinocytes, resulting in blebs of nuclear and cytoplasmic autoantigens on the cell surface (Caricchio et al. 2003). This mechanism may provide a source of antigens to drive autoantibody production (Casciola-Rosen and Rosen 1997).
1.2.1.3 Drug-induced lupus

Certain drugs, including procainamide, hydralazine, chlorpromazine, isoniazid, and minocycline have been shown to give rise to drug-induced lupus. However, this disease is associated with antibodies to ss-DNA and histones rather than ds-DNA. Although skin and joint disease are common, renal or central nervous system (CNS) pathology is virtually unknown, and the disease is reversed on curtailment of the drug involved (Knowles et al. 2003).

1.2.2 Genetic factors

Genetic predisposition plays a crucial role in susceptibility to SLE both in patients and animal models, reviewed by Tsao (2003) and Wakeland et al. (2001) respectively. The ‘threshold liability model’ attempts to explain the inheritance of complex multifactorial traits such as susceptibility to autoimmune diseases, including SLE. The model suggests that an individual will develop the disease when the disease liability exceeds a hypothetical threshold, determined by the cumulative content of susceptibility alleles, and modified by environmental exposures and chance occurrences (Wanstrat and Wakeland 2001). In SLE, the genes that determine sex, race and tissue type (human leucocyte antigen, HLA) are believed to influence pathogenicity. The evidence to support this hypothesis stems from: the higher rate of concordance for SLE in monozygotic twins (25%) compared to dizygotic twins (3%), the increased frequency of lupus and immunological abnormalities in relatives of lupus patients compared to healthy controls (Corporaal et al. 2002), and the fact that lupus occurs more frequently in certain ethnic groups (Johnson et al. 1995) and nine times more frequently in females than males. In addition other genetic predisposing factors including complement deficiencies, T and B cell receptor gene usage, and altered expression of phagocyte Fc-gamma receptor (Fcγ-R) alleles will be discussed.

1.2.2.1 Candidate genes

The list of candidate genes for lupus susceptibility is expanding fast, both in murine systems and human linkage studies (Wakeland et al. 2001; Tsao 2003). Several genome scans have been published for SLE, their results varying substantially, because of heterogeneity of sample populations and differing methods of analysis. These studies have identified seven chromosomal regions that have reached the
threshold for significant linkage to SLE [according to Lander and Kruglyak's criteria (Lander and Kruglyak 1995) for interpretation of linkage statistics] and have been confirmed in at least two cohorts of patients (Table 1.4, derived from a review of human genome scans, Tsao 2003).

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Location of loci</th>
<th>Human SLE susceptibility locus</th>
<th>Candidate genes within loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1q23</td>
<td></td>
<td>\textit{FcyRIIA}</td>
</tr>
<tr>
<td>1</td>
<td>1q25-31</td>
<td></td>
<td>\textit{FcyRIIB}</td>
</tr>
<tr>
<td>1</td>
<td>1q41-42</td>
<td>\textit{SELB1}</td>
<td>\textit{PARP}</td>
</tr>
<tr>
<td>2</td>
<td>2q35-37</td>
<td>\textit{SELB2}</td>
<td>\textit{PD-1}</td>
</tr>
<tr>
<td>4</td>
<td>4p16-15.2</td>
<td>\textit{SELB3}</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6p11-21</td>
<td>MHC haplotypes</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>16q12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (Tsao 2003)

Work is ongoing, using fine mapping, to narrow the linked loci to identify the susceptibility genes. In some cases, chromosomal regions containing genes already known to be associated were identified, such as \textit{FcyRIIA} on 1q23 (section 1.2.2.5). Programmed cell death 1 (PD-1 [or PDCD1]), is an inhibitory immunoreceptor with a pivotal role in peripheral tolerance (reviewed Nishimura and Honjo 2001), and mice deficient in PD-1 (Table 1.5) develop glomerulonephritis (Nishimura \textit{et al.} 1999). However, a recent study (98 patients with SLE and 84 patients with RA) suggests that although a PD-1 gene polymorphism may be important in the development of rheumatoid arthritis (RA), it was not associated with SLE (Lin \textit{et al.} 2004). Poly [ADP-ribose] polymerase (PARP) is a nuclear enzyme that mediates post-translational modification of proteins, and has a role in DNA strand break repair. Anti-PARP antibodies, from patients with SLE, prevent caspase-3 mediated
PARP cleavage during apoptosis thus prolonging autoimmune cell survival (Decker et al. 2000).

1.2.2.2 Major Histocompatibility Complex (MHC)

The human leukocyte antigen (HLA) region is a gene rich and transcriptionally active segment, encoding many immunologically important genes, including Major Histocompatibility Complex (MHC) class I and II. Among Caucasians, the haplotype A1 B8 DR3 is associated with an approximately tenfold increase in the risk of developing lupus (Walport et al. 1982). However, the primary link here may be the complement C4 allele, indeed two null alleles increases the relative risk by seventeen times. HLA associations in many non-Caucasian populations are not convincing, although in African-Americans DRw52b is reported to be positively associated with renal disease and negatively associated with anti-nRNP antibodies (Reveille et al. 1989). Interestingly, DR/DQ alleles show stronger association with the autoantibody profiles observed in SLE, than with the disease expression itself (Arnett and Reveille 1992). In patients with SLE, unlike some other autoimmune diseases such as type 1 diabetes and rheumatoid arthritis, the MHC-containing region is not the most prominent susceptibility locus detected by genome scans, suggesting that the MHC contribution to susceptibility is similar to that of non-MHC loci (Tsao 2003).

1.2.2.3 Ethnic background

SLE shows an ethnic bias, with more Afro-Caribbeans being affected than Orientals, who in turn are more affected than Caucasians. Black race is an independent risk factor for developing SLE. African-Americans and Afro-Caribbeans, living in the UK/USA, are at greatest risk of developing SLE, develop the disease earlier in life, and have an increased frequency and severity of renal pathology. A study in Birmingham, U.K, demonstrated major differences in the incidence and prevalence rates of SLE depending on ethnic group. The observed prevalence in females was 206/100 000 among Afro-Caribbeans, 91/100 000 among Asians and 36/100 000 among Caucasians. These results were irrespective of place of birth (Johnson et al. 1995).
1.2.2.4 Sex Hormones

SLE occurs nine times more often in females than in males (9:1 ratio) and sex hormones are known to influence autoimmune disease, in both mice and humans (reviewed McMurray 2001). Recent research, has suggested that there is an interdependence of the neuroendocrine and immune systems, involving many different hormones (Fox et al. 1996). Prior to puberty, the ratio of females:males is lower at approximately 3:1, and after menopause the ratio also falls. In humans when pregnancy occurs during active disease, exacerbations often occur as oestrogen levels rise. Abnormal oestrogen metabolism has been described in both women and men with SLE, causing an excess of 16-α-hydroxyestrone and oestrol metabolites (Lahita et al. 1981).

1.2.2.5 Defects in Fc gamma receptor alleles

The Fc gamma receptors (Fcγ-R), which are expressed ubiquitously on immune cells and bind immune complexes (ICs) containing IgG with low affinity, are encoded by the genes: FCGR2A, FCGR3A, FCGR3B and FCGR2B. These genes are clustered within 100kb at 1q23, a locus which shows linkage to susceptibility for SLE (Tsao 2003). Genetic polymorphisms of all four genes have been associated with SLE, and especially with alleles of FCGR2A and FCGR3A.

A single nucleotide polymorphism in genomic DNA encodes either the histidine (H) or the arginine (R) residue at codon 131 of FcγRIIa. FcγRIIa-R131 binds ICs containing IgG2a less efficiently (with lower affinity) than FcγRIIa-H131, and hence may delay clearance of such IgG2a ICs (Tsao 2003). Recently, FcγRIIa-R131 (but not H131) has been shown to bind C-reactive protein (CRP) with high affinity and is associated with proliferative lupus nephritis (WHO class II and IV). CRP is thought to act as an opsonin (coating of a cell/pathogen for recognition by phagocyte) and is often found in immune deposits involving IgG2a. FcγRIIa-R131 may contribute to the risk of proliferative lupus nephritis, via CRP triggering activation of phagocytes and the release of proinflammatory cytokines, whilst the low affinity binding of FcγRIIa-R131 to IgG2a, may also impair the clearance of the ICs (Zuniga et al. 2003). However, a meta-analysis of 17 studies (involving thousands of patients with SLE) has revealed that R131 only confers a 1.3-fold increased risk for developing
SLE and no increased risk among SLE patients of developing renal disease (Karassa et al. 2002).

The FcγRIIIa is expressed on natural killer cells, monocytes and macrophages and binds to both IgG1 and IgG3. A T-G polymorphism results in phenylalanine (F)-valine (V) at amino acid 158. Individuals homozygous for F-F bind IgG1 and IgG3 with lower affinity than those of the V-V genotype, which might result in less efficient clearance of ICs containing these IgG subclasses (Salmon and Pricop 2001). However, a recent meta-analysis (more than one thousand subjects) has concluded that the F158 allele confers only a 1.2-fold increased risk for developing lupus nephritis in some ethnic groups (Karassa et al. 2003).

FcγRIIb plays a crucial role in the maintenance of tolerance, since it contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and can negatively regulate immunoreceptor tyrosine-based activation motif (ITAM) dependent B cell activation and proliferation. Recently an association has been reported between the FcγRIIb-I-T 232 polymorphism and SLE susceptibility in Japanese patients (Kyogoku et al. 2002). FcγRIIb gene knock-out mice develop glomerular sclerosis and autoantibodies (Table 1.5). Mutations in this gene have obvious implications for the loss of tolerance and await further investigation.

1.2.2.6 The role of complement in the pathogenesis of SLE

The complement system consists of approximately twenty plasma proteins, whose effector functions include opsonisation, inducing phagocytosis by macrophages and neutrophils, B cell activation, chemotaxis and cell lysis. The relationship between SLE and complement is paradoxical since inflammation and tissue damage are usually complement mediated, yet it is believed that complement has a protective role in maintaining tolerance, by aiding the effective clearance of apoptotic material.

Immune complex mediated activation of the classical complement pathway results in inflammation and tissue damage in SLE. This activation is supported by experiments blocking the cleavage of C5, thus preventing the generation of potent proinflammatory factors C5a and membrane attack complex C5b-9. Female BWF1
### Table 1.5 Mouse models of lupus arising from gene targeting experiments

<table>
<thead>
<tr>
<th>Gene Targeted &amp; Background</th>
<th>Antibodies</th>
<th>Glomerular Nephritis</th>
<th>Postulated Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Autoimmunity through lack of clearance of apoptotic cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAP (KO) 129xC57BL/6</td>
<td>ANA, chromatin DNA, histone</td>
<td>Immune complex GN</td>
<td>Binds apoptotic cells/nuclear debris - aiding their clearance</td>
</tr>
<tr>
<td>DNase-I (KO) C57BL/6</td>
<td>ANA, nucleosome ds/ssDNA, histone</td>
<td>Immune complex GN IgG and C3 deposits</td>
<td>Removes DNA from antigenic nucleoprotein complexes</td>
</tr>
<tr>
<td>C1q (KO) 129xC57BL/6</td>
<td>ANA Sm</td>
<td>Proliferative GN, C3 &amp; apoptotic body deposits</td>
<td>Promotes clearance of dying cells</td>
</tr>
<tr>
<td>Serum IgM (KO) 129xC57BL/6</td>
<td>dsDNA, cardioliopin myeloperoxidase</td>
<td>Immune complex GN IgG and C3 deposits</td>
<td>Alters antigen clearance, binds nuclear debris</td>
</tr>
<tr>
<td><strong>Dysregulated B and T cell activation and proliferation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FcyRIIB (KO) 129xC57BL/6</td>
<td>ANA, chromatin DNA</td>
<td>Glomerular sclerosis IgG deposits</td>
<td>Inhibitory receptor gating of BCR, induces B cell apoptosis</td>
</tr>
<tr>
<td>hCD19 transgene B6SJLxC57BL/6</td>
<td>dsDNA</td>
<td>Lymphopaenia</td>
<td>Affects BCR signal transduction</td>
</tr>
<tr>
<td>CD22 (KO) 129xC57BL/6</td>
<td>dsDNA, cardioliopin Serum IgM, IgG</td>
<td>Immune complex GN</td>
<td>Negative regulator of BCR</td>
</tr>
<tr>
<td>Lyn (KO) E14xC57BL/6</td>
<td>ANA serum IgM</td>
<td>FSGS, crescentic GN IgG deposits</td>
<td>Negative signal transduction in BCR pathway, eliminates autoreactive B cells</td>
</tr>
<tr>
<td>IFN-γ transgene CBAXC57BL/10</td>
<td>ANA, histone dsDNA</td>
<td>Proliferative GN Mesangial GN, IgG deposits</td>
<td>Keratinocyte MHCII expression Langerhan cell migration</td>
</tr>
<tr>
<td>Eμ-bcl-2-22 (transgene) C57BL/6xSJL</td>
<td>ANA, histone dsDNA, Sm/RNP serum IgM/IgG</td>
<td>Immune complex GN IgM, IgG, C3 deposits</td>
<td>Expansion of B cell population ↑ Autoreactive B cell precursors</td>
</tr>
<tr>
<td>BlyS (BAFF / THANK/zTNF4/ TALL-1) (Transgene) C57BL/6xDBA</td>
<td>ssDNA, dsDNA ANA, Rh. Factor serum IgM/IgG cryoglobulin</td>
<td>Immune complex GN C3 and IgG deposits</td>
<td>Proliferation of activated autoreactive B cells, suppression of protective effect of dendritic cells against autoreactive T cells</td>
</tr>
<tr>
<td>CD45 (point mutation inhibitory wedge) 129xC57BL/6</td>
<td>dsDNA IgG2a IgA</td>
<td>Diffuse membranous proliferative GN</td>
<td>Inappropriate lymphocyte activation, polyclonal T and B cell activation</td>
</tr>
<tr>
<td>P21 (KO) C57BL/6 x 129/5V</td>
<td>dsDNA, ssDNA ANA, histone</td>
<td>Immune complex GN IgG deposits</td>
<td>Sustained CD4⁺ &amp; CD8⁺ T cell proliferation. Exposure to antinuclear antigens from excess apoptotic bodies</td>
</tr>
<tr>
<td>TGF-β</td>
<td>dsDNA, ssDNA</td>
<td>IgG glomerular deposits</td>
<td>Regulates T &amp; B cell cycling</td>
</tr>
<tr>
<td>Cbl-b (KO) C57BL/6</td>
<td>dsDNA</td>
<td>Interstitial nephritis Leucocyte infiltration</td>
<td>Negative molecular adaptor of lymphocyte activation pathway</td>
</tr>
</tbody>
</table>

Adapted from Lawman and Ehrenstein (2002). Abreviations: SAP, serum amyloid P; KO, knock-out; GN, glomerular nephritis; Rh. Factor, rheumatoid factor; BCR, B cell receptor; FSGS, focal segmental glomerular sclerosis; TCR, T cell receptor.
mice (First generation [F1] progeny of New Zealand Black and New Zealand White mice) treated with a monoclonal antibody against C5, showed significant amelioration of glomerulonephritis (Wang et al. 1996).

Inherited complement deficiencies are rare but these individuals may develop a form of SLE. Susceptibility for SLE is most strongly associated with deficiencies of the early classical complement pathway components C1, C4 and C2, implying a protective role for these components against disease development (reviewed Pickering and Walport 2000). In contrast SLE is very seldom associated with late complement component deficiencies. Complement deficiencies may be partial (heterozygous) or complete (homozygous). There is a hierarchy of disease susceptibility and also severity, since homozygous deficiency in C1q leads to a severe lupus-like syndrome (glomerulonephritis and skin manifestations) in 95% of cases, C4 deficiency results in moderate disease in approximately 75%, and C2 in up to 33% of individuals (Pickering and Walport 2000). C3 deficiency leads to a somewhat different clinical picture of recurrent pyogenic infections, membranoproliferative glomerulonephritis, and SLE is rare (Vyse et al. 1994). Partial C4A deficiency occurs in 50-80% of SLE patients but only 10-20% of controls. A C4A null allele is transmitted as part of the extended HLA-A1, B8, DR3 haplotype which is associated with SLE in Caucasians (Pickering and Walport 2000).

Gene-targeted C1q-deficient mice (Table 1.5) have also been shown to develop a syndrome reminiscent of SLE with antinuclear autoantibodies and proliferative glomerulonephritis (Botto et al. 1998). Although, recent papers demonstrate that interpretation of the effect of C1q deficiency in mouse models is strongly influenced by the genetic background and also by bacterial endotoxin (Mitchell et al. 2002; Robson et al. 2003).

The mechanism that links these deficiencies in the early components of complement to pathogenesis of SLE is not entirely clear. Perhaps the most favoured explanation is that complement proteins play a vital role in the processing and clearing of immune complexes and in the clearance of apoptotic cells, which as discussed in section 1.2.3, is thought to be defective in SLE. C1q has been shown to bind directly to apoptotic blebs (Korb and Ahearn 1997) and mice deficient in C1q or C4 have
defects in the clearance of injected apoptotic thymocytes. Apoptotic bodies are 
found in the glomeruli of the C1q deficient mice (Taylor et al. 2000). The binding of 
C1q to apoptotic blebs and the defective clearance and persistence of these cells, may 
explain why many patients with SLE have high titres of anti-C1q antibodies (Siegert 
et al. 1991). A recent study in MRL-lpr mice found that anti-C1q antibodies were 
already present at 2 months of age in both the sera and deposited in the kidney 
(Trouw et al. 2004). Mannose-binding lectin (MBL) is an acute phase protein that 
activates the lectin complement pathway. Variant alleles leading to MBL 
deficiencies have been associated with a predisposition for SLE. Antibodies to MBL 
are also present in patients with SLE (Seelen et al. 2003).

It is also believed that the innate immune system, including complement, is 
protective against SLE, maintaining tolerance by enhancing negative selection of 
self-reactive B cells. It is hypothesised that innate proteins such as serum amyloid P 
(SAP), deoxyribonuclease-1 (DNase I), natural IgM, C1q or C4 ensure efficient 
localisation and presentation of self-antigens such as dsDNA within the primary 
lymphoid compartment, thus resulting in the elimination of potentially self-reactive 
B cells. Therefore deficiency in these innate proteins could lead to an escape from 
negative selection and a lowered threshold for activation of self-reactive B cells in 
the periphery, given the presence of cognate T cell help and available antigens 
(Carroll 2001).

The levels of complement receptors, CR1 and CR2, are also altered in patients with 
SLE and the complement receptor genes are present within loci with linkage for 
susceptibility to SLE. CR1, present on peripheral B cells, erythrocytes, monocytes 
and tissue macrophages, binds and clears activated complement components 
C3b,iC3b and C4b (and possibly C1q) and their associated immune complexes. 
Decreased expression of CR1 on erythrocytes and peripheral blood leukocytes was 
described in patients with SLE and levels correlated with disease activity (Walport 
and Lachmann 1988). CR2 is expressed on B-cells and follicular dendritic cells, 
MRL/Mp-lpr/lpr (MRL/lpr) mice deficient in CR2 have more severe disease (Tolnay 
and Tsokos 1998).
1.2.3 The role of apoptosis in the pathogenesis of SLE

Apoptosis is the term used to describe programmed cell death. The process is initiated by ligand-receptor interaction, unlike cell necrosis which is caused by toxic insults. Apoptosis appears to have a dual role in lupus pathogenesis. It is an important mechanism for deletion of autoreactive lymphocytes during tolerance induction, but defective clearance of apoptotic products can result in the increased availability of autoantigens, allowing the persistence of autoimmune disease. It is the latter that has generated most interest in recent years, with defective clearance of apoptotic fragments summarised as the 'waste disposal hypothesis' (Walport 2000). Normally, apoptotic cells are rapidly cleared by macrophages, but recent evidence has demonstrated that there is a profound defect in this clearance pathway in lupus patients. The impaired uptake of early apoptotic cells by macrophages in both peripheral tissues and the germinal centres of patients with SLE may contribute to the loss of T and B cell tolerance. Apoptosis is allowed to continue into secondary necrosis with released autoantigens, bound by complement components, being retained on the surface of dendritic cells (Baumann et al. 2002; Herrmann et al. 1998).

Evidence from a number of animal models, suggests that numerous genetic defects may result in impaired clearance of apoptotic cells and bodies (membrane bound fragments, produced by apoptotic cell blebbing). A lupus phenotype develops in mice deficient in DNase I, SAP or complement component C1q, all of which are involved in the clearance of apoptotic material (Table 1.5). A recent publication, showed that C1q and DNase I cooperate in the degradation of chromatin from necrotic cells, and demonstrated that C1q was necessary for the effective uptake of degraded chromatin by monocyte-derived phagocytes (Gaipil et al. 2004).

Complement deficiencies and their role in mouse models and patients with SLE were described in section 1.2.2.6. It is thought that the acute phase molecules, SAP and CRP [pentraxin family of proteins], both function as opsonins, enhancing phagocytosis of apoptotic cells (Gaipil et al. 2003). Binding of SAP to chromatin is believed to mask potential autoantigens from the immune system, and SAP may also play a bridging role in clearing apoptotic cells (the role of SAP in chromatin clearance and maintenance of B cell tolerance is reviewed in (Paul and Carroll 1999).
The genes for SAP and CRP both map to 1q23.2, which shows linkage to SLE. Deletion of the gene encoding SAP, in \( SAP^{+/-} \) knockout mice, results in glomerulonephritis (Bickerstaff et al. 1999). There was delayed onset of lupus nephritis in BWF1 mice which also expressed a human CRP transgene (Szalai et al. 2003). The CRP response is defective in patients with acute flares of lupus and is linked to a polymorphism of the CRP locus (Russell et al. 2003).

A lupus phenotype is also observed in mice lacking secreted IgM (Ehrenstein et al. 2000) and (Boes et al. 2000)), however selective IgM deficiency is a rare occurrence in humans, although it has been associated with SLE (Takeuchi et al. 2001). Deficiency in IgA, is more commonly associated with SLE (Rankin and Isenberg 1997). The disease mechanisms involved in patients with SLE who have IgM or IgA deficiency are not clear, although they may include less efficient handling of immune complexes (as seen in SAP deficiency), defective clearance of apoptotic cells, or abnormalities in co-stimulation of B cell signalling (Ehrenstein et al. 2000).

Mice that lack the nuclease enzyme DNase I, develop anti-chromatin autoimmunity and glomerulonephritis (Napirei et al. 2000). This deficiency suggests that the ability of DNase I to digest DNA in extracellular chromatin, prevents an immune response. The levels of DNase I were found to be low in the serum of patients with SLE, especially those with renal disease; and the decrease in DNase I activity correlated with increased concentrations of the DNase I inhibitor, actin (Walport 2000).

Fas (CD95), is the cell surface protein receptor for ‘death factor’ Fas ligand (FasL). The binding of Fas ligand to Fas on the cell surface, induces apoptosis of the lymphocyte, through induction of several signalling pathways. Evidence from experimental models, has suggested that murine lupus can arise from a failure to delete autoreactive lymphocytes by Fas/Fas ligand mediated apoptosis (Nagata and Suda 1995). However, the majority of patients with SLE, do not appear to have mutations in the genes encoding either Fas or FasL (Kojima et al. 2000). A recent study demonstrated increased expression of Fas on activated B cells from patients with SLE. Fas expression correlated with disease activity (Bijl et al. 2001b).
Increased Fas expression would result in a higher susceptibility to Fas-mediated apoptosis and combined with defective clearance, the potential accumulation of apoptotic lymphocytes.

*Bcl-2* is a proto-oncogene which exerts a regulatory function during development and maintenance of adult tissue, by preventing apoptosis in specific cell types. Elevated *bcl-2* levels have been found in T lymphocytes derived from a proportion of lupus patients, and a number of cytokines (IL-2, IL-4, IL-7 and IL-15) can increase this expression and prevent cellular apoptosis (Graninger *et al.* 2000). B lymphocyte stimulator (BlyS) protein, also known as B-cell-activating factor of the TNF family (BAFF) among other names, enhances B cell survival and maturation, reviewed (Mackay and Browning 2002). Overexpression of BlyS results in severe autoimmune disorders in mice (Table 1.5), and elevated serum levels of BlyS occur in a subset of patients with SLE (Zhang *et al.* 2001), and over extended periods of time (Stohl *et al.* 2003). The proposed mechanism is that over expression of BlyS can overcome the strong death signals received by the B cell when autoantigen binds to the B cell receptor (BCR), and the autoreactive B cell can then survive and mature.

1.2.4 Dysregulation of cellular immune response in SLE

SLE is characterized by multiple functional defects among cells of the immune system including T and B lymphocytes (T and B cells) and accessory cells such as macrophages, dendritic cells (DCs) and natural killer (NK) cells. The number of circulating lymphocytes may be altered profoundly, hyperactive B cells are increased in number, with coexistent T lymphocytopenia (T cell death).

1.2.4.1 Loss of B and T cell tolerance

SLE appears to result from a series of interactions in the immune system that ultimately lead to the loss of self-tolerance to ubiquitous nuclear autoantigens. The immune system has checks in place to prevent autoimmunity, in the form of central and peripheral tolerance mechanisms. Central tolerance is established neonatally, T cells developing in the thymus that possess high affinity receptors for self-antigens are deleted. This process is known as negative selection and occurs if the self-antigens are adequately presented in the thymus. However, developing T cells with lower affinity for the same self-antigens are positively selected for export to the
periphery. During this process of positive selection, the T cell activation threshold may be 'tuned' to increase as the cells mature, so that the cells eventually can no longer be activated by self-antigens (peripheral tolerance), but can be activated by higher-avidity interactions with cross-reactive foreign antigens. Interference with the setting of this activation threshold may lead to autoimmunity. It has been shown that peripheral T cells from lupus-prone mice have a significantly lower threshold for T cell activation as compared with normal mice (Vratsanos et al. 2001). However, this is still a hypothesis with respect to human SLE.

Mechanisms of peripheral tolerance include induction of anergy or suppression. Anergy, functional unresponsiveness to antigen, can be either clonal anergy through lack of a second signal (co-stimulation) or adaptive anergy, due to repeated stimulation by self-antigen in the context of MHC class II. Some subsets of T cells specialise in suppression of immune responses. Recently CD4^CD25^ T\textsubscript{reg} cells have attracted considerable interest in this regard. CD4^CD25^ T\textsubscript{reg} cells develop in the thymus by a selection process which is opposite to that for typical CD4^ cells, high-avidity interactions with self-antigen/MHC II result in positive selection and export to the periphery, where the cells appear to be anergic but can still engage in immunosuppression of potentially self-reactive CD4^ cells (Itoh et al. 1999). It appears these cells may also be generated in the periphery (Akbar et al. 2003). A reduction in the number of these cells or an impairment of their regulatory function, could allow the development of autoreactive T cells. A recent paper shows that the number of CD4^CD25^ T cells is reduced in the peripheral blood of patients with SLE, however no correlation with disease activity was found (Liu et al. 2004).

A model has been proposed (included in Figure 1.1), which emphasises that autoimmunity is initiated by a loss of peripheral rather than central tolerance (Shlomchik et al. 2001). The presence of self-reactive lymphocytes is a prerequisite for autoimmunity, but the hypothesis of this model is that intact central tolerance eliminates high-affinity anti-self lymphocytes, but peripheral tolerance breaks down allowing expansion of lower affinity self-reactive T and B cells. Somatic hypermutation and selection can then result in the generation of high-affinity B cells, from low affinity precursors, producing the high affinity autoantibodies seen in SLE patients, provided that T cell help is available. In summary, the model proposed by
both Shlomchik et al. (2001) and Mamula (1998) assumes initial presentation of either foreign cross-reactive antigens or cryptic self-determinants (Lehmann et al. 1992), by professional antigen presenting cells (APCs, dendritic cells or macrophages) which activate T cells. Co-stimulation is required for this first step (aberrations in co-stimulation in lupus are described later). Normal T cells when exposed to self-antigen in the periphery would become tolerised, but either this is circumvented by molecular mimicry or lupus T cells have a lowered threshold of T cell activation in SLE (Vratsanos et al. 2001), which might allow normally cryptic self-peptides to initiate autoimmunity (reviewed Lehmann et al. 1998). Once activated the autoreactive T cells provide help to autoreactive B cells, allowing expansion of these B cell clones. Such B cells may be hyper-responsive in lupus, as shown in a lupus-prone mouse model (Mohan et al. 1997). In the second stage these autoreactive B cells, which have surface immunoglobulin receptors, can bind and process any available multideterminant autoantigens and present novel self-peptides to further T cells. This whole process results in a positive feedback cycle of activation and expansion of the autoimmune response. The consequent tissue damage provides further self-antigenic material to fuel the whole cascade. Activated T cells may also cause pathology directly, by migrating to the target organ and mediating cytotoxicity and releasing cytokines (Chan et al. 1999). This is an interesting model, however the actual mechanisms by which tolerance is overcome in patients with SLE have still to be fully elucidated.

1.2.4.2 B lymphocyte dysfunctions in SLE

The most marked abnormality in SLE, is the increased number of activated B cells. The role of autoantibodies in the pathogenesis of SLE is discussed in section 1.3. However, secretion of autoantibody is not the only role played by B cells in patients with SLE. B cells are extremely efficient APCs for antigens taken up specifically through the B cell receptor (Ig receptor). Thus B cells play an important role activating T cells, as shown by experiments using a strain of autoimmune prone mice that have B cells that cannot secrete antibodies. These mice still develop interstitial nephritis, vasculitis and glomerulonephritis (Chan et al. 1999). In addition, elimination of B cells in lupus prone mice results in a complete abrogation of pathology including T cell interstitial infiltration (Shlomchik et al. 1994). In a preliminary study 6 patients with SLE, who had failed conventional therapy, were
given a B cell depleting anti-CD20 antibody (Rituximab) and cyclophosphamide with encouraging clinical and serological benefit (Leandro et al. 2002).

Recently, there has been increasing understanding of the intracellular signalling pathways from the BCR that regulate the B cell immune response and tolerance. A group of molecules known as B cell inhibitory receptors are crucial in controlling B cell activation. The majority of these inhibitory receptors, such as FcγRIIb, CD22 and PD-1, belong to the immunoglobulin superfamily. Each inhibitory receptor contains one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which generate and transduce inhibitory signals. Ligation of the inhibitory receptor to an immunoreceptor tyrosine-based activatory motif (ITAM)-containing activatory molecule, results in tyrosine kinase phosphorylation of the tyrosine residue within the ITIM by (src family kinase) lyn. Tyrosine phosphorylation of the ITIM allows it to bind and activate phosphatases containing an src homology 2 (SH2) domain, including SHP-1, SHP-2, SHIP and SHIP2.

Deficiency or mutations in many of these molecules causes lupus-like symptoms in mouse models (as shown in Table 1.5), reviewed Pritchard and Smith (2003). As described earlier (section 1.2.2.5) a polymorphism in FcγRII has been linked to a group of Japanese patients with SLE (Kyogoku et al. 2002). However, to date there is no data to support a role for abnormal function of PD-1, CD22, lyn or SHP-1 in patients with SLE.

1.2.4.3 T lymphocyte dysfunctions in SLE
A diverse range of T cell dysfunctions result in an imbalance in the effector functions of T cell subsets in SLE. In patients with SLE there is generally an exaggerated activity of CD4+ T helper cells and diminished function of CD8+ T suppressor cells. This effect is accompanied by reduced antibody-dependent cytotoxicity and imbalanced cytokine production. T lymphocytopenia is common in patients with SLE, especially of cells bearing the CD8+ phenotype. The T lymphocytopenia is partly attributable to anti-T cell antibodies and also to increased apoptosis. The increases in the anti-T cell titre are paralleled by increases in disease activity (Yamada et al. 1993). CD8+ cells, and NK cells may behave aberrantly by providing help, rather than suppression, to B cells and hence stimulating production of
autoantibodies (Linker-Israeli et al. 1990). This abnormality may be due to defective production of the cytokine, transforming growth factor-beta [TGFβ] (Ohtsuka et al. 1998).

The presence of double-negative T cells (CD4⁻ CD8⁻) is a feature of patients with SLE and of murine lupus models. As double-negative cells, these T cells probably escape thymic deletion. Only 30% of double-negative T cells have the classical αβ T cell receptor (TCR) chains, while 70% express the alternative γδ TCR. These double-negative T-helper (Th) cells, expressing γδ TCR, are believed to augment pathogenic anti-dsDNA autoantibody production (Rajagopalan et al. 1992).

Studies in lupus-prone mouse models and patients with SLE suggest that T cell dysfunction is primarily caused by multiple biochemical defects in T cell signalling pathways, resulting in a significantly lowered threshold for T cell activation and enhanced T cell help to B cells, for the production of autoantibodies (reviewed Kammer et al. 2002 and Ohashi, 2002). Whether a lymphocyte is activated, anergic or undergoes apoptosis, depends on the summation of the signals which it receives after binding of antigen to its cell surface receptors. Signals are either delivered by cytokines or engagement of co-stimulatory molecules with T cell surface receptor molecules. Such antigen-receptor signalling events are abnormal in lupus lymphocytes, manifested by increased calcium responses and hyperphosphorylation of several cytosolic protein substrates (Tsokos et al. 2003). Several defects of the signalling cascade have been identified which can be roughly divided into three groups based on their position in the signalling pathway, proximal (to the TCR/CD3 complex), middle and distal. These T cell signalling abnormalities are summarised in Table 1.6.
<table>
<thead>
<tr>
<th>Defect in pathway segment</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Altered CD45 tyrosyl phosphatase activity</td>
<td>Defective signal transduction via TCR/CD3</td>
<td>Takeuchi <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>Reduced/absent TCR ζ-chain homodimer tyrosyl kinase activity</td>
<td>↑ tyrosine phosphorylation &amp; ↓ AICD (T cells) &amp; NK cell activity</td>
<td>Kammer <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Enhanced tyrosyl phosphorylation and lck</td>
<td>Altered signal transduction but not yet fully defined</td>
<td>Matache <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>Heightened [Ca(^{2+})](_i)</td>
<td>Multiple effects including ↑ expression of CD40 &amp; FasL</td>
<td>Kammer <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><strong>Middle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient PKA-I and PKA-II activities</td>
<td>Defective cAMP-dependent protein phosphorylation</td>
<td>Mandler <em>et al.</em> (1982)</td>
</tr>
<tr>
<td>Reduced PKC-catalysed phosphorylation</td>
<td>Altered signal transduction but not yet fully defined</td>
<td>Tada <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>Altered mitochondrial hyperpolarisation &amp; reactive O(_2) intermediates</td>
<td>Increased T cell apoptosis</td>
<td>Gergely <em>et al.</em> (2002a)</td>
</tr>
<tr>
<td><strong>Distal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diminished MAPKK-catalysed ERK phosphorylation</td>
<td>DNA hypomethylation &amp; ↑ LFA-1 expression leads to ↓ threshold for T cell activation and ↑ T cell homing to target Ags.</td>
<td>Deng <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>Increased PKR-dependent phosphorylation of eIF2α</td>
<td>Impaired translational &amp; proliferative response to mitogens</td>
<td>Groleau <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>Reduced Dnmt1 activity</td>
<td>DNA hypomethylation (mechanism in drug-induced lupus)</td>
<td>Deng <em>et al.</em> (2001)</td>
</tr>
</tbody>
</table>

TCR, T cell receptor; AICD, activation-induced cell death; [Ca\(^{2+}\)]\(_i\), intracellular calcium concentration; PKA-I, type I isozyme of protein kinase A; FasL, Fas ligand; MAPKK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; LFA-1, lymphocyte function-associated antigen 1; Ags, antigens; eIF2α, eukaryotic initiation factor 2α; Dnmt1, DNA methyltransferase 1.
1.2.4.4 Accessory cells

Dendritic cells (DCs) play a key role in inflammation and immunity against microbes, and also in the maintenance of peripheral tolerance to self-antigens, silencing potentially autoreactive T cells by either deletion or the generation of regulatory T cells (Steinman and Nussenzweig 2002). Recent work has shown that sera from patients with SLE, but not healthy controls, can stimulate CD14+ monocytes to mature and act as DCs, the serum factor which stimulated the monocytes in the SLE serum was identified as interferon-alpha (IFN-α) (Blanco et al. 2001). It has been proposed, that there is excess production of IFN-α in patients with SLE, due either to genetic defects or driven by persistent viral stimulation, leading to unabated DC activation, breakdown of tolerance and autoimmunity (Pascual et al. 2003). Support for this theory comes from studies showing that IFN therapy of cancer or viral infections is frequently associated with autoantibody production, and some of these patients also have SLE-like symptoms (Ehrenstein et al. 1993; Schilling, et al. 1991). However, only a fraction of SLE patients have increased levels of IFN-α in their serum, although oligonucleotide microarrays demonstrated an IFN-α gene expression signature in peripheral blood mononuclear cells from the majority of patients (Bennett et al. 2003; Baechler et al. 2003).

Neutropenia in the absence of cytotoxic drugs has been reported in at least 20% of patients with SLE (Keeling and Isenberg 1993). This neutropenia is thought to be caused by increased apoptosis of neutrophils in patients with SLE (Courtney et al. 1999), which may be due to the presence of anti-neutrophil antibodies. In one study anti-neutrophil antibodies were found in 20% of patients with SLE, these antibodies bound to the 50KD La antigen on the neutrophil surface and could penetrate the cells (Hsieh et al. 2003). Neutrophils dying in the periphery could be a source of apoptotic material for presentation by IFN-α activated DCs; and the proteolytic enzymes and oxygen metabolites released by dying neutrophils, may explain much of the tissue damage seen in vasculitis and glomerulonephritis (Johnson et al. 1987; Johnson et al. 1988). Impaired phagocytic clearance of apoptotic neutrophils by macrophages was found in patients with SLE (Ren et al. 2003). The impaired clearance of apoptotic cells by macrophages was discussed in section 1.2.3 (Baumann et al. 2002; Herrmann et al. 1998).
1.2.4.5 Costimulation

Activation of antigen-specific T cells requires at least two distinct signals. The first is antigen-specific, involving presentation of antigen peptides in conjunction with MHC on the surface of the APC, binding to the TCR-CD3 complex. The second costimulatory signal, is provided by engagement of T cell surface receptors with their specific ligands on the surface of the APC. The lack of a second signal can lead to T cell unresponsiveness, termed anergy, or trigger apoptosis of the T cell.

The most prominent costimulatory pathway involves CD28, expressed on mature T cells, and its ligands the B7 family of molecules, B7-1 (CD80) and B7-2 (CD86), expressed on the APC, including B cells. Costimulation via CD28-B7 is critical for T cell activation resulting in cytokine production, clonal expansion, enhanced T cell survival and provision of B cell help. CD28/B7 engagement enhances the expression of CTLA-4 (CD152) on the activated T cells. CTLA-4 is structurally similar to CD28 and negatively regulates T cell function by binding to B7-1 or B7-2, subsequently providing self-regulation of the immune response and maintaining peripheral tolerance. The complexities of these pathways in autoimmunity have recently been reviewed (Salomon and Bluestone 2001). The role of CD28/B7 costimulation has been analysed in the murine lupus models. CTLA-4 deficient mice develop severe lymphoproliferative autoimmune disease. Treatment with CTLA-4Ig, to block the CD28/B7 interaction, prevented disease in the BWF1 model, as did a combination of antibodies against both B7-1 and B7-2. MRL-lpr mice, injected with both anti-B7-1 and anti-B7-2, expressed significantly lower anti-snRNP and anti-dsDNA autoantibodies than untreated mice (Liang et al. 1999).

The production of pathogenic antibodies also requires cognate interaction between CD40 ligand (CD40L or CD154) on T cells and CD40 on B cells. CD40L-CD40 interaction provides a bi-directional signal for T and B cell activation, which drives differentiation of mature B cells to long-lived plasma cells that home to the bone marrow and secrete high affinity autoantibodies in SLE patients (Grammer and Lipsky 2002). Patients with SLE exhibit hyperexpression of CD40L on their B and T cells, this mediates excessive B cell activation which may promote autoantibody production (Desai-Mehta et al. 1996).
Chapter One

Introduction

The expression of costimulatory molecules on the peripheral blood lymphocytes of patients with SLE, during active and inactive disease, has recently been reported (Bijl et al. 2001a). Almost all CD4+ cells expressed CD28 both in patients and controls. B7-1 expression on CD19+ B cells was low in both patients and controls and did not correlate with disease activity. In contrast the percentage of CD19+ B cells expressing B7-2 was increased in patients with SLE, even when disease was inactive. However, the highest expression of B7-2 was in active disease and levels correlated with the SLEDAI score of disease activity and with levels of anti-dsDNA antibodies. No changes were found in the levels of CD40 or CD40L.

1.2.4.6 Cell Adhesion Molecules

Adhesion molecules are classified into selectin, integrin and immunoglobulin supergene family groups. Leucocyte adhesion is regulated by changes in adhesion molecule expression and avidity, dependent on the level of cellular activation. During the inflammatory or autoimmune response, adhesion molecules mediate the interactions between lymphocytes and vascular endothelial cells during extravasation and homing, as well as allowing local retention of cells, such as neutrophils, in the extracellular matrix. Adhesion molecules also play an important role in the interaction between APC's and T cells, ensuring effective T cell help or cytotoxic T cell function. In patients with SLE E-selectin, lymphocyte function-associated antigen-1 (LFA-1)/ intercellular adhesion molecule-1 (ICAM-1) and very late antigen-4 (VLA-4)/ vascular cell adhesion molecule-1 (VCAM-1) appear to provide the predominant adhesive interactions at inflammatory sites. There is evidence in murine models and patients with SLE, of increased expression of many of the adhesion molecules, both endothelial cell surface and soluble forms in serum. In some cases, the levels of adhesion molecules directly correlated with disease activity and decreased with clinical improvement (reviewed by McMurray 1996).

1.2.4.7 Cytokines

The aberrant cellular effector mechanisms seen in patients with SLE, are closely connected to the interaction of cells with their extracellular environment, which critically involves protein cellular messengers known as cytokines. Traditionally, T-helper cells are characterised into different subsets dependent on their cytokine profile. In general Th1 cells support cell-mediated immunity and produce
proinflammatory cytokines, such as interferon γ (IFNγ), tumour necrosis factor-α (TNFα) and interleukin -12 (IL-12) whereas Th2 cells provide B cell help and suppress cell-mediated immunity via cytokines including IL-4, IL-5 and IL-10. With the caveat that a rigid distinction between Th1 and Th2 diseases is probably incorrect, one might predict that SLE would be a predominantly Th2 cell mediated disease, resulting in excess help for B cells, polyclonal B cell activation and production of pathogenic autoantibodies. The shift from a predominantly Th0 population of T cells, producing both Th1 and Th2 cytokines towards a Th2 predominance, might arise as a result of altered regulation or expression of genes, due to a biochemical or signalling defect. A further subset of CD4+ T cells has recently been characterised, Th3 cells produce mainly TGFβ, but also IL-4 and IL-10. Th3 cells play a regulatory role, down regulating APCs, and are important in induction of immune tolerance. However, the Th1/Th2/Th3 paradigm is limited because not all cytokines are produced by T cells, in fact IL-6 and IL-10 are mainly synthesised by monocytes and IFN-α by DCs.

The balance of cytokines may determine disease activity in general and the particular organ involvement. Serum levels of cytokines are difficult to interpret as they may be affected by soluble cytokine receptors, which are shed from cells. Serum levels may not reflect the local levels of cytokines produced in the tissues where they are acting. When looking at in vitro cytokine production by immune cells it is important to differentiate between spontaneous and stimulated cytokine production. Experiments and potential treatments that simply target a particular cytokine in isolation may not provide meaningful data, since the cytokine network is so interrelated and cytokines may compensate for each other. All of these factors have lead to many contradictory reports in this field. The major findings regarding the function of cytokines in SLE, are summarised in Table 1.7 and reviewed by (Dean et al. 2000).
Table 1.7 Summary of the role of cytokines in the pathogenesis of SLE

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Clinical and experimental observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Increased spontaneous production by monocytes from lupus patients, but decreased stimulated production. T cells unresponsive, possibly defective IL-1R or biochemical defect.</td>
</tr>
<tr>
<td>IL-2</td>
<td>Production decreased in both mice and humans with SLE, probably due to genetic and/or TCR signalling defects. Patients show decreased response to IL-2 due to decreased expression of β-chain of IL-2R. Deficiency in IL-2 may result in defective T cell differentiation and apoptosis.</td>
</tr>
<tr>
<td>IL-4</td>
<td>Current consensus is that IL-4 production is not increased in SLE patients, probably due to the multiple conflicting roles of IL-4, it may enhance B cell production of autoantibodies but also enhance T cell suppressor activity. Local action of IL-4 may promote tissue damage in lupus nephritis, blockade of IL-4 or its signalling pathway ameliorates GN in a mouse model. IL-4 reduces expression of FcγRII and CD22 on B cells, decreasing their inhibitory function.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Ratio of IL-6: sIL-6R increased in lupus nephritis. Increased IL-6 protein and mRNA in kidney biopsies and urine of patients, indicates increased local production. IL-6 may maintain B cell hyperactivity via autocrine stimulation of IL-6R constitutively expressed on B cells of SLE patients but not healthy controls. Anti-IL-6 decreases anti-dsDNA Abs and renal damage in MRL-lpr/lpr mice.</td>
</tr>
<tr>
<td>IL-10</td>
<td>IL-10 is increased in the sera of patients with SLE, it stimulates B cell proliferation and differentiation, but suppresses Th1 cells and impairs APC function. Like IL-6, IL-10 may enhance B cell production of pathogenic autoantibodies, by an autocrine pathway. IL-10 treatment of T cells from SLE patients, causes an increase in reactive O2 intermediates and cell death, which could contribute to circulating nuclear material. Anti IL-10 blocks anti-DNA Ab production by PBMCs from lupus patients in SCID mice.</td>
</tr>
<tr>
<td>IL-12</td>
<td>Not clear cut, decreased in SLE (most reports) especially in glomerulonephritis patients, the addition of IL-12 to SLE PBMCs in vitro, reduced B cell production of anti-dsDNA.</td>
</tr>
</tbody>
</table>

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### Introduction

**IL-15**
Elevated in 40% patients with SLE (one study), correlated with IL-2R α-chain (CD25) & BCL-2 expression, therefore immune abnormalities may be partly due to excessive production of IL-15, rather than IL-2α.

**IL-18**
Increased in SLE, correlates with disease activity. Lymphocytes hyperresponsive to IL-18, MRL-lpr/lpr lymphocytes have increased expression of IL-18Rβ. Increases in IL-18, correlate with increased nitric oxide in patients with renal disease.

**IFNα**
Now believed to play central role in lupus pathogenesis, activates immature dendritic cells and increased APC function may drive B/T cell autoimmunity.

**IFNγ**
Increased production in patients, but macrophage & NK cell-mediated cytotoxicity is impaired. Recombinant IFNγ exacerbated disease in patients and lupus prone mice.

**TNFα**
TNFα production increased in active SLE, but ratio of TNFα:TNFα receptors is lower, so relative deficiency in bioactive TNFα. TNFα may have a protective role in lupus, dependent on dose and timing, this is still controversial. MHC linked production: Increased TNFα seen in haplotypes, DR3 & DR4 along with decreased incidence of lupus nephritis; decreased TNFα seen in haplotypes, DR2 & DqW1, associated with lupus nephritis.

**TGFβ**
Decreased in lupus, low level may contribute to renal pathology since TGFβ regulates T and B cell cycling & suppresses IgG production. IL-10 decreases TGFβ production and blockade of IL-10 increases TGF-β and restores regulatory T cell function.

**Abreviations:** IL, interleukin; R, receptor; sIL-6R, soluble interleukin-6 receptor; GN, glomerular nephritis; Ab, antibody; PBMC, peripheral blood mononuclear cell; APC, antigen presenting cell; IFN, interferon; NK, natural killer cell; SCID, severe combined immunodeficient mice; MHC, major histocompatibility complex; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

**References:** ¹Mageed and Prud'homme (2003); ²Crispin and Alcocer-Varela (1998); ³Singh (2003); ⁴Rudge et al. (2002); ⁵Dean et al. (2000); ⁶Tsokos et al. (2003); ⁷Gergely et al. (2002b); ⁸Llorente et al. (2000); ⁹Aringer et al. (2001); ¹⁰Wong et al. (2002); ¹¹Crow (2003).
1.3 The Role of Anti-dsDNA Autoantibodies in Lupus Nephritis

1.3.1 The spectrum of autoantibodies in SLE

A major serological feature of SLE is the presence of circulating autoantibodies against a multiplicity of nuclear, cytoplasmic and membrane antigens. Intriguingly, although there are at least 2000 potential intracellular targets the autoimmune response in SLE is confined to 30-40 of these (Duncan and McConkey 1982; Gharavi et al. 1988). These antibodies are predominantly targeted at intracellular nucleoprotein particles, with over 90% of patients having anti-nuclear antibodies (ANA). These include the 70% (approximately) of patients who have antibodies against dsDNA, high serum titres of which are virtually confined to SLE, reviewed Isenberg et al. (1997b). Anti-dsDNA antibodies have also been eluted from affected kidney and skin samples. Antibodies to both native/dsDNA and denatured/single-stranded (ssDNA) can be present in SLE patients, but the former are more strongly associated with the renal pathology. Although attention has focused on anti-DNA antibodies in renal disease, other autoantibodies have been implicated in the pathogenesis of SLE. The presence of certain autoantibodies has been linked to the expression of particular clinical subsets of the disease, this is summarised in Table 1.8.

1.3.2 Evidence of the pathogenicity of anti-dsDNA in lupus nephritis

Autoantibodies that bind to dsDNA are of paramount important in patients with SLE and especially in those with nephritis. The pathogenic role of these antibodies is indicated by correlation between disease activity and the level of anti-dsDNA antibodies in many patients with SLE (Ravirajan et al. 2001), the elution of anti-dsDNA antibodies from the kidneys of patients (Kofler et al. 1967; Sabbaga et al. 1990) and the fact that these antibodies are hardly ever found in healthy relatives of patients with SLE (Isenberg et al. 1985). Anti-dsDNA antibodies with the ability to fix complement, human IgG subclasses IgG1 and IgG3, correlate with the disease activity in lupus nephritis (Ravirajan et al. 2001). It has been shown that patients may have ANA present for years prior to the onset of clinical disease, strongly implicating them in the aetiology of SLE, rather than simply a side effect of tissue damage. It is unclear whether these earliest autoantibodies are non-pathogenic,
### Table 1.8 Autoantibodies associated with clinical subsets of SLE

<table>
<thead>
<tr>
<th>Location</th>
<th>Antibody Specificity</th>
<th>Antigen/epitope</th>
<th>Prevalence (%)</th>
<th>Clinical Associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular</td>
<td>dsDNA</td>
<td>dsDNA</td>
<td>40-90</td>
<td>Renal &amp; cardiovascular/pulmonary disease</td>
</tr>
<tr>
<td></td>
<td>Histones</td>
<td>H1,2A,2B,H3 &amp; H4</td>
<td>30-80</td>
<td>Drug induced lupus (&gt;95% positive)</td>
</tr>
<tr>
<td></td>
<td>Sm</td>
<td>B/B' &amp; D</td>
<td>~30 (Afro-Caribbeans)</td>
<td>SLE specific (Afro-Caribbeans associated with DR2 &amp; nephritis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5-10 (Caucasians)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ro</td>
<td>60 &amp; 52KD protein</td>
<td>25-40</td>
<td>Sjögren's syndrome, photosensitivity &amp; neonatal lupus syndrome</td>
</tr>
<tr>
<td></td>
<td>La</td>
<td>47KD protein</td>
<td>10-15</td>
<td>Sjögren's syndrome &amp; neonatal lupus syndrome</td>
</tr>
<tr>
<td></td>
<td>Heatshock proteins</td>
<td>Hsp 90 (Hsp 70)</td>
<td>50 (40 mainly IgM)</td>
<td>Cardiovascular/pulmonary disease</td>
</tr>
<tr>
<td></td>
<td>Ribosomal P proteins</td>
<td>P0,P1 &amp; P2</td>
<td>15-35</td>
<td>Link to lupus psychosis is controversial</td>
</tr>
<tr>
<td>Cell Membrane</td>
<td>Cardiolipin</td>
<td>Phospholipids &amp; DNA</td>
<td>20-40</td>
<td>Recurrent abortion &amp; thrombosis</td>
</tr>
<tr>
<td></td>
<td>Neuronal</td>
<td>Neuronal antigen</td>
<td>70-90 (+CNS) 10 (-CNS)</td>
<td>In serum &amp; central nervous system</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte</td>
<td>HLA/CD4/CD8 markers</td>
<td>~74 (IgM) ~47 (IgG)</td>
<td>80% are cytotoxic (T cells affected more than B cells)</td>
</tr>
<tr>
<td></td>
<td>Red cells &amp; platelets</td>
<td>Non Rhesus related</td>
<td>&lt;10</td>
<td>Haemolytic anaemia &amp; ITP respectively</td>
</tr>
<tr>
<td>Extracellular</td>
<td>Complement</td>
<td>C1q</td>
<td>~56</td>
<td>Rising titres indicative of proliferative glomerular nephritis</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid factor</td>
<td>Fc region of IgG</td>
<td>~25</td>
<td>Usually IgM (may be linked to erosive disease)</td>
</tr>
</tbody>
</table>

Prevalence data based on the approximate range from several series published in the literature and the first 300 patients at the Bloomsbury Rheumatology clinic (1978-2000, Isenberg D.A, personal communication)

Abreviations: dsDNA, double-stranded DNA; RNP, ribonucleoprotein; ITP, idiopathic thrombocytopenic purpura.
require some time to induce clinical symptoms, or play a role in development of pathogenic antibodies.

In addition to the clinical evidence from patients, anti-dsDNA antibodies are also strongly implicated in the pathogenesis of many spontaneous murine models of SLE. These mouse models include the New Zealand black (NZB); the F1 progeny of NZB and New Zealand white mice ([NZBxNZW] F1 or shortened to BWF1); MRL/Mp-+/+ (often shortened to MRL/+); MRL/Mp- lpr/lpr (often shortened to MRL/lpr); BXSB and the F1 progeny of Swiss-Webster and NZB ([SWRxNZB] F1 or in short, SNFl). Most of these exhibit varying degrees of glomerulonephritis, especially the females (oestrogens may accelerate disease in most models), and are valuable tools for studying renal presentations of lupus; although it is important to remember the contrasts between experimental models and human lupus. These models are summarised in Table 1.9 and extensively reviewed by Peutz-Kootstra et al. (2001) and Stoll and Gavalchin (2000). A recent study used a novel elution protocol to elute antibodies deposited in the kidneys of nephritic SNFl mice. Clonally restricted, complement fixing, cationic IgG anti-dsDNA antibodies were the most prevalent species in the renal deposits, and heavily enriched relative to the corresponding serum levels (Xie et al. 2003). Lupus-like disease can also be induced in mice by various means, including the generation of chronic graft versus host disease by injection of DBA/2 mouse lymphocytes into the F1 progeny of C57BL/10 x DBA/2, this lupus-like disease is characterised by glomerulonephritis and circulating autoantibodies including anti-dsDNA (Bruijn et al. 1992).

The number of mouse models has rapidly increased with the introduction of many gene-targeted mice such as single gene knockouts, gene mutants, and transgenic models (Table 1.5). Antibodies to dsDNA develop in the majority of the mouse models arising from these gene targeting experiments, although there are many different disease mechanisms operating in these mice and the role of anti-dsDNA is not yet clear in all of them (reviewed Lawman and Ehrenstein 2002). Further evidence of the role of anti-dsDNA antibodies, has been obtained from in vivo studies of monoclonal anti-dsDNA antibodies, derived from both lupus-prone mouse models and patients with SLE, and implanted into immunodeficient mouse strains (discussed further in section 1.4.8).
<table>
<thead>
<tr>
<th>Murine model</th>
<th>Autoantibodies</th>
<th>Clinical Features</th>
<th>Mean survival (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZB</td>
<td>Anti-ssDNA</td>
<td>Haemolytic anaemia</td>
<td>Males: 67</td>
</tr>
<tr>
<td>New Zealand black</td>
<td>Anti-dsDNA (low)</td>
<td>Mild glomerulonephritis</td>
<td>Females: 63</td>
</tr>
<tr>
<td></td>
<td>Anti-erythrocyte</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Natural thymotoxic antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BWF1</td>
<td>Anti-dsDNA</td>
<td>Severe glomerulonephritis</td>
<td>Males: 58</td>
</tr>
<tr>
<td>(NZB x NZW) F1</td>
<td>Natural thymotoxic antibody</td>
<td></td>
<td>Females: 35</td>
</tr>
<tr>
<td>SNF1</td>
<td>Anti-ssDNA</td>
<td>Severe glomerulonephritis</td>
<td>Males: 64</td>
</tr>
<tr>
<td>(SWR x NZB) F1</td>
<td>Anti-dsDNA</td>
<td></td>
<td>Females: 29</td>
</tr>
<tr>
<td>MRL/Mp-lpr/lpr</td>
<td>Anti-dsDNA</td>
<td></td>
<td>Males: 22</td>
</tr>
<tr>
<td></td>
<td>Anti-ssDNA</td>
<td>Severe glomerulonephritis, synovitis, polyarteritis &amp; lymphadenopathy.</td>
<td>Females: 20</td>
</tr>
<tr>
<td></td>
<td>Anti-Sm/RNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rheumatoid factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRL/Mp - +/+</td>
<td>Anti-dsDNA</td>
<td>Late-onset glomerulonephritis</td>
<td>Males: 98</td>
</tr>
<tr>
<td></td>
<td>Anti-ssDNA</td>
<td></td>
<td>Females: 73</td>
</tr>
<tr>
<td></td>
<td>Anti-Sm/RNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BXSB/Mp</td>
<td>Anti-ssDNA</td>
<td>Severe glomerulonephritis, haemolytic anaemia, vasculitis &amp; lymphadenopathy.</td>
<td>Males: 20</td>
</tr>
<tr>
<td></td>
<td>Anti-dsDNA</td>
<td></td>
<td>Females: 68</td>
</tr>
<tr>
<td></td>
<td>Anti-erythrocyte</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Modified from Stoll and Gavalchin (2000)
Although the evidence places anti-dsDNA antibodies at the ‘scene of the crime’, only a subset of circulating anti-DNA antibodies deposit in the kidney and are likely to be nephritogenic. Indeed only 30-50% of SLE patients with such antibodies develop lupus nephritis (ter Borg et al. 1990). It is not clear at present, which features distinguish pathogenic and non-pathogenic anti-dsDNA antibodies. This area is the focus of the work in this thesis and will be reviewed in sections 1.4 - 1.5.

1.3.3 The origin of anti-DNA antibodies

The stimulus for production of anti-DNA antibodies remains one of the greatest enigmas of SLE, reviewed Kalsi et al. (1999) and Hahn (1998). Antibodies to ssDNA are part of the normal repertoire of natural autoantibodies, although most of these are low affinity IgM antibodies which react weakly with self-antigens. Spontaneous murine models of SLE (Table 1.9) have a background of random polyclonal B cell activation. However, due to their relatively restricted nature (Gharavi et al. 1988), and to the presence of antigen selected mutations in the complementarity determining regions (CDR) of monoclonal anti-dsDNA antibodies, the anti-dsDNA autoantibodies found in human SLE patients are thought more likely to arise by an antigen driven T helper cell dependent response, possibly in combination with limited polyclonal activation. The polyreactive nature of anti-DNA antibodies, together with the fact that mammalian DNA is non-immunogenic, suggests that naked DNA itself is unlikely to be the original immunogen for what we observe as anti-DNA antibodies.

1.3.3.1 The polyreactivity of anti-DNA antibodies

The anti-DNA antibody response in patients with SLE is heterogeneous, with anti-DNA antibodies recognising multiple epitopes on diverse molecules, such as different nucleic acids, phospholipids, proteins, polysaccharides and cell membrane structures, reviewed by Stollar (1986). This diverse binding of anti-DNA antibodies is not simply due to charge interaction, since anti-DNA antibodies do not necessarily bind to polynucleotides that have similar patterns of ionic charge (Stollar 1986). This polyreactivity of anti-DNA antibodies may be due to multiple variable binding sites or to shared epitopes on different antigens. An example of a possible shared epitope, was provided when a monoclonal antibody derived from mice immunised with conjugates of a phospholipid, cardiolipin, and protein, bound to both cardiolipin
and DNA. The putative shared epitope was thought to be one or more of the phosphodiester phosphate groups which occur in both polynucleotides and phospholipids (Rauch et al. 1984). There are many other examples of possible shared epitopes, especially between autoantigens and viral or bacterial antigens, which lead to theories of molecular mimicry, and such cross-reactivity may drive the autoimmune response through a process known as epitope spreading.

1.3.3.2 Molecular mimicry and epitope spreading

Many microorganisms possess cell surface antigens that mimic self-antigens. If the immune system recognises and processes these foreign antigens there is also the possibility of autoimmunity against the mimicked self-antigens. In mice immunised with phosphorylcholine (PC), a bacterial cell wall component, autoantibodies which cross-reacted with dsDNA were generated as part of the immune response against PC (Shefner et al. 1991).

Epitope spreading is a hypothetical mechanism, which may explain how autoantibodies develop against the multiple antigen specificities, which are found in patients with SLE. The hypothesis of epitope spreading, suggests that if a single epitope on a self-protein is targeted the immune response can spread to other epitopes on the same molecule, and subsequently to epitopes on other closely related molecules (reviewed by Craft and Fatenejad 1997; Mamula 1998). Autoantibodies, to different components of the same nuclear particle, such as the nucleosome or the spliceosome, often occur in linked sets (clusters) and in an ordered hierarchy of expansion with time, in the sera of individual patients with SLE (Lerner and Steitz 1979; Mattioli and Reichlin 1973). Clusters of autoantigens, and indeed clusters containing both self and viral antigens, have been demonstrated in apoptotic blebs (Casciola-Rosen et al. 1994; Rosen et al. 1995) available for presentation by APCs to self-reactive T cells (see Figure 1.1).

New Zealand white (NZW) rabbits immunised with a spliceosomal peptide, PPPGMRPP, not only produced antibodies specific for the immunising peptide but developed autoantibodies to related spliceosomal antigens and to dsDNA, as well as clinical features suggestive of lupus, such as proteinuria and seizures (James et al. 1995). The sequence PPPGMRPP, occurs three times towards the carboxyl terminal
end of Sm B/B', has been demonstrated to be the predominant antigenic epitope in anti-Sm positive sera, and is potentially one of the early targets of the autoimmune response in SLE (James and Harley 1992). This peptide was also of interest due to its striking resemblance to the PPPGRRP region of the Epstein-Barr nuclear antigen-1 (EBNA-1), and due to evidence suggesting a possible role for Epstein-Barr virus (EBV) in the etiology of SLE (James et al. 1997). However other laboratories, including ours, were unable to reproduce this model fully, showing only limited epitope spreading (Mason et al. 1999; Vlachoyiannopoulos et al. 2000). If spreading occurred from a component of the spliceosome to DNA, a component of the nucleosome, as suggested by this model, it is in contradiction to the appearance of linked sets of antibodies in patients. Epitope spreading has been demonstrated in many lupus-prone mouse models, injection of core histone peptides into lupus prone mice accelerated both anti-dsDNA antibody production and the occurrence of glomerulonephritis (Kaliyaperumal et al. 1996).

B cells can process their own surface immunoglobulin and present it to T cells, which provide help to sustain production of pathogenic autoantibodies (Weiss and Bogen 1989; Yurin et al. 1989). Several papers have demonstrated that idiopeptides derived from anti-DNA antibodies themselves, thus trigger reciprocal T-B determinant (epitope) spreading, resulting in expansion of cells producing autoantibodies. This has been extensively shown in lupus-prone mouse models (Ebling et al. 1993; Beger et al. 2002 and reviewed Singh and Hahn 1998). Immunisation with a peptide surrogate for DNA, DWEYSVWLSN, results in anti-dsDNA production and glomerular deposition in normal BALB/c mice (Putterman and Diamond 1998). This peptide surrogate was used since part of its sequence, DWEYS, was found to be a consensus motif in several peptides recognised by a pathogenic murine monoclonal anti-DNA antibody. Interestingly DWEYS is 100% homologous to sequences of bacterial proteins from Haemophilus influenzae and Streptococcus pneumoniae. Experimental evidence that epitope spreading occurs in humans is limited, although there is some evidence that it may be occurring to a limited extent in patients with SLE (McNeilage et al. 1990; Scofield et al. 1996). As hypothesised in Figure 1.1, once tolerance breaks down and autoimmunity is triggered, epitope spreading may be of greatest importance in the expansion and persistence of the autoimmune response.
1.3.3.3 Environmental and other stimuli for anti-DNA antibody production

DNA has unique immunological properties that may affect its immunogenicity. Thus depending on sequence and base methylation, DNA can be stimulatory, inhibitory or inert. Naked native mammalian DNA is not normally immunogenic. Injection of mammalian DNA results only in low titre anti-ssDNA antibodies (Madaio et al. 1984; Schwartz and Stollar 1985). The majority of DNA in mammalian cells exists as native double-stranded B-DNA (right-handed helix), but under certain conditions, such as the presence of naturally occurring polyamines or exposure to the drug hydralazine, short stretches of DNA may exist in other forms, including Z-DNA (left-handed helix). Sera from patients with SLE contain antibodies against B-DNA, Z-DNA and ssDNA. Z-DNA, ssDNA and alkylated DNA are more immunogenic than native B-DNA, producing higher antibody titres (Stollar 1997). Mammalian DNA has also been shown to be inhibitory, especially during infection and can suppress immune responses to bacterial DNA, such as macrophage production of cytokines (Pisetsky 2000).

Mammalian DNA may be modified and become immunogenic by exposure to reactive oxygen species (ROS), such as the hydroxyl radical. ROS may be generated by UV irradiation or released by dying phagocytes like neutrophils. Several studies have shown that sera from patients with SLE can bind preferentially to ROS modified bases of DNA as compared with native DNA (Garg et al. 2003; Tasneem and Ali 2001). It has also been suggested that activated phagocytic cells can release ROS which can penetrate cell membranes and alter nuclear DNA causing it to become antigenic (Berden 1997). Chemical irritants such as pristane can also cause ANA production and immune complex nephritis in non-autoimmune BALB/c mice, however the disease mechanism is not clear and the ANA response was limited to IgM anti-ssDNA and IgM anti-histone antibodies (Satoh et al. 1995).

Bacterial DNA has been shown to be much more antigenic than mammalian DNA. Bacterial DNA contains unmethylated CpG motifs, which are immunostimulatory and more common in bacterial DNA than in mammalian DNA and can elicit cytokine production and polyclonal B cell activation (Pisetsky 1996). However in studies injecting bacterial DNA into healthy mice, despite a strong antibody response, the antibodies lacked both specificity for mammalian dsDNA, and
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Introduction

costistics typical of the high affinity anti-dsDNA antibodies found in patients with SLE (Gilkeson et al. 1993; Wloch et al. 1997).

For many years autoantibodies to DNA were thought to arise as natural autoantibodies by polyclonal B cell activation. The conceptual problems against antigen driven T cell dependent antibody production, included the poor immunogenicity of mammalian DNA, and the lack of evidence that the TCR-MHC complex could bind DNA, or in fact whether MHC could even present nucleic acids to T cells. This was overcome by the realisation that T cells in murine and human lupus bind to DNA in complex with a protein hapten, such as a histone-derived peptide, which forms a bridge between T cells that recognise the peptide, and B cells with DNA binding surface immunoglobulin (reviewed Datta 2003). Such DNA-histone complexes occur naturally in nucleosomes and may become available for antigen presentation, to T cells in patients with SLE, due to the defective 'waste-disposal' of debris from apoptotic or necrotic cells (sections 1.2.3 and 1.3.3.4).

DNA may also be presented to T cells in association with foreign proteins or peptides. Immunisation with a complex of native B-DNA and a DNA binding peptide (Fus1), derived from the internal domain of a ubiquitin fusion protein from Trypanosoma cruzii, led to the production of six monoclonal anti-DNA antibodies in Balb/C mice (Krishnan and Marion 1993). These monoclonal anti-DNA antibodies shared specificity and variable region structural similarity with monoclonal anti-DNA antibodies derived from lupus-prone mice. Polyomavirus transcription factor T antigen can induce anti-DNA antibodies in mice and antibodies to dsDNA were found in patients with frequent polyomavirus reactivations (Rekvig et al. 1997). In these cases, it is believed that immunogenic immune complexes are formed between the host chromatin and the DNA-binding large T antigen of polyoma virus BK.

1.3.3.4 The role of nucleosomes in anti-DNA antibody production

Within eukaryotic cells, DNA is packaged with histones in particles known as nucleosomes. The nucleosome is the fundamental repeating unit of chromatin and consists of a core particle composed of an octamer of histones (2 copies each of H2A, H2B, H3 and H4), which is wrapped with approximately 146 base-pairs of helical dsDNA, Figure 1.2. DNA linked to histones, as found abundantly in
apoptotically released nucleosomes, is the prime suspect to be the stimulating antigen in the production of anti-DNA autoantibodies (reviewed by Berden 1997).

Nucleosomes are generated during apoptosis by endonuclease cleavage of the internucleosomal linker DNA of chromatin. Defects in this process may result in the apoptotic products being modified from those recognised as ‘self’ by the immune system. Thus cryptic epitopes, previously unknown to the immune system might emerge which are more immunogenic and overcome tolerance mechanisms (reviewed Dieker et al. 2002; Utz and Anderson 1998). Various post-translational modifications of self-protein antigens have been shown to occur specifically in apoptosis including histone acetylation or deacetylation of chromatin, histone phosphorylation, histone methylation as well as citrullination, transglutaminase cross-linking, poly(ADP-ribosyl)ation and ubiquitination. There is evidence to suggest that proteins phosphorylated during apoptosis are targets of autoantibodies in patients with SLE (Utz et al. 1997), and autoimmunity to ubiquitin and ubiquitinated histones has been reported in lupus-prone mouse models (Elouaai et al. 1994). Caspase cleavage of proteins during apoptosis often results in products that are more immunogenic than the original protein (Utz and Anderson 1998). Unique fragments of many autoantigens have been demonstrated after protease cleavage by granzyme B in cytotoxic lymphocyte granule-induced death (Casciola-Rosen et al. 1999). Recent work also shows that nucleic acids are modified during apoptosis, such as abnormal DNA methylation of CpG motifs, and caspase-dependent cleavage of nucleic acids, reviewed by (Degen et al. 2000). Recent work has shown that hypomethylated CpG motifs in chromatin containing ICs can activate autoreactive B cells by dual engagement of the BCR and Toll-like receptor 9 (TLR9) (Leadbetter et al. 2002; Viglianti et al. 2003). This may be an important mechanism for initiation of SLE by either bacterial DNA or unmethylated mammalian DNA.

Nucleosomes and other autoantigens are found in blebs on the surface of apoptosing cells. As discussed earlier (section 1.2.3), it is believed that defective removal of apoptosing cells and their products in patients with lupus, due to disruption of molecules involved in phagocytosis (including C1q, SAP, DNase I, c-mer, IgM and CRP) may lead to the availability of autoantigens which can then initiate and drive the autoimmune response (Walport 2000). Polynucleosomes have been also been
A nucleosome consists of an octameric histone core, containing two molecules each of H2A, H2B, H3 and H4, surrounded by two turns of the DNA helix. H1 binds to the nucleosome where DNA enters and leaves the nucleosome (A). The nucleosomes are like ‘beads on a string’ joined by histone-free linker DNA. The DNA and histones packaged into nucleosomes are further condensed to form a chromatin fibre so that there are 6 nucleosomes per turn (B). H1 is thought to aid packaging by holding adjacent nucleosomes together. Adapted from Cooper (2000)
shown to bind activated monocytes which can act as APCs (Emlen et al. 1992). A recent report shows that at high concentrations nucleosomes can induce necrosis of lymphocytes, which could cause inflammation and provide amplification of the autoimmune response (Decker et al. 2003).

High affinity anti-dsDNA antibodies of the IgG isotype (especially IgG1 and IgG3) are widely believed to be the major culprits in the pathogenesis of lupus nephritis (Ravirajan et al. 2001). However, as discussed earlier (section 1.3.3.1) many of these anti-dsDNA antibodies do have narrow cross-reactivity for antigens other than naked DNA. The question can therefore be posed, are anti-dsDNA really anti-dsDNA? The answer may be yes, if they are positive binders in a validated anti-dsDNA assay, as in approximately 70% of patients with SLE. However, it seems likely that many of these pathogenic anti-dsDNA antibodies are not actually elicited by pure mammalian DNA but are actually anti-nucleosome antibodies, binding conformational epitopes of DNA complexed with histones, and that also bind to dsDNA. In fact some antibodies may actually be anti-nucleosome antibodies which bind DNA by virtue of already being complexed to histones or intact nucleosomes (Di Valerio et al. 1995; Kramers et al. 1994).

Antibodies against chromatin are found in 88% of patients with SLE (Burlingame et al. 1994). Many antibodies referred to as anti-dsDNA, have a higher affinity for intact nucleosomes, than either DNA or histones. One study (120 patients with SLE), suggests that nucleosome specific antibodies have a higher prevalence in patients with SLE, as well as a better correlation with lupus nephritis (Amoura et al. 2000). However, another smaller study (33 patients with SLE, including 11 with lupus nephritis) found that anti-dsDNA and anti-heparan sulphate antibodies were more prevalent than anti-nucleosome antibodies and that it was the anti-heparan sulphate antibodies which correlated with disease activity, in patients with lupus nephritis (Ravirajan et al. 2001). Most recently, a large study of 199 patients with SLE found that the levels of anti-dsDNA, anti-histone, anti-nucleosome and anti-C1q antibodies were all increased in patients with active lupus nephritis, especially those with proliferative glomerulonephritis, compared to those with active SLE without nephritis (Cortes-Hernandez et al. 2004). It has been proposed that measurement of anti-chromatin or anti-nucleosome antibodies may be a more sensitive diagnostic
marker of disease activity than anti-dsDNA (Burlingame et al. 1994; Bruns et al. 2000; Simon et al. 2004), although anti-nucleosome antibodies are also present in inactive patients. However, anti-nucleosome antibodies of the IgG3 isotype do appear to be a specific marker for active SLE, especially lupus nephritis (Amoura et al. 2000).

Increased levels of oligonucleosomes have been found in the plasma of patients with SLE (Amoura et al. 1997; Rumore and Steinman 1990). Many monoclonal anti-dsDNA antibodies derived from patients with SLE, or murine models, also bind to nucleosomes or histones (discussed further in section 1.4.8). Several studies in mouse models of lupus, have indicated that the formation of nucleosome specific antibodies may precede the development of both anti-dsDNA and anti-histone antibodies (Amoura, et al. 1994; Burlingame et al. 1993). In human patients such studies are hampered by a lack of samples prior to disease onset. However, a recent study evaluated serum samples from 130 patients before they received a diagnosis of SLE (Arbuckle et al. 2003). Anti-nuclear antibodies were present at least 3.4 years before diagnosis, whereas anti-dsDNA antibodies had a mean onset of 2.2 years before diagnosis, supporting the hypothesis of progressive accumulation of autoantibodies leading to onset of disease. It is important not to over simplify or under estimate the importance of anti-dsDNA specific antibodies, as compared with anti-nucleosome antibodies. A recent study in patients with SLE and their relatives indicated that anti-dsDNA specific antibodies may have a greater disease association with renal involvement and also have different genetic origins than anti-H2A/H2B/dsDNA (anti-nucleosome) antibodies (Mohan et al. 2001). This subject remains controversial, the major difficulty is that the cross-reactivity of anti-dsDNA and anti-nucleosome antibodies and the presence of interfering antigens (such as DNA, histones, nucleosomes or complexes of these) in the samples tested, makes it difficult to be sure which subset of antibodies were actually measured.

It has been shown in vitro that autoreactive T cells from lupus patients recognise nucleosomes preferentially to DNA or histones alone, and that 50% of pathogenic $T_H$ cells are nucleosome specific (Mohan et al. 1993). In an elegant series of papers (reviewed Datta, 2003), nucleosomes have been demonstrated as a major immunogen for the induction of $T_H$ cell mediated production of cross-reactive pathogenic autoantibodies, including anti-dsDNA in lupus prone mice. Five major epitopes
were found in nucleosomal histones (H1'22-42, H2B10-33, H385-105, H416-39 and H471-94), and it was demonstrated that the dominant pathogenic autoepitope, H1'22-42 (from a region of histone which contacts DNA in the nucleosome) was processed and presented on MHC class II molecules, by APC which had been fed crude chromatin (Kaliyaperumal et al. 2002). These histone peptide autoepitopes have also been shown to be promiscuously presented by diverse MHC alleles (Shi et al. 1998), and have been used in tolerogenic therapy to halt the progression of glomerulonephritis in lupus-prone mice. H416-39 peptide had the greatest effect, inactivating a broad spectrum of autoimmune T and B cells, by 'tolerance-spreading' (Kaliyaperumal et al. 1999). The results from Datta's group and others involving peptides from anti-DNA antibodies (Singh et al. 1995; Hahn et al. 2001) increase the possibility of the use of peptides to vaccinate patients with established SLE, reviewed Singh (2000).
1.4 Experimental Investigation of the Factors Affecting the Binding and Pathogenicity of Anti-dsDNA Antibodies

1.4.1 Monoclonal anti-DNA antibodies

Studying anti-DNA antibodies in the sera of patients with SLE, it is difficult to draw conclusions as to whether any observed cross-reactivity is due to the range of antibodies or to polyreactivity of individual antibodies. The use of monoclonal antibody technology yields sufficient quantities of individual antibodies derived from patients, to allow detailed analysis of their structural and pathogenic characteristics. The only caveat being that such monoclonal antibodies, might not accurately represent the pathogenic antibodies in all patients (Brinkman et al. 1990). The chances of obtaining such antibodies can improved by screening of the antibody producing clones and selecting antibodies with the highest affinity for dsDNA.

1.4.1.1 Techniques for production of monoclonal antibodies

Monoclonal antibody producing cell clones are obtained by the fusion of antibody producing B cells, from the peripheral blood or spleen of a patient with SLE (or a lupus-prone mouse), with a non-secreting myeloma cell (a cancerous plasma cell), to produce a hybrid cell (called a hybridoma). The fused cells are selected by growth in hypoxanthine-aminopterin-thymidine (HAT) medium, which kills unfused myeloma cells (due to their lack of the gene encoding hypoxanthine:guanine phosphoribosyl transferase [HGPRT]), any unfused spleen cells die after a few days, and only the fused cells survive due to the B cell possessing the HGPRT gene. Hybridoma cells possess the immortal growth properties of the myeloma cells and the ability to secrete the antibody produced by the original B cells. The resulting clones are screened for the desired antibody specificity and isotype, and sub-cloned (by dilution) to ensure each hybridoma cell line is obtained from a single progenitor cell. The resulting hybridoma cell lines secrete large quantities of monoclonal antibody and can be cultured indefinitely. This method was originally used to produce murine monoclonal antibodies (Kohler and Milstein 1975) but then later adapted to produce human monoclonal antibodies (Olsson and Kaplan 1980; Teng et al. 1983).

Human monoclonal anti-DNA antibodies have also been produced using EBV transformation of peripheral blood lymphocytes (PBL) (Manheimer-Lory et al. 1991;
van Es et al. 1991). However, when direct fusion and EBV transformation were compared using PBLs from the same patients, EBV transformation resulted in a higher proportion of IgM antibodies (Ehrenstein et al. 1993). Proliferating activated B cells, but not resting B cells, have been shown to be resistant to EBV transformation (Casali and Notkins 1989), whereas direct fusion of B cells selects from a pool of activated proliferating B cells, which will become plasma cells, and thus this method is more likely to select primed B cells from patients with SLE, which will potentially produce more representative pathogenic antibodies (Olsson et al. 1983).

Human monoclonal anti-DNA antibodies have also been produced using repertoire cloning by phage display (Barbas et al. 1995). This approach is described in section 1.4.2.1. The advantage of repertoire cloning is that it can produce several anti-DNA antibodies at one time, without the need to make cell lines. The disadvantage is that the $V_H/V_L$ combinations are generated randomly, hence it is possible that they do not truly represent the heavy and light (H/L) chain combinations, which would occur in antibodies found in patients with SLE. A monoclonal antibody from a hybridoma cell, derived by direct fusion, does contain the exact H/L chain combination that originally occurred in that B cell in vivo.

1.4.1.2 Human monoclonal anti-DNA antibodies

Murine monoclonal anti-DNA antibodies have been extensively produced from lupus-prone mice, including MRL-Ipr, BWF1 or SNF1. Studies involving some of these monoclonal antibodies will be discussed in later sections (1.4.7.1 and 1.4.8.2). Human anti-DNA monoclonal antibodies, especially high affinity antibodies of the IgG isotype, have been much harder to produce than their murine counterparts. Most published work describes low affinity IgM antibodies. Problems include rare availability of human spleen cells, therefore having to rely on PBLs, lower yields of hybrid cells per fusion and a higher degree of chromosomal instability than is seen with murine monoclonal antibody production (Olsson et al. 1983). However, some groups have produced high affinity, IgG secreting human hybridomas from patients with active lupus, including a total (by direct fusion of PBLs) of thirteen published IgG clones from the laboratories of Kalden and Isenberg (Winkler et al. 1991; Ehrenstein et al. 1993; Ravirajan et al. 1998).
The first six human IgG anti-DNA monoclonal antibodies were produced by Winkler et al. in 1991, by fusion of the heteromyeloma cell line CB-F7 with peripheral blood mononuclear cells from SLE patients with highly active disease. The resulting hybridomas produced antibodies of various IgG subclasses: 19.E7 (of isotype IgG1κ [kappa light chain]), 32.B9 (IgG3λ [lambda light chain]), 33.F12 (IgG1κ), 33.H11 (IgG1λ), 33.C9 (IgG2κ) and 35.21 (IgG2λ). Subsequently our group generated five hybridomas producing IgG anti-DNA antibodies, B3 (IgG1λ), D2 (IgG), E7 (IgG), F8 (IgG), and D5 (IgG1κ) from a patient with active SLE (Ehrenstein et al. 1993). This study suggested that obtaining high affinity IgG secreting hybridomas, appears to be more likely when the peripheral blood lymphocytes are derived from a patient with very active disease, since fusion of the same patient when her disease was inactive, resulted only in IgM anti-DNA hybridoma cells (Ehrenstein et al. 1993). This switch possibly reflects the increased proportion of IgG antibodies during active disease. Two further human monoclonal anti-DNA antibodies were produced by our group, RH-14 (IgG1λ) and DIL-6 (IgG3λ) (Ravirajan et al. 1998). The human anti-dsDNA antibodies B3, 33.H11, RH-14 and DIL-6 are all utilized in the experiments described in this thesis (the derivation of these antibodies is described in section 2.2, and their properties are summarised in Table 2.1).

1.4.2 Expression of recombinant monoclonal anti-DNA antibodies

Cell lines producing monoclonal antibodies, especially human hybridomas, are often unstable, may shed chromosomes, or lose the ability to secrete antibody. In order to preserve monoclonal antibodies and to test hypotheses regarding the effects of sequence changes on their binding and functional properties, it has been necessary to develop expression systems to produce recombinant monoclonal anti-DNA antibodies, as well as mutated antibodies or antibody fragments, reviewed (Rahman et al. 2002b).

1.4.2.1 Bacterial expression systems

The simplest prokaryotic expression systems, produce single chain Fv (scFv) molecules. In scFv expression plasmids, the $V_h$ and $V_l$ sequences are cloned on either side of a sequence encoding a short flexible linker molecule. After bacterial
transfection with these plasmids, the bacteria produce proteins containing the \( V_H \) and \( V_L \) regions of the antibody, joined by the linker, which allows formation of an antigen-binding site. The structure of scFvs does not resemble any molecule occurring in nature, but the binding properties are retained (Polymenis and Stollar 1994; Brigido et al. 1993). ScFvs provide a powerful tool to study the effects of gene sequence on antigen binding when they are expressed in phagemid vectors. These vectors are modified bacteriophages (viruses which infect bacteria). The scFvs are expressed linked to a surface protein on a phage particle which is produced by the transfected bacteria. This technique is known as phage display. A library of many different phages, displaying millions of combinations of \( V_H \) and \( V_L \), can be rapidly created (by polymerase chain reaction [PCR] applied to PBLs) using this method. The phages can then be panned against surfaces carrying the antigens of interest, thus screening for the \( V_H \) and \( V_L \) combinations with, for example, the greatest affinity for dsDNA. The bound phages are then eluted, amplified and screened through several cycles of panning to enrich for anti-DNA specific phage clones. This technique is known as repertoire cloning (Roben et al. 1996).

However, it is not certain that the \( V_H \) and \( V_L \) combinations obtained using repertoire cloning, would actually have existed \textit{in vivo}. To investigate this issue, Roben \textit{et al} used anti-idiotypic antibodies (Roben \textit{et al}. 1996). Idiotypic determinants are antigenic determinants found in the variable region of antibodies. Antibodies with the same idiotypic sequence should have the same amino acid sequence at the part of the sequence that encodes the idiotypic sequence. Therefore two antibodies that have the same \( H \) chain idiotypic sequence and the same \( L \) chain idiotypic sequence, are likely to have very similar \( H \) and \( L \) chain sequences. Roben \textit{et al}, obtained the antigen-binding fragments (Fab) of IgG anti-dsDNA antibodies from the PBL of two clinically active SLE patients by repertoire cloning. One of these Fab, AD4-37, carried the \( H \) chain idiotypic sequence of B6 and the \( L \) chain idiotypic sequence of \( \lambda IIIa \). In an inhibition ELISA, both B6 and \( \lambda IIIa \) anti-idiotypic antibodies were added to the serum of the patient from whom AD4-37 was derived. It was found that inhibition of the dsDNA binding activity of the patients serum, was virtually the same using both of these anti-idiotypic antibodies (46%) as it was with the \( \lambda IIIa \) anti-idiotypic antibody alone (47%). This result suggested that a large number of the serum IgG anti-dsDNA autoantibodies in the serum of this patient co-
expressed the H chain idiotype B6 and the L chain idiotype λ.IIIa and therefore could have been similar to the Fab AD4-37 that was derived by PCR of her PBLs. This supports the theory that repertoire cloning can generate combinations of heavy and light chains that also occur in the anti-dsDNA antibodies found in vivo.

An alternative to scFvs is to express Fab fragments, which contain the whole light chain (\(V_L\) and \(C_L\)) covalently bound to half of the heavy chain (\(V_H\) and \(C_{H1}\)). Once again the Fab can be free in solution or displayed on the surface of a phage, allowing repertoire cloning. Bacterial expression of Fab fragments gives lower yields than sFv. However, Fab maintain the structure of an antibody binding site, and so are the most appropriate fragments to be used in crystallisation studies as described in section 1.4.6. However, since neither scFv nor Fab fragments possess the effector Fc region, these molecules are not suitable for studies of pathogenicity. The disadvantage of using prokaryotic expression systems is that they cannot express whole immunoglobulin molecules, possibly because they cannot fold these large molecules properly, and bacteria do not possess all of the enzymes necessary for important post-translational modifications, such as glycosylation. The functional properties of scFv and Fab fragments specific for DNA and produced using bacterial systems, are described in section 1.4.7.2.

1.4.2.2 Eukaryotic expression systems

A number of techniques have been described for the expression of anti-DNA antibodies in mammalian cells and these fall into three groups: the simultaneous transfection of separate heavy and light chain expression vectors, the transfection of a single vector containing both heavy and light chain sequences (super-vector), and the transfection of heavy chain vector into hybridoma cells which only secrete light chains (a heavy chain loss variant). The former two can also be either transient or stable expression systems. The binding and functional properties of recombinant anti-DNA immunoglobulin, produced in eukaryotic expressions systems are discussed in section 1.4.7.3.

In transient expression systems, the genes that are inserted into the plasmid vector are not integrated into the genome of the transfected cell. Therefore the cell only produces the expression product for a short amount of time, 3-6 days, since the
foreign genes are soon lost from the cell (Gluzman 1981), and consequently only small amounts of IgG can be produced in this way. COS-7 cells (an immortal African green monkey kidney cell line), which secrete no endogenous immunoglobulin, have been used for transient expression of anti-DNA antibodies (Zack et al. 1995; Rahman et al. 1998; Rahman et al. 2001).

In stable expression systems the transfected DNA is incorporated into the genome of the host cell and is passed on to all subsequent cell generations. However, it is essential to select those cells that have successfully integrated the DNA by incorporating genes conferring drug resistance into the expression plasmid (culture in selection media), and also by sub-cloning, select those cells that are expressing the highest amount of product. Stable expression of anti-DNA antibodies has been demonstrated in Chinese hamster ovary (CHO) cells (Haley et al. 2004) and in F3B6, a non-secreting human-mouse heteromyeloma cell line (Li et al. 2000).

The H chain expression vectors used in both transient and stable eukaryotic expression systems, contain the $V_H$ DNA sequence 5’ to the appropriate $C_H$ DNA sequence (i.e. encoding the entire H chain constant region and the hinge region). The L chain expression vectors contain the $V_L$ DNA sequence 5’ to the appropriate $\kappa$ or $\lambda$ $C_L$ DNA sequence. The separate H and L chain expression vectors are simultaneously transfected into eukaryotic cells and whole fully glycosylated immunoglobulin molecules are expressed. Care has to be taken in subsequent assay systems to measure whole immunoglobulin molecules, since the use of separate H and L chain vectors can yield production of just heavy or light chains, as well as whole IgG. Alternatively, the $V_H$, $C_H$, $V_L$ and $C_L$ DNA sequences may all be cloned into the same expression vector (supervector). This method has the advantage that simultaneous acquisition of two separate plasmids is not required, which may increase the yield of cells producing whole immunoglobulin. However, the simultaneous transfection of separate H and L chain expression vectors does make it easier to produce a wide range of immunoglobulin molecules with different H/L chain combinations.

Alternatively, H chain expression vectors may be transfected into H chain loss variants, which are hybridoma cell lines that secrete L chains but have lost their
ability to secrete H chain. This method has been used extensively to study the importance of changes in the heavy chain sequence on properties of murine anti-DNA Ig molecules (Katz et al. 1994; Radic et al. 1991; Radic et al. 1993). The converse method, of transfecting light chain vectors into cells that only produce heavy chains, has not been widely used in the study of anti-DNA antibodies. Heavy chain loss variants have not been used to study human anti-DNA antibodies, due to the inherent difficulties in the production and maintenance of human hybridomas producing IgG anti-DNA antibodies, compounded by the fact that loss variants will only form a small proportion of each clone.

1.4.3 General immunoglobulin structure and gene rearrangement

1.4.3.1 Structure of IgG

Immunoglobulin molecules consist of two identical heavy chains (50 KD each) and two identical light chains (25 KD each). The heavy chains comprise four domains and the light chains have two domains (Figure 1.3). There are five different heavy chains in human immunoglobulin molecules IgM (μ), IgG (γ), IgA(α), IgD(δ), and IgE(ε). The IgG γ heavy chain is 330 amino acids in length. The C-terminus of the heavy chains forms the constant or Fc (crystallisable fragment) region of the antibody. The Fc region is conserved in all IgG molecules barring minor sequence changes that determine the subclass of the antibody (IgG1, IgG2, IgG3 and IgG4 in humans, or IgG1, IgG2a, IgG2b and IgG3 in mice). The Fc region determines the effector properties of the immunoglobulin molecule, such as Fc receptor binding and complement activation. The light chain has two basic sequences kappa (κ) or lambda (λ), in humans 60% of light chains are κ and 40% are λ, whereas in mice 95% are κ and only 5% are λ. The N-terminal 100-110 amino acids of the heavy and light chains, are variable in sequence (variable regions, V_H and V_L) and together they form the antigen binding site (Janeway et al. 2001; Roitt et al. 1996).

Detailed comparison of the amino acid sequences of the V_L and V_H domains revealed that the sequence variability is concentrated in hyper-variable regions also referred to as the complementarity determining regions (CDRs). Mouse and human heavy and light chains each have three such regions, CDR1, CDR2 and CDR3, which together form 15-20% of the variable domain. The remaining 80-85% of each domain is
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Antigen binding site

Light chain

hinge region

Variable region
(N-terminus)

hypervariable loops
disulphide bonds
carbohydrate

Heavy chain

Constant region (Fc)
(C-terminus)

Figure 1.3  Schematic diagram of the structure of an IgG molecule

Immunoglobulin molecules consist of two identical heavy chains (50 KD each) and two identical light chains (25 KD each), which are linked by disulphide bridges. The heavy chains comprise four domains and the light chains each have two domains. Three of the heavy chain domains ($C_hI$, $C_h2$ & $C_h3$) are constant in all antibodies of the same isotype and one ($V_h$) is highly variable between individual antibody molecules. The light chain has one constant ($C_l$) and one variable ($V_l$) domain. The variable domains, and in particular the hypervariable regions (also termed complementarity determining regions, CDRs), of the amino terminal domains (N-terminus) of both the heavy and light chains form the antigen binding site of the antibody. The carboxyl terminal domains (C-terminus) of the heavy chains form the Fc (crystallisable fragment) region, this region determines the isotype of the antibody molecule and controls the antibody’s effector functions.
much less variable in amino acid sequence and are called the framework regions (FRs). The CDRs are separated in the primary amino acid sequence by the framework regions, but folding of the molecule to form the secondary structure brings together the CDRs to form the antigen binding site. The precise sequence of the variable regions determine the antigen binding specificity of each IgG antibody. Antibody-antigen binding is achieved by hydrogen bonds, salt bridges and Van der Waal's forces. Charged residues on the antigen are frequently neutralised by opposing charged residues on the antibody, such charge neutralisation appears to be particularly important at the centre of the binding region (Janeway et al. 2001).

1.4.3.2 Generation of immunoglobulin diversity

Antibodies can differentiate between molecules which differ only by a single amino acid; the human immune system generates at least $10^{11}$ different antibody specificities (Roitt et al. 1996). At the most simple level, antibody diversity is determined by the random recombination of 'variable', 'diversity' and 'joining' (V, D and J) gene segments to form the heavy chain V-region gene, and V and J gene segments for the light chain V-region gene, during differentiation of lymphoid stem cells into virgin B cells. A productive rearrangement of the heavy chain genes is followed by rearrangement of the kappa light chain, and if that is not productive the lambda chain is rearranged. There are two loci so two chances to rearrange each chain, if the heavy chain or both light chain rearrangements are not productive the cell will die by apoptosis. Each type of gene segment is present in multiple copies in the germline genome (approximately 50 $V_h$, 40 $V_\kappa$ and 30 $V_\lambda$ functional gene segments, plus fewer D and J segments).

Diversity is further increased, by a number of mechanisms which cause sequence differences, by deletion and insertion of nucleotides, at the joining regions (junctional diversity) of the VDJ of the heavy chain, and the VJ segments of the light chain. However, although they increase diversity, sequence changes at junctions may incorporate frameshifts, resulting in a non-productive recombination. In the heavy chain the CDR1 and CDR2 are encoded by a V gene and CDR3 by the region of VDJ joining; in the light chain, CDR1 and CDR2 are again encoded by one of the V genes, and CDR3 by a V gene and the VJ junction, thus the CDR3 has the potential to be the most variable region.
The human $V_H$, $V_\kappa$ and $V_\lambda$ gene segments, are found on chromosomes 14, 2 and 22 respectively. The human V gene segments, are grouped into families, the members of which, all share at least 80% nucleotide sequence homology with each other. There are seven families each of human $V_H$ and $V_\kappa$ gene segments, and 10 families of $V_\lambda$ gene segments (Cook and Tomlinson 1995; Williams et al. 1996). Analysis of the usage of $V_H$ genes, have shown that genes belonging to the larger families $V_H1$, $V_H3$ and $V_H4$, are the most commonly expressed in humans, especially $V_H3$, with 3-23, previously known as $V_H26$, being the most frequently used segment (Huang et al. 1996). Similarly, the larger $V_\lambda1$, 2 and 3 families are more commonly expressed in the light chains of human antibodies, particularly $V_\lambda2$, with 2a2 the most frequently used segment (Ignatovich et al. 1997).

Following gene rearrangement in the pre-B cell, immature B cells can undergo maturation in response to activation by antigen. Antibody heavy chain constant regions undergo isotype class switching. Initially all antibodies are expressed with $C_H$ heavy chains, but these can be replaced sequentially by $C_\delta$, $C_\gamma$, $C_\varepsilon$ or $C_\alpha$, so that B cells from a single clone (specific for the same antigen) can exhibit a variety of effector functions (Janeway et al. 2001). On repeated stimulation by antigen, B cells will switch from co-expression of low affinity IgM and IgD (only small amounts secreted) to produce higher affinity IgG.

Normally, if a B cell encounters a self-antigen in the germinal centre, it will be deleted to maintain tolerance, however in humans a mechanism exists for it to make a second productive rearrangement of the light chain. This process is known as receptor editing (Prak and Weigert 1995). Second rearrangements at the light chain loci, may be detectable since they tend to utilise the distal J genes, such as $J_{\kappa}4$ and $J_{\kappa}5$. It has been suggested that deficiencies in receptor editing, may play a role in breakdown of tolerance in autoimmune diseases (Dorner and Lipsky 2001). Receptor editing has been demonstrated as a possible mechanism preventing the formation of anti-DNA antibodies, in mice transgenic for the heavy and light chains of a murine anti-DNA antibody (Gay et al. 1993). However, improper editing of autoreactive B cells may contribute to the breakdown in tolerance in MRL-$lpr$ mice which express the 3H9H/56R heavy chain transgene (derived from a murine anti-dsDNA antibody).
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(Li et al. 2002). In a similar way, heavy chain revision (secondary rearrangement of the heavy chain) involving increased usage of atypical rearrangements (use of upstream D genes and downstream J(H) genes and D-D fusions) has been implicated in the development of autoreactive B cell precursors in MRL-+/+ mice (Klonowski and Monestier 2000).

Antibody diversity and specificity, is further increased during late stage B cell development by the occurrence of somatic hypermutation of antigen activated B cells, in the germinal centres. Somatic hypermutation generates single base mutations in the \( V_H \), \( V_L \) and flanking sequences. The precise mechanisms of somatic hypermutation are not fully understood. The \( V_H \), \( V_K \) and \( V_\lambda \) gene segments which encode these hypermutated areas, are known as 'hotspots'. However, it is known that mutations are not targeted randomly along the length of the \( V \) gene and that somatic mutations tend to occur at particular consensus sequences such as AGC, AGT, GGC and GGT. These mutational ‘hotspots’ are more commonly found in the CDRs rather than the FRs (Wagner et al., 1996), so CDRs have an increased tendency to mutate. The mutations can be either replacement, causing a change in the amino acid sequence, or silent, no change in sequence. Replacement mutations are clustered in the CDRs.

Somatic hypermutation is the basis for increasing antibody affinity for an antigen during maturation of the immune response. The sequential accumulation of replacement somatic mutations may lead to the gradual increase in specificity and binding affinity (affinity maturation) of the antibody produced by a clone of B cells (Tomlinson et al. 1996). Prior to antigen selection, immunoglobulin sequence diversity of the primary antibody repertoire is focussed at the centre of the antigen binding site. Following antigen selection, through the process of somatic hypermutation, the antigen binding affinity is increased and diversity spreads to regions at the periphery of the binding site, which were highly conserved in the primary antibody repertoire (Tomlinson et al. 1996). The somatic hypermutation rate is \( 10^{-3} \) to \( 10^{-4} \) changes per base pair, per cell division, as compared with the spontaneous mutation rate of \( 10^{-7} \) or less (Janeway et al. 2001). B cells producing antibodies which have the greatest specificity and affinity for the driving antigen, proliferate faster than other B cell clones (antigen-driven clonal expansion) and have
a high ratio of replacement to silent mutations in the V region CDRs (but not FRs), since these regions constitute the majority of the antigen binding site (Shlomchik et al. 1987).

1.4.4 The influence of immunoglobulin sequence and structure on the binding of anti-dsDNA antibodies.

Sequence analysis of the variable regions of monoclonal antibodies that share the same binding or pathogenic characteristics, can be used to determine if these antibodies have any sequence characteristics in common. Due to the recent publication of maps of the entire human Vκ, Vλ and Vh gene loci, it is now possible to begin to determine whether monoclonal antibodies show preferential usage of Vh or Vl gene segments, may have undergone receptor editing, or whether they possess a certain pattern of somatic mutations (Williams et al. 1996; Cook et al. 1994; Schable and Zachau 1993). The amino acid sequences of many murine and human monoclonal anti-DNA antibodies, have been determined in an attempt to identify structural features which might distinguish pathogenic from non-pathogenic antibodies (reviewed Rahman et al. 2002a).

1.4.4.1 Variable region gene usage by monoclonal anti-DNA antibodies

The sequences of over 300 murine monoclonal anti-DNA antibodies have been reviewed, from both autoimmune strains such as MRL/lpr and BWF1, as well as normal mice immunised with protein-DNA complexes or antibodies carrying particular idiotypes, and also transgenic mice producing anti-DNA antibodies (Radic and Weigert 1994). It is striking that monoclonal antibodies derived from these widely differing mouse models often use the same Vh and/or Vl genes. Vh genes of the J558 and 7183 families are commonly used. The J558 family is a very large gene family, but this is not sufficient to account for the observed degree of preferential usage. In addition, some pairings of Vh and Vk genes occur more frequently among murine anti-DNA antibodies.

The picture is less clear cut for human anti-DNA antibodies. Although over 50 human monoclonal anti-DNA antibody sequences have been published, most of these are polyreactive IgM antibodies which bind DNA with low affinity. Relatively few human IgG anti-DNA antibodies have been sequenced, these were analysed for gene
usage in a recent review (Rahman et al. 2002a). Most human IgG anti-DNA antibodies use genes of the $V_{H}3$ or $V_{H}4$ families, although the significance of this finding is uncertain since most functional human $V_{H}$ genes are members of $V_{H}1$, $V_{H}3$ or $V_{H}4$ families. The light chain gene usage by human anti-DNA monoclonal antibodies, also seems to reflect the same proportional usage seen in normal recombination. As yet there are no data to show preferential use of a particular human $V_{H}$ or $V_{L}$ gene family in human monoclonal anti-DNA antibodies. This observation fits with data obtained in a study using single cell PCR of peripheral B cells from patients with SLE and healthy donors, which found no difference in $V,D$ and $J$ gene segment use, somatic mutations, or heavy and light chain pairings, between one normal (209 B cells) and two SLE (156 B cells) patients (de Wildt et al. 2000). One of the IgG monoclonal antibodies studied in this thesis, B3, was derived from an SLE patient and is encoded by the most commonly rearranged human $V_{H}$ and $V_{\lambda}$ genes, V3-23 and 2a2 respectively (Rahman et al. 2001).

1.4.4.2 The importance of somatic mutations and charged residues in the CDRs of monoclonal anti-DNA antibodies.

It is now quite easy to determine the location and nature (replacement or silent) of somatic mutations in an antibody by comparing it with the published germline gene that it most closely resembles. VBASE, a database of all known human immunoglobulin V region genes enables rapid identification of the most similar germline gene. Each variable region sequence can be aligned to the closest germ-line counterpart in the VBASE Sequence Directory using DNAplot software, thus allowing subsequent analysis of nucleotide homology and distribution of somatic mutations. Statistical analysis can be applied to the distribution of replacement and silent mutations in an antibody sequence to determine whether this pattern of somatic mutation is a consequence of antigen-drive. Two statistical methods exist at present: a binomial distribution method (Chang and Casali 1994) and a multinomial distribution method (Lossos et al. 2000). The multinomial approach is thought to be more accurate, since unlike the binomial method, it takes into account all four possible outcomes of a particular mutation, i.e. R and S mutations in the CDRs and/or FRs of the gene.
MacCallum et al proposed a set of rules to predict which amino acids within an antibody sequence are most likely to form contacts with antigen (MacCallum et al. 1996). The defined contact sites are mainly contained within the CDRs as defined by (Wu and Kabat 1970), but some adjacent FR residues are also involved. The positions of the CDRs and the proposed contact sites within the amino acid sequence of the heavy and light chains are shown in Table 1.10. Evidence suggests that somatic mutation leads to an increase in positively charged amino acid residues such as arginine and lysine, in anti-DNA antibodies, especially within CDR3 (Radic and Weigert 1994; Tillman et al. 1992; Winkler et al. 1992; Rahman et al. 2002a). It has been postulated that the presence of these positively charged residues might enhance binding to the DNA molecule, which carries an array of negative charges. Arginine appears to be particularly important in the binding to DNA (Radic et al. 1993), it can form hydrogen bonds with base-paired guanine, as well as unpaired and base-paired cytosine. Arginine can fit into the major or minor DNA groove through extensive interactions with the DNA sugar-phosphate backbone, its flexible side chain facilitates binding (Seeman et al. 1976). The presence of uncharged asparagine residues, also appears to enhance binding to DNA, this is probably due to its ability to function as a donor or recipient of hydrogen bonds.

Antigen-driven accumulated replacement mutations have been described in the CDRs of monoclonal anti-DNA antibodies derived from mouse models of lupus, and these monoclonal antibodies showed a high frequency of arginine, asparagine and lysine residues in the CDRs (Radic and Weigert 1994). Tillman et al sequenced over 100 monoclonal anti-DNA antibodies derived from BWFl mice (Tillman et al. 1992). Some of these antibodies derived from the same expanded clone of B cells, since they had V_H and V_K sequences which only differed in the number and position of somatic mutations. Within a single clone, the IgG antibodies with the most mutations were more likely to bind dsDNA, whilst the IgM antibodies and IgG antibodies with fewer mutations mainly bound ssDNA. It was confirmed that the antibodies with greatly enhanced binding to dsDNA, were those with somatic mutations that increased the numbers of arginine residues in the CDRs, especially within CDR3 of the heavy chain. A recent review of the published sequences of ANAs from 35 studies, on 9 different mouse strain backgrounds, concluded that anti-dsDNA antibodies differed from anti-ssDNA antibodies, in having more negatively
RANGE OF AMINO ACID RESIDUES WITHIN EACH CONTACT REGION / CDR

<table>
<thead>
<tr>
<th>CONTACT REGION</th>
<th>CDR as defined by Wu and Kabat (1970)</th>
</tr>
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<tbody>
<tr>
<td>L1</td>
<td>L30-L36</td>
</tr>
<tr>
<td>L2</td>
<td>L46-L55</td>
</tr>
<tr>
<td>L3</td>
<td>L89-L96</td>
</tr>
<tr>
<td>H1</td>
<td>H30-H35b</td>
</tr>
<tr>
<td>H2</td>
<td>H47-H58</td>
</tr>
<tr>
<td>H3</td>
<td>H93-H101</td>
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</table>

Table 1.10 Range of amino acid residues in each contact region / complementarity determining region (CDR)

MacCallum et al analysed 26 antibody Fab and Fv crystal structures that were each complexed with an antigen and determined sites at which the antigen most commonly made contact with the antibody (MacCallum et al. 1996). These sites were consequently termed ‘contact regions’. Contact regions were defined as the sites of the heavy and light chain variable regions at which the antibody was most likely to contact its antigen. The defined contact sites are mainly contained within the CDRs as defined by Wu and Kabat (1970), but some adjacent FR residues are also involved. The positions of the CDRs and the proposed contact sites within the amino acid sequence of the heavy and light chains are shown in the table above.
charged aspartic acid and uncharged tyrosine residues in the $V_h$ CDR1, significantly
different distributions of charged or polar residues in $V_h$ CDR2, and more arginine
residues in $V_h$ CDR3 (Chen et al. 2002). The differences in the CDR1 and 3 regions
were irrespective of $V_h$ family, but the differences in CDR2 were dependent on $V_h$
family of origin. This study also found enrichment of $V_{h1}/J558$ genes.

The accumulation of arginine, asparagines and lysine residues by somatic
hypermutation in the CDR regions of six human anti-DNA monoclonal antibodies
was reported by Winkler et al (1992). Using the multinomial method of Lossos et al
(2000), a recent review (Rahman et al. 2002a) found a significant probability that
particular $V_h$ or $V_l$ sequences, occurring in the majority of published human IgG
anti-DNA antibody sequences, had resulted from the antigen-driven accumulation of
somatic mutations. The IgM anti-DNA antibodies that were also analysed did not
show such antigen-driven accumulation of mutations, and antibodies derived by
phage display had relatively little mutation (Rahman et al. 2002a). This observation
was in common with a study which showed that high affinity for dsDNA was not
associated with extensive mutation of the $V_h$ regions of these Fab (Barbas et al. 1995).

The occurrence of mutations resulting in sequence motifs, such as the accumulation
of arginine, asparagine or lysine residues at sites which may contact antigen (‘contact
regions’ as defined by (MacCallum et al. 1996)), was systematically analysed in the
sequences of published human anti-DNA antibodies (Rahman et al. 2002a). This
analysis confirmed the accumulation of somatic mutations encoding arginine,
asparagine and lysine residues at these contact sites, particularly in the CDR1 and
CDR2 areas of the IgG heavy and kappa light chains. However, the CDR3 contact
sites were not included in this analysis, due to the difficulty in determining the
presence of somatic mutations in CDR3, due to junctional diversity.

There are multiple somatic mutations in human IgG anti-DNA antibody B3, both $V_h$
and $V_k$, consistent with antigen driven affinity maturation (Ehrenstein et al. 1995).
For example, in both B3 and 33.H11, the residue at position 53, which is located in
the centre of $V_h$ CDR2, is a product of somatic mutation. At position 53, the germ-
line serine is mutated to a positively charged arginine in B3$V_h$ and to a neutral
glycine in 33.H11V\textsubscript{H}. The effects of these mutations on the structure and function of B3 are discussed further in the context of computer modelling, and \textit{in vitro} binding properties, sections 1.4.5 and 1.4.7.3 respectively.

It is clear from the studies of human and murine monoclonal anti-DNA antibodies, that the presence of certain residues, including arginine, asparagine and lysine, may be important in binding to DNA. However, not all such residues enhance the binding to DNA, some may cause steric hindrance to binding, it is important to see the positions of such residues relative to the DNA molecule in order to predict and explain antibody binding in \textit{in vitro} or \textit{in vivo} systems, this can be achieved using computer modelling.

\textbf{1.4.5 Computer modelling of anti-DNA antibodies}

To predict antibody-DNA interaction, sequence data can be employed in computer modelling of the three-dimensional structure of anti-DNA antibodies. Based on crystal structures, the modeller can then hypothesise which amino acid groups may enhance or reduce binding to DNA. This technique has been used to model several human anti-dsDNA monoclonal antibodies, including B3. Modelling of B3, revealed a binding groove along the V\textsubscript{H}-V\textsubscript{L} axis, which was flanked by three arginines at positions R27a (V\textsubscript{L} CDR1) and R54 (V\textsubscript{L} CDR2) of the light chain, and position R53 (V\textsubscript{H} CDR2) of the heavy chain. Two of these arginine residues, V\textsubscript{L}R27a and V\textsubscript{H}R53, were derived by somatic mutation. Interactive docking of dsDNA helix in the binding groove confirmed that the presence of these arginines, at antibody-antigen contact sites, might stabilise the binding of B3 to dsDNA, as shown in Figure 1.4 (Kalsi \textit{et al}. 1996). Interestingly, the model also allowed docking of Z-DNA in a groove at right-angles to that for B-DNA, which may provide a hypothesis to explain the ability of a single antibody to bind two different antigens. Further modelling has been carried out, to test the binding of mutagenised forms of B3 to dsDNA, as described in section 1.4.7.3 (and Figures 1.5, 1.6, 1.7). Computer modelling of the variable fragment (Fv) of human monoclonal IgA anti-dsDNA antibody 412.67 also revealed a groove, which could accommodate a DNA double helix. The computer model of 412.67 predicted the interaction of arginines from the V\textsubscript{H}CDR3 (at positions 105 and 107) with the dsDNA helix (Li \textit{et al}. 2000).
The double helix of the DNA molecule is shown in the centre of the figure. $B3V_H$ is shown in light blue whilst $B3V_\lambda$ is shown in dark blue. The three arginine residues at the periphery of the site (R27a [$V_\lambda$ CDR1] and R54 [$V_\lambda$ CDR2] of the light chain, and R53 [$V_H$ CDR2] of the heavy chain), predicted by the model to stabilise the interaction with the dsDNA helix, are shown in yellow. The only arginine that is derived from a somatic mutation is in $V_\lambda$ CDR1 at position 27a (R27a).

Figure 1.4  Computer generated model of $B3V_H/B3V_\lambda$ with dsDNA

Modelled by Dr Sylvia Nagl
1.4.6 \textit{X-ray crystallography of anti-DNA antibodies}

Few crystal structures of murine anti-DNA antibodies have been published, because of difficulties in purifying sufficient quantities of the monoclonal antibody for X-ray crystallography analysis. A crystal of an antibody complexed with its antigen is known as a co-crystal. Co-crystals show the precise locations at which amino acid residues of the antibody make contacts with the antigen, and also changes in conformation of an antibody, if any, on binding to an antigen. The only published co-crystal involving an anti-DNA antibody, has been made of a murine monoclonal anti-DNA antibody (BV04-01, that binds ssDNA) binding to a trinucleotide of deoxythymidic acid \([d(pT)_3]\) \cite{Herron1991}. When complexed with \(d(pT)_3\), BV04-01 undergoes conformational changes in order to incorporate the trinucleotide. The co-crystal also showed, that the trinucleotide is held in a large irregular groove between the \(V_H\) and \(V_L\) regions on the surface of the antibody, and that all six CDRs of the H and L chains contribute to this binding site. Only one arginine residue in \(V_H\)CDR2 at position 52, was seen to form an electrostatic contact with the phosphodiester backbone of the trinucleotide. Although other arginine and lysine residues were present in the binding site, they were unable to directly interact with the antigen, due to either participating in ionic bonds in order to stabilise the topography of the antigen binding site, or due to not being in the appropriate locations to contact the phosphodiester backbone.

A crystal without its antigen has been made of the Fab of murine anti-dsDNA antibody Jel 72, this antibody also binds triple stranded DNA. The crystal of Jel 72 reveals a flat binding site with knob-like ridges containing basic amino acid residues, the \(V_H\) CDR3 loop is particularly rich in arginines \cite{Mol1994}. Computer modelling of Jel 72 suggests that although this antibody binding site does not possess a cleft, as seen with the model of B3, the protruding knobs may penetrate the major and minor grooves of the DNA double (or triple) helix. An arginine residue at position 99 of the heavy chain of Jel 72 may interact with guanine bases of the DNA \cite{Barry1994}.

One exciting use of bacterial expression systems, is their potential to produce a large enough quantity of human antibody Fab, to allow X-ray crystallographic analysis of human anti-DNA antibodies. This has been attempted with B3 Fab, however there
were technical difficulties in purifying B3 Fab from cross-reactive debris in the bacterial periplasm (Kumar et al. 2002), so as yet no crystal structure exists of a human anti-dsDNA antibody complexed with its antigen.

1.4.7 Investigation of the effect of sequence, on the function of anti-DNA antibodies in vitro

1.4.7.1 The in vitro function of monoclonal anti-DNA antibodies produced by hybridoma cells

The important effect of sequence on function, is emphasised by the fact that the binding of an antibody to its antigen can be altered by changing just one amino acid in the sequence of the variable region. For example, in a murine monoclonal antibody which recognised the bacterial antigen phosphocholine (PC), a single amino acid substitution, due to somatic hypermutation, resulted in the loss of PC binding and the acquisition of binding specificity for dsDNA (Diamond and Scharff 1984).

Consideration of three antibodies encoded by the same V\textsubscript{H} gene (V4-34), E3 (Longhurst et al. 1996), D5 (Ehrenstein et al. 1993) and RT-79 (Ravirajan et al. 1992), demonstrates that the exact position rather than the mere presence of basic or positively charged amino acid residues in the CDRs, is vital for determining anti-DNA binding ability. RT-79 V\textsubscript{H} CDR3 possesses five arginines whilst D5 V\textsubscript{H} CDR3 only has two, but D5 binds dsDNA with high affinity whilst RT-79 only binds ssDNA; E3 is also rich in asparagine and arginine residues but doesn’t bind DNA at all (Stevenson et al. 1993).

In the literature, thirteen human anti-DNA IgG monoclonal antibodies (19.E7, 32.B9, 33.F12, 33.H11, 33.C9, 35.21, D2, E7, F8, D5, B3, RH-14 and DIL-6) have been produced by direct fusion as described in section 1.4.1.2. Of these thirteen antibodies, 11/13 bound dsDNA by ELISA (E7 and F8, bound only ssDNA) and of these 11, 10/11 bound to *Crithidia luciliae*, 33.H11 did not and was also negative in the Farr assay, implying a low affinity anti-dsDNA reactivity. This fits with the fact that 33.H11 has fewer CDR mutations than B3 and its dissociation constant is higher suggesting a weaker binding to DNA (see Table 2.1). Investigation of these thirteen human IgG anti-DNA monoclonals showed varying ability of these antibodies to also bind histones, nucleosomes, heparan sulphate and laminin. The detailed binding specificities of RH-14, B3, DIL-6 and 33.H11, which are studied in this thesis, are
shown in the methods, Table 2.1. The effect of fine changes in the sequence on antigen binding function of B3 and 33.H11, has been examined in recombinant mutants of these monoclonal anti-DNA antibodies produced by eukaryotic expression systems, as described in section 1.4.7.3.

1.4.7.2 The in vitro function of anti-DNA specific scFv and Fab fragments produced by bacterial expression systems

As described earlier, the use of expression systems allows 'in depth' study of the effect of changes to the sequence, on the function of anti-DNA antibodies. Repertoire cloning, was used to produce six high affinity human anti-dsDNA Fab, from PBLs of a patient with SLE (Barbas et al. 1995). Interestingly, the genes encoding the Vh regions of these antibodies displayed only minimal evidence of somatic hypermutation, they were 98-99% homologous to germline. The heavy chains of the SLE Fabs, were characterized by a predominance of basic residues, arginine and lysine, toward the N terminus of the CDR3. The crucial role of Vh CDR3 in high-affinity binding to DNA, was further suggested by the creation of DNA binding in an unrelated antibody, by Vh CDR3 transplantation from the SLE antibodies. The authors proposed that high-affinity anti-DNA antibodies might arise in SLE, without extensive somatic hypermutation in the variable-region genes, because of the expression of inappropriate Vh CDR3s.

Roben et al, derived two high-affinity anti-dsDNA Fab from a patient with SLE, but none from her healthy identical twin sister. Both of these Fab, AD4-37 and AD4-18, had accumulations of basic residues in Vh CDR3, however AD4-37 was almost unmutated in both Vh and VL, whereas AD4-18 contained multiple somatic mutations in both. The importance of the Vh CDR3 region in AD4-37, was demonstrated through combination of the light and heavy chain of AD4-37 with a library of phages expressing a multitude of heavy and light chains from both the SLE patient and her healthy twin, Fab combinations of H and L chains which bound to DNA were only obtained using the heavy chain of AD4-37, or chains with very similar Vh CDR3 sequences, and also only with lambda light chains derived from the SLE patient (Roben et al. 1996).
The human anti-DNA antibodies B3 and 33.H11 were both produced as human hybridomas by fusion of PBLs from patients with lupus (Ehrenstein et al. 1993; Winkler et al. 1991), the cDNA sequences of the binding sites from both of these monoclonal antibodies have been expressed in the form of Fab in a bacterial expression system. Our group has previously shown the importance of the B3 heavy chain, since dsDNA binding is reduced when the heavy chain of B3 is replaced by that of 33.H11 (Kumar et al. 2000).

Although much research has focussed on the role of the $V_h$CDR3 of anti-DNA antibodies, bacterial expression systems have been used to show that light chains also contribute to the DNA binding affinity of both murine and human anti-DNA antibodies. Both $V_h$ and $V_l$ were important in the binding of murine monoclonal antibody Z-22 to left-handed Z-DNA, when the $V_l$ of a scFv of Z22 was replaced with a library of splenic $V_k$ cDNA from a mouse immunized with Z-DNA, only a $V_k$ closely resembling that of the original Z22 (differing at six amino acid positions) yielded scFv with Z-DNA-binding activity. In addition, $V_h$ chain swapping and site directed mutagenesis of the $V_h$ CDR3 region confirmed its importance in binding to Z-DNA, and especially the importance of particular asparagine residues (Brigido et al. 1993; Polymenis and Stollar 1994). Work by Jang et al using scFvs demonstrated that for murine (MRL- Ipr) monoclonal anti-DNA (native right-handed B form) antibody 2C10, the heavy chain alone was sufficient for DNA binding to either B or Z-DNA. However, the selectivity of binding to B rather than Z DNA, and the fine specificity of binding were determined by residues in the CDR3 light chain (Jang et al. 1996; Jang and Sanford 2001). The importance of arginine residues in the $V_h$ CDR3 and an asparagine residue in $V_l$ CDR3 was also shown in antibody 2C10 (Jang et al. 1998).

The importance of both the $V_h$ and $V_l$ was also shown in human monoclonal anti-DNA antibody D5. Phage bound D5 Fab could bind both ssDNA and dsDNA, but if the D5 $V_h$ sequence was replaced by that of a cold agglutinin, encoded by the same germline $V_h$ gene but lacking basic residues in the $V_h$ CDR3, there was a complete loss of DNA binding. Somatic mutations in the $V_k$ of D5 were also shown to be important, as replacement with the germline sequence, reduced binding to DNA (Mockridge et al. 1996). In some anti-DNA antibodies the variable light chain
sequence plays the major role in antigen binding. Expression of different $V_H$ and $V_L$ combinations as soluble Fab, demonstrated that binding to DNA of human anti-dsDNA antibodies B3 and 33.H11 is primarily dependent on the presence of their $V_L$ sequences (Kumar et al. 2000; Kumar et al. 2001). This finding was consistent with predictions of computer models of these antibodies (Kalsi et al. 1996; Rahman et al. 2001), as discussed in section 1.4.5. The systems described in these studies all illustrated the importance of not just the presence but also the precise position of residues such as arginine, lysine and asparagine on DNA-binding.

1.4.7.3 The in vitro function of recombinant monoclonal anti-DNA antibodies produced by eukaryotic expression systems

Heavy chain loss variants (HCLVs) have been used to demonstrate the importance of arginine residues in the $V_H$ CDR2 and CDR3, of murine monoclonal anti-DNA antibody 3H9. 3H9$V_H$ contains three somatic mutations in CDR2, one being an arginine at position 53. When this R53 was reverted to glycine, binding to dsDNA was virtually eliminated whilst reversion of the other somatic mutations in CDR2 had little or no effect on binding (Radic et al. 1993). Thus, the heavy chain was dominant in determining 3H9’s ability to bind DNA, however the light chain was important in determining the specificity of binding. This distinction was demonstrated by pairing 3H9 anti-DNA heavy chain with different light chains, which altered the resulting antibodies ability to bind to dsDNA, cardiolipin and ribonucleic acid (Radic et al. 1991). The importance of arginines in the $V_H$ CDR3, and to a lesser extent somatic mutations in $V_H$ CDR1 and CDR2, were also shown in HCLVs secreting $V_L$ of murine anti-DNA antibody D42, transfected with mutagenised versions of $V_H$ from D42 (Pewzner-Jung et al. 1996). However, arginine residues are not always beneficial, indeed mutagenic loss of two arginines from the FR3 of $V_H$ of murine anti-DNA antibody R4A, expressed in HCLVs secreting R4A $V_L$, actually increased binding to DNA (Katz et al. 1994). The importance of residues within the FRs has been shown for other anti-DNA antibodies. For example, Radic et al predicted from a computer model of a murine anti-dsDNA antibody, that $V_H$FR3 is positioned so as to contribute contacts with the minor groove of the DNA (Radic and Weigert 1994).
Overall, these experiments using HCLV and bacterial expression systems underline the importance of $V_H$ CDR3 and arginine residues, but show that mutations in other areas of the sequence, including some framework regions, can be involved in the fine-binding specificity of anti-DNA antibodies. As discussed previously, HCLVs have not been used to study human anti-DNA antibodies, and the use of mammalian expression systems for both murine and human monoclonal antibodies, has considerable advantages over the use of bacterial expression systems. The murine anti-dsDNA antibody 3E10, has been expressed in eukaryotic COS-7 cells. Mutations in all $V_H$ CDRs altered affinity for DNA, particularly the loss of an arginine in CDR3 which reduced affinity, and the gain of an asparagine in CDR1 which increased affinity (Zack et al. 1995).

Li et al., demonstrated stable expression of recombinant IgG1κ anti-dsDNA antibodies in F3B6 human-mouse heteromyeloma cells (Li et al. 2000). This method was used to produce sufficient IgG for affinity purification. Several recombinant IgG1κ producing stable clones were made, including the $V_H$ and $V_κ$ sequences from the ‘wild-type’ human monoclonal IgA anti-dsDNA antibody 412.67 and the germline revertant of this antibody. In addition site directed mutants were made replacing either two arginines in the $V_H$ CDR3 (derived by N segment additions), or somatic mutation derived arginines, with neutral glycine or tryptophan residues. ELISA testing of the binding affinity of the ‘wild-type’ and germline revertant to ss and dsDNA, found that all of the somatic mutations in both $V_H$ and $V_L$ could be reverted to germline without effect. This result implied that the affinity maturation of this antibody was not dependent on binding to DNA, but was more likely directed by another antigen. However, the importance of arginine residues in binding to dsDNA was demonstrated by site directed mutagenesis of two arginines (derived by N segment additions) in the $V_H$ CDR3 of 412.67. Removal of these arginine residues abolished binding to DNA, and removal of either of these arginines singly allowed binding only to ssDNA not dsDNA. In addition, the importance of the precise location of the arginine residues was demonstrated by the lack of involvement in dsDNA binding, of an arginine residue in $V_κ$ CDR3. These findings were consistent with a computer model of 412.67 (as discussed 1.4.5), which showed the two
arginines in V_h CDR3 were exposed on the antibody surface, but that the arginine in V_k CDR3 was inward facing from the antigen binding site.

Our group achieved the first transient eukaryotic expression of human IgG anti-DNA antibody molecules, using human IgG anti-dsDNA monoclonal antibody B3 and human IgM anti-DNA antibody WR1176, which only binds ssDNA not dsDNA. There are more somatic mutations in B3 than WR1176, consistent with the fact that B3 is a high affinity IgG antibody whereas WR1176 is a lower affinity IgM antibody. However, both antibodies show some areas of accumulated positively charged residues, particularly in V_h CDR3 of WR1176 and V_L CDR1 of B3. COS-7 cells were co-transfected with pairs of heavy and light chain vectors to produce whole IgG molecules containing 4 different combinations of V_h and V_L from B3 and WR1176 (WR1176V_h / WR1176V_L, B3V_h / B3V_L, WR1176V_h / B3V_L and B3V_h / WR1176 V_L) (Rahman et al. 1998). Only those IgG molecules containing both B3V_h and B3V_L bound dsDNA, demonstrating the importance of both the heavy and light chains in the binding of B3 to dsDNA.

The COS-7 transient expression system, was also used to produce variants where B3 heavy chain was paired with five different \( \lambda \)-light chain sequences derived from 2a2, thus these IgG combinations only vary at sites of mutation (Rahman et al. 2001). The variants were produced by pairing B3V_h with either unmutated B3V_\lambda ; with one of two B3V_\lambda sequences altered by mutagenesis (B3V_\lambda a and B3V_\lambda b); or with the V_\lambda sequences derived from either anti-dsDNA IgG monoclonal antibody 33.H11; or from IgG antiphospholipid antibody UK4 (described in section 2.2, Table 2.1). The first mutant B3V_\lambda a, had an arginine at position 27a in V_\lambda CDR1, which was derived by somatic hypermutation. This arginine was reverted to the 2a2 germline serine residue by site directed mutagenesis. The second mutant B3V_\lambda b, contains an additional second mutation, produced by PCR error which converted a germline encoded glycine at position 29 to a serine, a mutation which actually exists as a somatic mutation in UK4. Both 33.H11 and UK4 V_\lambda sequences are encoded by 2a2, but differ from B3V_\lambda and the germline sequence due to somatic mutation. The following combinations were expressed in COS-7 cells: wild type B3V_h/B3V_\lambda, B3V_h/B3V_\lambda a, B3V_h/B3V_\lambda b, B3V_h/33H11V_\lambda and B3V_h/UK4V_\lambda. After treatment
with DNase I, the COS-7 cell supernatants containing the expressed antibodies were assessed for binding to dsDNA in ELISA. The wild type B3V\textsubscript{H}/B3V\textsubscript{\lambda} bound dsDNA and ssDNA more than B3V\textsubscript{H}/33H11V\textsubscript{\lambda}, which in turn bound slightly better than B3V\textsubscript{H}/B3V\textsubscript{\lambda}a, which again bound better than B3V\textsubscript{H}/B3V\textsubscript{\lambda}b, and the combination B3V\textsubscript{H}/UK4V\textsubscript{\lambda} did not bind ss or dsDNA at all. The arginine to serine point mutation in B3V\textsubscript{H}/B3V\textsubscript{\lambda}a resulted in a 50% reduction in dsDNA binding, indicating the importance of this arginine in the binding site, and the second accidental mutation from glycine to serine found in B3V\textsubscript{H}/B3V\textsubscript{\lambda}b reduced binding further, possibly due to increasing unfavourable electostatic interactions in the binding site.

As discussed in section 1.4.5, computer modelling of the molecular structure of B3 has shown the presence of a binding site for dsDNA, and the stabilization of the interaction with dsDNA by the presence of three arginine residues in the V\textsubscript{H} and V\textsubscript{\lambda} (Kalsi et al. 1996), as shown in Figure 1.4. The V\textsubscript{H}CDR2 arginine (shown in yellow in Figure 1.4) at the periphery of the site along with the other two light chain arginines is predicted to be essential in stabilising the B3/dsDNA interaction. The importance of the arginine at position 27a of the V\textsubscript{\lambda} CDR1, as shown by in vitro binding to dsDNA by ELISA (Rahman et al. 2001), was predicted by modelling of the relative interaction of the dsDNA helix, with either an arginine (as occurring in ‘wild-type’ B3) or serine residue (as occurring in the B3V\textsubscript{H}/B3V\textsubscript{\lambda}a combination) at this position (Figure 1.5).

Further modelling also predicted that B3V\textsubscript{H}/33.H11V\textsubscript{\lambda} was likely to bind dsDNA, as was indeed shown by ELISA (Rahman et al. 2001), due to the presence of an arginine residue at position 92 (R92) of 33.H11 V\textsubscript{\lambda}CDR3, as shown in Figure 1.6. It is possible that the presence of R92 in V\textsubscript{\lambda}CDR3 of 33.H11V\textsubscript{\lambda} compensated for the fact that 33.H11V\textsubscript{\lambda} lacked an arginine residue in V\textsubscript{\lambda} CDR1, as found at position 27a in B3V\textsubscript{\lambda}. Modelling also predicted that the presence of an arginine group at position 94 (R94) of UK4 V\textsubscript{\lambda}CDR3 would be likely to block binding of B3V\textsubscript{H}/UK4V\textsubscript{\lambda} to dsDNA due to steric hindrance Figure 1.7. This prediction was also proved to be correct by ELISA (Rahman et al. 2001).
Figure 1.5 Computer-generated model to show the interaction of the amino acid residue at position 27a of V_\text{CDR1} with dsDNA helix in B3V_\lambda and B3V_\lambda a

The upper diagram shows the interaction between an arginine residue (R27a) at position 27a of B3V_\lambda CDR1 (shown in blue) and the dsDNA helix in B3V_{\text{H}}/B3V_\lambda. The lower diagram shows the same region of B3V_{\text{H}}/B3V_\lambda a. The serine residue (S27a) at position 27a of B3V_\lambda a (shown in green) is unable to interact with the DNA molecule.

Modelled by Dr Sylvia Nagl
The computer model predicts that 33.H11V_\lambda is able to create a DNA binding site in combination with B3V_H. B3V_H is shown in light blue whilst 33.H11V_\lambda is shown in dark blue. The V_\lambda residues that differ from those found at the same positions in B3V_\lambda are shown in red. Unlike B3V_\lambda, 33.H11V_\lambda does not have an arginine residue at position 27a in CDR1, it has the germline-derived serine (S27a). However, the presence of an arginine residue at position 92 (R92) in 33.H11V_\lambda CDR3 may compensate for this, since the model predicts that R92 can interact with the backbone of dsDNA.

Figure 1.6  Computer-generated model of B3V_H/33.H11V_\lambda with dsDNA

Modelled by Dr Sylvia Nagl
Figure 1.7  Computer generated model of B3V\(_H\)/UK4V\(_\lambda\)

B3V\(_H\) is shown in light blue whilst UK4V\(_\lambda\) is shown in dark blue. The V\(_\lambda\) residues that differ from those found at the same positions in B3V\(_\lambda\) are shown in red. No complex with DNA could be modelled. The model predicts that UK4V\(_\lambda\) is unable to bind DNA in combination with B3V\(_H\), due to steric hindrance resulting from the introduction of an arginine residue at position 94 (R94) and a serine residue at position 29 (S29) in UK4V\(_\lambda\) by somatic mutation.

Modelled by Dr Sylvia Nagl
These molecular modelling studies therefore concurred with the differences seen in the binding to dsDNA of IgG molecules formed from B3 heavy chain combined with the five different 2a2 derived light chains (Rahman et al. 2001). Work in our laboratory has resulted in the development of a stable expression system, to produce recombinant IgG anti-DNA antibodies in CHO cells, which will allow the further evaluation of the importance of $V_h$ and $V_l$ sequence motifs, on anti-DNA antibody function both in vitro and potentially in vivo. The results reported in chapters four and five of this thesis, further the investigation of the importance of sequence features of B3, 33.H11 and UK4 on their in vitro binding to dsDNA and their function in vivo.

### 1.4.8 Investigation of the pathogenicity of anti-dsDNA antibodies in vivo

There is substantial evidence of the role of anti-DNA antibodies in lupus in both spontaneous lupus-prone mouse models (Table 1.9), and in models of lupus arising from gene targeting of molecules of interest in the pathogenesis of lupus (Table 1.5). However, creation of transgenic mice expressing anti-DNA antibodies allows study of the regulation of anti-DNA antibody expression as well as the pathogenesis of the anti-DNA antibodies. There has also been extensive study of monoclonal anti-DNA antibodies injected or implanted into normal and immunodeficient mice.

#### 1.4.8.1 Anti-DNA transgenic mice

Transgenic mice have been produced, which contain the genes encoding the heavy and light chains of pathogenic murine IgG anti-dsDNA antibodies, thus facilitating longer-term studies of the regulation and pathogenic mechanisms of these particular antibodies, reviewed (Ravirajan and Isenberg 2002). The difficulty in making anti-DNA IgG transgenic mice, in normal strain and also to varying extents in lupus-prone mice, is that once the transgenes are incorporated the expression of the transgenes and the production of high affinity anti-dsDNA antibodies by B cells, is hampered by allelic exclusion, receptor editing and other tolerance mechanisms.

Despite extensive receptor editing in both normal and lupus-prone mice strains, a reduced B cell receptor signalling threshold was found in BWF1 mice as compared to C57BL/6 mice expressing the heavy chain transgene of a murine IgM anti-DNA antibody (Wellmann et al. 2001). Another study in normal and lupus prone mice
expressing the heavy chain transgene of anti-DNA antibody R4A, showed negligible anti-DNA activity in the normal strains but anti-DNA activity was seen in BWF1 mice which utilised a range of light chain genes. In the non-autoimmune mice two populations of B cells were observed, those expressing V\(_k\)1 were anergic and the other non-V\(_k\)1 subset were deleted. The authors suggested that the fate and regulation of these B cells is determined by their light chain’s secondary cross-reactive specificity for an antigen other than dsDNA (Spatz et al. 1997). Subsequently, a third population of dsDNA-binding B cells was identified in these mice, which produced germline encoded antibodies with a much lower affinity for dsDNA than the antibodies made by anergic or deleted B cells. This non-tolerised population escaped regulation and could be recruited to an immune response and undergo somatic mutation to become high-affinity anti-DNA B cells (Bynoe et al. 1999).

Very similar results were obtained in a series of experiments in the laboratory of Dan Eilat. They demonstrated tolerance on a non-autoimmune background, but very high titres of anti-dsDNA antibodies were obtained in transgenic BWF1 mice constructed with \(V_H\) genes derived from murine anti-DNA monoclonal D42 (Friedmann et al. 1999). Once again anergy and receptor editing were seen in normal mice but in lupus-prone mice, anergy was abrogated and although receptor editing was still in place, the edited B cells were not deleted, but these low affinity precursors underwent activation, class-switching and somatic mutation to produce B cells making high affinity anti-DNA antibodies (Yachimovich-Cohen et al. 2003).

Thus anti-DNA transgenic mice have been used to demonstrate that receptor editing plays an important part in tolerance induction, but also in the generation of high affinity anti-DNA B cells in lupus-prone mice. However, a transgenic mouse has been successfully made on a normal background, which exhibited failure of tolerance in terms of allelic exclusion, and allowed development of mild nephritis. These mice were created by inserting transgenes allowing expression of both the H and L chains, of a known pathogenic IgG2a anti-ssDNA and anti-dsDNA monoclonal antibody, derived from a lupus prone mouse (Tsao et al. 1992). These experiments illustrate some of the difficulties experienced in obtaining transgenic mice expressing anti-DNA antibodies, and to date there are no transgenic mice expressing human anti-DNA antibodies.
DNA antibodies. Hence the majority of \textit{in vivo} studies of anti-DNA antibodies, are carried out using monoclonal murine or human anti-DNA antibodies which are inoculated into mice.

\textit{1.4.8.2 Pathogenicity of murine monoclonal anti-DNA antibodies} \textit{in vivo}

Murine monoclonal anti-DNA antibodies have been extensively produced and studied. Vlahakos \textit{et al} studied 24 murine monoclonal anti-DNA antibodies produced from MRL-\textit{lpr}, SNFl or NZW lupus-prone mice. When these antibodies were administered (either intravenously [i.v] or intraperitoneally [i.p]) to normal mice, the pattern of immune deposit formation and resulting nephritis varied depending on specific properties of the individual antibodies. Three general immunofluorescence patterns were observed: extracellular immune deposits with the glomeruli and blood vessels, intranuclear immunoglobulin deposition within cells of the kidney and other organs, or no immune deposits, the occurrence of immune deposits was irrespective of the quantity of circulating anti-dsDNA antibody. It was especially interesting that the deposits seen with four of the anti-DNA antibodies, derived from MRL-mice, showed four distinct disease profiles, and that these four antibodies utilised different \(V_H\) genes and exhibited markedly different pI values (isoelectric point determined by charges of the antibody's amino acid residues) as well as distinguishable antigen binding profiles. In contrast, four monoclonal antibodies derived from SNF1 mice, which were almost identical in sequence and structure produced mesangial and capillary wall deposits that were indistinguishable. This suggested that subsets of pathogenic anti-DNA antibodies with distinct cross-reactive antigen binding properties may form immune deposits at distinct locations within the glomerulus, resulting in different clinical expression of disease (Vlahakos \textit{et al.} 1992b).

Itoh \textit{et al} also showed selective deposition of murine (MRL/\textit{lpr} derived) monoclonal antibodies, in the kidneys of normal mice that had been implanted (i.p) with hybridoma cells. Identical lesions were observed when the hybridomas were implanted into Severe Combined Immunodeficient (SCID) mice, suggesting that the observed glomerular lesions were due entirely to the injected antibodies. Once again variations in the type of deposition were observed, one clone (2B11.3) resulting in cell-proliferative lesions associated with IgG3 and C3 deposits with macrophage
infiltrates and the other clone (7B6.8) resulting in severe subendothelial hyaline deposits of IgG3 resembling wire-loops and inflammatory cell infiltration (Itoh et al. 1993). Interestingly, the clone 2B11.3 was reported not to bind DNA, whilst 7B6.8 bound ssDNA and dsDNA.

In a comparison of three pathogenic and two non-pathogenic BWF1 derived high affinity monoclonal anti-DNA antibodies, Ohnishi et al showed the presence of arginine residues in the Vh CDR2 and CDR3 of the pathogenic antibodies and arginine was absent in the CDRs of the non-pathogenic antibodies (Ohnishi et al. 1994). Both positive and negatively charged amino acids were more frequent in the pathogenic antibodies. These findings fitted with the in vitro data, which showed that 2 of the 3 pathogenic antibodies bound nucleosomes, histones (when antibody complexed with DNA) and heparan sulphate in renal basement membranes (when complexed with DNA/histone).

An interesting study by Mostoslavsky et al (2001), studied the in vivo pathogenicity of murine anti-DNA monoclonal antibodies in immunodeficient Rag-1−/− mice. The pathogenic anti-DNA antibody subset, as defined by glomerular deposition and proteinuria production, and the non-pathogenic antibody subset were indistinguishable in terms of the prevalence of arginine residues in the Vh CDR3 and the pI values within each group were not significantly different, both subsets bound dsDNA equally well with no difference in their affinity constants. However, the two groups of antibodies did differ in their cross reactive binding to α-actinin (as discussed further in section 1.5.3.2), only the pathogenic antibodies binding α-actinin in a western blot of rat kidney glomerular components (Mostoslavsky et al. 2001).

1.4.8.3 Pathogenicity of human monoclonal anti-DNA antibodies in immunodeficient mice

Studies assessing the pathogenicity of human anti-DNA antibodies are limited due to the paucity of human IgG monoclonals and difficulties with their use in most murine strains. The use of immunodeficient mice, which lack an intact immune system, can avoid the problem of rejection of the human hybridoma cells, making them useful for studying the pathogenicity of human dsDNA antibodies.
In severe combined immunodeficient (SCID) mice, a defect in V(D)J recombinase means that antigen receptors (TCR and BCR) are not expressed and the lymphocytes do not mature. 15-25% of young adult SCID mice are 'leaky' in that they produce readily detectable numbers of mature T and B cells, although with a limited repertoire of antigen receptors (Bosma and Carroll 1991). 'Leakiness' increases with age and can easily be detected by assaying serum for murine immunoglobulin (Vladutiu 1993). Another immunodeficient mouse, the \textit{RAG-1} gene knockout mouse (Rag-1\textsuperscript{-/-}), was created by the introduction of germline mutations in embryonic stem cells in 1992 (Mombaerts \textit{et al}. 1992). The recombination activation genes, \textit{RAG-1} and \textit{RAG-2}, mediate V(D)J recombination of Ig and TCR loci. Loss of either the \textit{RAG-1} or the \textit{RAG-2} gene results in the total inability to rearrange V(D)J segments and lymphocyte development is arrested at this early stage. These mice can be described as having a 'non-leaky' SCID phenotype. They have small lymphoid organs which contain absolutely no mature B or T lymphocytes, and no serum IgM.

As with the murine monoclonal antibodies, not all human anti-dsDNA monoclonal antibodies bind to the kidney when implanted into SCID mice and individual antibodies exhibit different localisation of binding and varying degrees of pathogenicity. Investigation of thirteen human IgG anti-DNA monoclonals from the two laboratories of Isenberg and Winkler, showed varying ability of these antibodies to also bind histones, nucleosomes, heparan sulphate and laminin. Five of these hybridomas, B3, D5, 32.B9, 33.C9 and 32.21, were implanted into 9 week old SCID mice (Ehrenstein \textit{et al}. 1995). 33.C9 was the only antibody which bound to the glomeruli, forming extracellular deposits in the capillary wall and mesangium, whilst B3 and 35.21 penetrated cells and bound to the nuclei both in the kidney and in other organs. Although the mice implanted with B3, 35.21 and 33.C9 had proteinuria, they showed no pathological evidence, under light microscopy, of glomerulonephritis. It is notable, that although only three of the antibodies deposited in the kidney, all five of the antibodies showed reactivity to dsDNA by ELISA. It is particularly interesting that the three antibodies which did bind in the kidney, bound strongly to nucleosomes by ELISA. 33.C9 and B3 also bound histones (B3 only bound histones slightly). It is also worth noting that none of the five antibodies bound to heparan sulphate or laminin, although it is hypothesised that histones may bind the glomerulus via charge interactions to such molecules and thus provide a mechanism
for the binding of anti-DNA antibodies which are cross reactive for histones (discussed further in section 1.5).

Previously our group have used SCID mice to show for the first time that deposition of a human IgG anti-dsDNA monoclonal antibody, RH-14, was sufficient to induce renal damage observable by electron microscopy (Ravirajan et al. 1998). Hybridoma cells secreting RH-14 were implanted into the peritoneum of the SCID mice, which subsequently developed proteinuria and immunofluorescence staining showed the presence of human IgG in the kidney. Electron microscopy of kidney sections from RH-14 implanted SCID mice, showed that human IgG was deposited on the glomerular capillary basement membrane and in the mesangial matrix. Electron microscopy also showed changes in the glomerular structures of these SCID mice, thickening of the basement membrane and ‘footpad’ fusion, which resemble the early pathological changes in patients with lupus nephritis. However, light microscopy of the RH-14 implanted kidneys showed no evidence of leukocyte infiltration or fibrotic change. This might have been due to the lack of functional T and B cells in the SCID mice, failure of RH-14 to activate complement or to the short duration (4-5 weeks is usually the maximum prior to ascites formation, at which time the mice must be sacrificed) of antibody exposure.

The experiments described in chapter three of this thesis, were conducted to compare whether implanting RH-14 in older and hence ‘leakier’ SCID mice with a few functional T and B cell clones, would result in pathology observable by light microscopy. For comparison, totally ‘non-leaky’ Rag-1−/− mice were also studied, since these mice had been successfully used in the study of murine anti-DNA monoclonal antibodies (Mostoslavsky et al. 2001).
1.5 Antigenic targets and kidney localisation of anti-dsDNA Antibodies

1.5.1 Deposition of anti-dsDNA antibodies in lupus nephritis

Tissue damage can occur in multiple organs/systems in patients with SLE, and is often initiated by the deposition of immune complexes containing autoantibodies, which trigger inflammation. The type of autoantibodies present in an individual patient, among other factors, may determine which organs are involved. In this thesis I am going to concentrate on the role of anti-dsDNA antibodies in causing lupus nephritis. In lupus nephritis several factors determine the type and severity of the kidney lesions, including the quantity and location of the immune deposits, the systemic response to immune deposition, the local inflammatory and fibrogenic response, and the local autoimmune cellular response. In order to be classed as nephritogenic an autoantibody should form immune deposits in the kidney and directly initiate an inflammatory response (including complement activation, and release of chemo-attractants, proteases and oxygen radicals), or induce proteinuria, or cause changes in the physiological function of kidney cells, or cause cell death, or fibrosis (reviewed Couser 1998)

The different forms of glomerulonephritis, and the roles played by the recruited components of the immune system (monocytes, neutrophils, lymphocytes and platelets) were described in sections 1.1.4 and 1.2, however the kidney cells themselves also play a role in tissue damage and proteinuria production, by release of oxidants, proteases, prostaglandins and various cytokines and growth factors. The glomerulus is a capillary tuft within the kidney mesangium, supported on the glomerular basement membrane (GBM) lined by podocytes (specialised epithelial cells) on the urine-space aspect. On route to the urine, the plasma ultrafiltrate passes out from the blood and sequentially through the fenestrated endothelium, the collagenous network of the GBM, and finally the filtration slits of the slit diaphragm formed by the podocyte foot processes. The structure of the glomerulus with its component cells is shown in Figure 1.8. Deposition of immune deposits, consisting of immunoglobulin with or without cognate antigen and/or complement components can occur at three sites, subepithelial (adjacent to podocyte foot processes), subendothelial or intramembranous (within the GBM). It is not known why some anti-dsDNA antibodies deposit preferentially in the kidney. The nature of the target
Figure 1.8 The structure of the glomerulus in health and disease

Glomeruli are highly specialised structures found within the kidney. Glomeruli are responsible for ultrafiltration of the plasma to ensure that essential proteins are retained in the blood. The normal glomerulus (shown on the right of figure A) is a capillary tuft supported on the glomerular basement membrane (GBM). The GBM is secreted by podocytes (glomerular epithelial cells) that line the urine-space (US) aspect. Podocytes consist of three parts, the cell body, major processes and foot processes. The foot processes interdigitate with the foot processes of neighbouring podocytes to form filtration slits, which are bridged by an extracellular meshwork of proteins known as the slit diaphragm. The plasma ultrafiltrate passes out of the glomerular capillaries which are lined by fenestrated endothelium, through the collagenous network of the GBM and finally through the filtration slits and slit diaphragm.

Figure B shows an electronmicrograph of the normal glomerular ultrastructure. The fenestrations of the glomerular endothelium are indicated by arrows and the filtration slits between the podocyte foot processes are indicated by arrow heads. The presence of actin filaments in the podocyte foot processes is indicated by the white asterisks (*).

In nephritis the foot processes may show effacement and fusion (as shown on the left of figure A). Figure C shows an electronmicrograph of effaced foot processes forming a continuous band of cytoplasm containing a dense band of actin filaments (*) running parallel to the GBM.

Foot process fusion, accompanied by proteinuria, is an early event in glomerular disease. In lupus nephritis one would also expect to see immune deposits, with or without complement components, located either between the GBM and the podocytes (sub-epithelial), within the GBM itself or between the GBM and the endothelial cells (sub-endothelial). In severe forms of proliferative glomerular nephritis one would also find hypercellularity and crescent formation due to the infiltration of mononuclear cells.

Figures modified from Somlo and Mundel (2000).

Abbreviations: MC, mesangial cell; US, urine space.
antigen(s) and the binding interactions involved, remain a matter of conjecture, although a number of hypotheses have emerged, reviewed (Berden et al. 1999; Lefkowith and Gilkeson 1996). These hypotheses are not mutually exclusive, and include simple glomerular deposition of circulating immune complexes, direct cross-reaction binding to glomerular antigens and indirect binding to glomerular antigens, via a nucleosome (DNA-histone) bridge.

1.5.2 Glomerular deposition of circulating immune complexes

This hypothesis was derived when SLE was first recognised as an immune complex disease. It was originally thought that circulating immune complexes of DNA-anti-DNA antibodies became passively trapped in the glomerulus, but the presence of such complexes in human lupus sera is controversial at best. One study found such complexes in only 6% of patients (Izui et al. 1977). It has also been demonstrated in vitro that DNA/anti-DNA complexes bind poorly to the GBM (Izui et al. 1976), and that in mice injected with such complexes, they are rapidly cleared by the liver (Emlen and Mannik 1982). However, it seems likely that some nucleosome/anti-nucleosome antibody complexes may deposit in the glomeruli as discussed in 1.5.4.

1.5.3 Direct binding of cross-reactive antibodies to glomerular antigens

The premise of the cross-reactive antigen hypothesis is that anti-DNA antibodies are broadly reactive, and bind directly to a wide array of molecules such as GBM components. This theory depends on the existence of multiple variable binding sites or recognition of shared epitopes. The observations which led to this hypothesis, were that many murine and human monoclonal anti-dsDNA antibodies appeared to bind directly to several non-nuclear antigens, in enzyme linked immunosorbent assays (ELISAs), western blots of cell lysates, kidney perfusion systems and in vivo following injection (Sabbaga et al. 1990; Madaio et al. 1987; Faaber et al. 1986; Raz et al. 1989). The antigens recognised by these cross-reactive anti-dsDNA antibodies, included cardiolipin and cell surface proteins, such as myosin I and alpha-actinin (alpha-actinin); and also components of the GBM, such as heparan sulphate and laminin. When mice were injected intraperitoneally with mouse (Vlahakos et al. 1992b; Itoh et al. 1993; D’Andrea et al. 1996) or human (Ehrenstein et al. 1995; Ravirajan et al. 1998) monoclonal anti-dsDNA antibodies, different types of glomerular binding were observed, sometimes in conjunction with proteinuria and inflammation. Sera
and affinity purified anti-dsDNA antibodies, from both patients with SLE and lupus-prone mice, also bound directly to some kidney antigens (Faaber et al. 1986). Autoantibodies isolated from the sera of patients with lupus nephritis, but not from SLE patients without nephritis, bound \textit{in vitro} to isolated glomeruli (Budhai et al. 1996), and formed immune deposits after perfusion in the isolated rat kidney model (Raz et al. 1989).

\textbf{1.5.3.1 Cross-reactive binding to components of the glomerular basement membrane}

The GBM is a thin sheet of extracellular matrix, composed of laminins, type IV collagens, heparan sulphate proteoglycans (such as perlecan and agrin) and entactins, which act as a molecular filter, preventing the passage of macromolecules from the blood to the urine. Laminins constitute a family of heterotrimeric glycoproteins, which are composed of three polypeptide chains. At recent meetings, Naparstek \textit{et al} have reported the cross-reactivity of some anti-DNA antibodies with laminin, reviewed Mageed and Zack (2002). Murine and human monoclonal anti-DNA antibodies were shown to bind to the extracellular matrix of mesangial cells in culture, and the binding was localised to a 21-mer peptide (TVS100) located in the globular region of the alpha chain of laminin (Naparstek \textit{et al.} 2003). Serum from lupus-prone mice and patients with SLE, but not healthy controls, also bound to this peptide. Moreover there was a correlation between anti-TV5100 antibody titre and disease activity in patients with SLE. Three of five murine monoclonal anti-DNA antibodies tested (A52, C72 and 3E7), bound strongly to both DNA and the laminin peptide (TV1500), and also caused nephritis when implanted in SCID mice. It is interesting to note that a monoclonal antibody which only recognised TV1500 but not DNA, was also pathogenic in SCID mice. Repeated injection of TV1500 peptide in SCID mice, which had been implanted with cells producing anti-DNA antibody (C72), prevented the development of nephritis, which was seen in mice that received a control peptide. In MRL-\textit{lpr} mice treated with TV1500 peptide, survival was increased and only mild focal changes could be seen in the kidneys, equivalent to histology observed after treatment with beta-methasone (Naparstek \textit{et al.} 2003). Finally Naparstek \textit{et al} reported immuno-absorption of patients’ sera on a TV1500 peptide affinity column. However, although the reactivity to TV5100 peptide was reduced by 60%, intriguingly the anti-DNA activity was unchanged. Sabbaga \textit{et al}, also demonstrated specific binding of a nephritogenic murine monoclonal anti-DNA
antibody to laminin (Sabbaga et al. 1990). Other workers report a role for antibodies which bind to laminin in experimental lupus nephritis (chronic graft-versus-host disease), but did not investigate cross-reactivity of these antibodies with DNA (Peutz-Kootstra et al. 2000).

1.5.3.2 Cross-reactive binding to alpha-actinin

A recently proposed candidate antigen for cross-reactive binding by anti-DNA antibodies, is the 100 KD actin-binding and cross-linking protein, alpha-actinin (α-actinin). There are four known isoforms of human α-actinin genes, which are highly homologous and highly conserved among species (Kaplan et al. 2000). The expression of ACTN2 and ACTN3 is limited to skeletal and cardiac muscle (Beggs et al. 1992), whereas ACTN1 and ACTN4 encode the widespread non-muscle forms of actinin (Honda et al. 1998). Alpha-actinin-4 (but not α-actinin-1) is present in human kidney tissue lysates and immunofluorescent staining localised the α-actinin-4 to the podocytes (glomerular epithelial cells), to some blood vessels (Kaplan et al. 2000), and also mesangial cells (Drenckhahn and Franke 1988). Within the podocyte, which is comprised of a cell body, major processes and foot processes, α-actinin is predominantly localised to the foot processes (Kaplan et al. 2000).

Alpha-actinin-4 is an integral part of the contractile system within the podocyte foot processes (Figure 1.9), linking the actin cytoskeleton with components of the slit diaphragm (Khoshnoodi and Tryggvason 2001; Somlo and Mundel 2000). Disruption of the morphology of the actin cytoskeleton may cause foot process effacement and fusion (Figure 1.8), which results in proteinuria, as seen in nephrotic syndrome. Indeed, mutations in ACTN4 encoding defective α-actinin-4 cause an autosomal dominant form of focal segmental glomerulosclerosis (FSGS). The mutant α-actinin-4 was shown to bind actin more strongly than the wild-type α-actinin-4 in vitro (Kaplan et al. 2000). A recently developed mouse model of human ACTN4-associated FSGS suggested a relationship between actin cytoskeleton dysregulation by mutant α-actinin-4, and the deterioration of the nephrin-supported slit diaphragm (Michaud et al. 2003). The upregulated expression of α-actinin in podocytes was demonstrated to precede foot process effacement and proteinuria in an experimental nephrotic syndrome induced by puromycin aminonucleoside in rats.
Figure 1.9  Schematic diagram of the location of α-actinin-4 in the cytoskeleton of the podocyte foot process

The podocyte foot processes contain a contractile system composed of actin, myosin-II, α-actinin-4, talin, vinculin and synaptopodin. The cytoskeleton is connected to the GBM via α3β1 integrin and linkage to the slit diaphragm components, nephrin and P-cadherin, may be mediated by CD2-associated protein (CD2AP) or by a complex of ZO-1, α-, β-, and γ-catenin. Disruption of one of these cytoskeleton signalling pathways, may lead to reorganisation of the actin cytoskeleton and foot process effacement, and/or alter the integrity of the slit diaphragm resulting in leakage of protein into the urinary space (proteinuria). Adapted from Somlo and Mundel (2000).
(Smoyer et al. 1997), although another group found expression of α-actinin-4 was unchanged in this model (Luimula et al. 2002). Increased expression of α-actinin associated with cytoskeletal changes and foot process effacement, were also seen in experimental Masugi nephritis (Shirato et al. 1996). The essential role played by α-actinin-4 in cell movement and normal podocyte function, has been also been demonstrated by the development of severe glomerular disease in homozygous α-actinin-4 knock-out mice (Kos et al. 2003). Based on these studies, and studies of other podocyte proteins, it seems likely that the podocyte slit-diaphragm is as important as the GBM as a protein restrictive filtration barrier, reviewed (Tryggvason and Wartiovaara 2001; Mathieson 2003). It is therefore logical that the proteinuria seen in lupus nephritis might also result from disruption of the cytoskeletal proteins and slit-diaphragm integrity.

Two groups have reported that pathogenic (as defined by glomerular deposition and proteinuria production), but not non-pathogenic murine anti-DNA monoclonals bind directly to α-actinin (Mostoslavsky et al. 2001; Deocharan et al. 2002). Both groups confirmed the identity of the 100 KD molecule, as α-actinin, by matrix associated laser desorption ionisation (MALDI). Mostoslavsky et al report direct binding to α-actinin by several pathogenic murine anti-DNA antibodies (described in section 1.4.8.2), as opposed to no cross-reactivity with α-actinin by their non-pathogenic counterparts (Mostoslavsky et al. 2001). Mostoslavsky et al suggest that α-actinin is a potential structural mimic of the DNA backbone (Mostoslavsky et al. 2001). The crystal structure of α-actinin has recently been confirmed as a twisted anti-parallel dimer that contains a negatively charged acidic surface (Ylanne et al. 2001).

Although α-actinin is thought to be an intracellular protein, there is evidence showing that α-actinin is also expressed on the cell surface, in the plasma membrane. A membrane associated 100KD protein, identified as non-muscle α-actinin, was shown to cross-react with a monoclonal antibody which was specific for the human immunodeficiency virus type 1 (HIV-1) transmembrane protein gp41 (Spehar and Strand 1995). Focal adhesions are an elaborate network of interconnecting proteins linking actin stress fibres to the extracellular matrix substrate, α-actinin-1 but not α-
actinin-4, has been shown to be present in focal adhesion plaques, however this work was carried out in a breast cancer cell line (Honda et al. 1998). Deocharan et al, showed by flow-cytometry and immunofluorescence, that R4A binds to the surface of both live mesangial cells derived from MRL/lpr mice and to a kidney podocyte cell line (Deocharan et al. 2002). This was confirmed using murine anti-chicken α-actinin serum, to stain the surface of murine mesangial cells (Mostoslavsky et al. 2001). Both of these groups (Mostoslavsky et al. 2001; Deocharan et al. 2002), also showed (by western blotting) that pathogenic murine anti-dsDNA monoclonal antibodies only bound to the membrane and membrane cytoskeletal fractions of glomerular cell lysates.

Deocharan et al demonstrated that pathogenic murine anti-DNA antibody R4A (but not a non-pathogenic mutant of R4A), binds directly to α-actinin in lysates of MRL-lpr kidney mesangial cells (Deocharan et al. 2002). This α-actinin binding was greatly diminished in BALB/c mesangial cell lysates, suggesting that availability or expression of α-actinin may be a factor determining susceptibility to lupus nephritis. The sera from lupus prone MRL-lpr and BWF1 mice, but not BALB/c mice, were shown to bind to commercial α-actinin and glomerular cell extracts (Mostoslavsky et al. 2001; Deocharan et al. 2002). Several reports suggest that the expression or distribution of α-actinin may be modulated by cytokines such as TGFβ, fibroblast growth factor-1 and insulin-like growth factor (Hsu et al. 1996; Brooks et al. 1997). This is especially interesting because the level of mRNA for all of these cytokines increases with age in murine lupus nephritis (Nakamura et al. 1993). These findings are consistent with the report that α-actinin was readily detectable in a 7 month old BWF1 mouse with nephritis but not in a pre-nephritic 3 month old mouse (Mostoslavsky et al. 2001). The α-actinin staining was co-localised with deposited murine IgG, in the glomerular capillary loops of nephrotic lupus prone BWF1 mice, and in Rag-1^-^-^-^- mice that had been inoculated with hybridoma cells producing a pathogenic murine anti-dsDNA antibody (Mostoslavsky et al. 2001).

Deocharan et al also reported preliminary studies (no data was shown), claiming that R4A binds to α-actinin in human mesangial cells and that sera from active lupus patients have high titres of anti-α-actinin antibodies (Deocharan et al. 2002). A
meeting abstract, reported three IgM human monoclonal anti-dsDNA antibodies binding 100 KD \( \alpha \)-actinin by ELISA and in a western blot of human mesangial cell lysates (Marambio et al. 2002). This binding was inhibited by pre-incubation with \( \alpha \)-actinin, and the binding was unaffected by treatment of the cell lysates or antibodies with DNase I, indicating direct cross-reactive binding. Thus there is sufficient evidence to suggest that \( \alpha \)-actinin may be a target for murine pathogenic anti-DNA antibodies. The experiments reported in Chapter Six of this thesis, seek to investigate whether human anti-DNA antibodies also bind to \( \alpha \)-actinin.

**1.5.3.3 Is the binding of anti-DNA antibodies cross-reactive or direct?**

Several authors have claimed binding of monoclonal anti-DNA antibodies to glomerular constituents, is cross-reactive binding rather than binding via a 'DNA bridge', since binding is unaffected by DNase I treatment of the target cells (Deocharan et al. 2002; Madaio et al. 1987; Mostoslavsky et al. 2001; Raz et al. 1989). Also binding is often increased by DNase I treatment of the antibody preparations, and absorption with DNA partially or completely inhibits antibody binding (Raz et al. 1993). In addition, glomerular binding of the pathogenic murine monoclonal anti-dsDNA antibody (R4A), was inhibited by a peptide mimotope of DNA (DWEYS), which was injected along side R4A in normal mice (Gaynor et al. 1997).

However, purification of antibodies by protein A or G affinity chromatography or DNase I treatment, is not sufficient to dissociate all complexes (Kramers et al. 1994). Nucleosomal material is present in the supernatant of apoptotic hybridoma cells during culture, potentially resulting in complexes of nucleosomes with the secreted autoantibodies. In addition, when purified antibodies are injected into the peritoneum of mice, it is likely that such complexes will form.

In conclusion it has not been conclusively proved that direct binding of anti-dsDNA to constitutive glomerular antigens, in the complete absence of nucleosomes, actually takes place. Due to the high affinity specific autoantibodies produced by antigen-driven maturation, it is thought that broad polyreactivity of antibodies may be unlikely to occur, although for cross-reactive binding to occur the antibody only
needs to be bireactive. An alternative suggestion is that anti-dsDNA antibodies only bind to DNA, and subsequently bind in the glomerulus by electrostatic interactions of the bound DNA, or DNA containing complexes, with glomerular antigens.

1.5.4 Nucleosome-mediated binding to components of the glomerular basement membrane

Early research suggested that anti-dsDNA antibodies bound to DNA that was deposited in the kidney (Izui et al. 1976). However, intravenously administered DNA does not generally bind to GBM components or deposit in the glomeruli unless histones are pre-deposited (although DNA of low molecular size, may lodge in the GBM). Subsequently, it was suggested that circulating non-complexed cationic anti-DNA antibodies, bound to in-situ nuclear antigens such as DNA, histones or nucleosomes. In support of this theory, histones were shown to bind well to GBM components (Schmiedeke et al. 1989), enabling GBM deposition of DNA in the form of nucleosomes. This theory was referred to as the 'planted antigen' hypothesis (Lefkowith and Gilkeson 1996), but other studies suggested that direct binding of pre-formed complexes of anti-DNA (or anti-nucleosome) antibodies and nucleosomes (complexes of DNA and histones), to GBM components is more likely, reviewed (Berden et al. 1999). Indeed, intact nucleosomes were demonstrated to bind to glomeruli (Coritsidis et al. 1995), both in vivo and in vitro, so which components were the nucleosomes binding to?

Anti-dsDNA antibodies were shown to bind heparan sulphate (HS [the anionic polysaccharide side chain of HS proteoglycans]) in ELISA. Initially this was believed to be cross-reactive binding of anti-DNA to HS, but high salt/DNase I treatment abrogated binding, leading to the hypothesis that binding was nucleosome mediated (Berden 1997). This finding was reinforced, and the theory of cross-reactivity challenged, by renal perfusion studies in which highly purified non-complexed anti-DNA antibodies did not bind to the GBM (Kramers et al. 1994). This study showed that protein A affinity purification, even under high-salt conditions, was not sufficient to remove all complexed material from anti-dsDNA antibodies without prior treatment with DNase I. It was further elucidated that binding of these anti-DNA monoclonal antibodies to the GBM, was due to the cationic histone part of the nucleosome binding to anionic HS. Binding of the anti-
DNA antibodies in the renal perfusion system, was inhibited by the removal of HS from the GBM (Kramers et al. 1994), the use of anti-histone antibodies to mask the cationic regions (van Bruggen et al. 1997b), and binding of heparan to the cationic regions of the nucleosome (van Bruggen et al. 1996).

It is notable that for binding to occur in the kidney perfusion system, pre-formed complexes of nucleosomes and anti-DNA antibodies seem essential, since no binding was observed when free nucleosomes were perfused prior to perfusion of the antibodies. This would argue against formation of the complexes in-situ in the kidney, as suggested by the 'planted-antigen' hypothesis (Lefkowith and Gilkeson 1996), although once the immune complex is deposited, further autoantibodies may then bind to it in-situ, thus amplifying the ensuing inflammation. Staining of kidney biopsies from patients with lupus nephritis, demonstrated glomerular deposits containing histones and nucleosomes (van Bruggen et al. 1997a). A reduction in the staining of HS was found in the GBM of both human and murine nephritis, possibly caused by a masking of HS by nucleosome-containing immune complexes and this correlated with proteinuria (Berden et al. 1999). Nucleosome-mediated binding of autoantibodies was also demonstrated to the epidermal basement membrane in the skin of patients with lupus nephritis, although binding via HS could not be proven (Grootscholten et al. 2003).

Removal of HS from the GBM in the perfused kidney system, using heparitinase, reduced but did not completely abrogate anti-DNA antibody binding, suggesting that HS was not the only GBM ligand for nucleosome-mediated IgG deposition (Kramers et al. 1994). The major GBM component, collagen type IV, has also been identified as a molecule that nucleosomes can bind to with high affinity (Di Valerio et al. 1995) reviewed (Lefkowith and Gilkeson 1996). Antibodies from murine lupus, recognise the nucleosome complex bound to the helical domain of collagen type IV (meeting abstract (Bernstein and Lefkowith 1995)). Subsequent experiments, using MRL-\textit{lpr} serum showed that collagenase treatment of the GBM, prevented binding of the nucleosome/IgG complexes to isolated glomeruli, suggesting that collagen type IV was a ligand for this binding mechanism (Bernstein et al. 1995). Treatment of the GBM with heparitinase, did not inhibit binding of the MRL-\textit{lpr} serum (Bernstein et al. 1995). However, an earlier paper showed the methodology used to isolate the
glomeruli would have resulted in almost complete depletion of HS from the GBM (Termaat et al. 1992).

A large study (63 patients with lupus nephritis, 15 active non-renal lupus and 33 inactive SLE), used an ELISA coated with extracts of human GBM, to confirm the clinical significance of glomerular-binding antibodies in patients with SLE (Lefkowith et al. 1996). This study found that most patients with active nephritis had glomerular binding activity (GBA), as did some of the active non-renal patients, although at lower levels, patients with inactive lupus did not exhibit GBA. High GBA, correlated with increasing severity of nephritis in patients in this study, although the renal histopathology was the same as those patients with lower GBA. In addition, GBA significantly decreased in patients who had responded to therapy. Thus is appears that nucleosome (or chromatin) mediated binding of anti-DNA (and anti-nucleosome) antibodies to HS and collagen IV is an established mechanism for IgG deposition in the kidney. It is likely that other GBM components are also targets.

However, if binding is always mediated by binding to nucleosomes, why are different patterns of deposition observed when different murine and human monoclonal anti-dsDNA antibodies are injected into mice (Vlahakos et al. 1992b; Itoh et al. 1993; Ehrenstein et al. 1995; Ravirajan et al. 1998)? Therefore, it seems likely that both cross-reactive binding and nucleosome-mediated binding of anti-DNA antibodies, may contribute to the pathogenicity of lupus nephritis, since these hypotheses are not mutually exclusive.

1.5.5 Cellular penetration by anti-DNA antibodies
There are a number of in vivo studies suggesting that anti-DNA, anti-U1 RNP and anti-ribosomal P antibodies can penetrate living cells and potentially induce renal disease by intracellular effects, such as inducing loss of tolerance to self by modification of apoptotic events, reviewed Cabral and Alarcon-Segovia (1997). When injected in normal mice, a subset of murine anti-DNA antibodies, localised in the nuclei of cells in multiple organs, including the kidney, and caused glomerular hypercellularity, podocyte foot process fusion and proteinuria (Vlahakos et al. 1992a). It was subsequently shown in vitro, that these anti-DNA antibodies traverse
the cytoplasm and enter the nucleus in a time and temperature dependent manner (Yanase et al. 1994); this was later demonstrated to be mediated by cross-reactive binding of the antibodies to myosin 1 on the cell surface (Yanase et al. 1997). Other researchers suggest that antibody penetration depends on binding to an extracellular matrix protein in the presence of DNA or a membrane determinant precisely resembling DNA (Zack et al. 1996). Human anti-DNA monoclonal antibody B3, has been shown to penetrate glomerular cells and bind to the nucleus, when implanted in SCID mice (Ehrenstein et al. 1995). It thus appears that a subset of cross-reactive anti-DNA antibodies, might exert their pathogenic effect by cellular penetration and nuclear localization, reviewed (Madaio and Yanase 1998).
1.6 HYPOTHESES AND AIMS OF THIS THESIS

1.6.1 Hypotheses

The previous implantation of RH-14 into two month old SCID mice, resulted in proteinuria and pathogenic changes in the glomeruli of these mice (Ravirajan et al. 1998). However, the morphological changes in the glomeruli of these mice, which resembled the early changes seen in patients with lupus nephritis, were only observable by electron microscopy. It could be hypothesised that the lack of functional T and B cells in these SCID mice might limit the development of pathology in these mice. On the other hand, the presence of even a few functional lymphocyte clones, as found in 15-25% of two month old SCID mice, might interfere with the observation of pathology.

Cumulative evidence from the published literature discussed in this Introduction suggests that the antibodies, which are pathogenic in patients with lupus nephritis, are those with high affinity for dsDNA or dsDNA containing complexes (nucleosomes). Sequence analysis of these antibodies suggests the antigen driven accumulation of somatic mutations in the CDRs of both heavy and light chain variable regions. These mutations appear to result in an increase in certain amino acid residues, especially positively charged arginine residues, in the antigen contact sites of the antibody binding site. Work both by others and also in our laboratory, has suggested that these arginine residues may be important in the binding to dsDNA and for the pathogenicity of both murine and human anti-dsDNA monoclonal antibodies.

It is not known why some anti-dsDNA antibodies deposit preferentially in the kidney. The nature of the target antigens and the binding interactions involved, remain a matter of conjecture. It has been suggested that some anti-dsDNA antibodies do not bind directly to DNA deposited in the kidney, but are cross-reactive and bind directly to kidney antigens. Previous studies have suggested that pathogenic murine anti-dsDNA antibodies bind directly to α-actinin present on kidney podocytes and mesangial cells (Mostoslavsky et al. 2001; Deocharan et al. 2002).
1.6.2 Aims of this thesis

1. The aim of chapter three was to compare the pathogenic effect of implanting human hybridomas, secreting anti-dsDNA monoclonal antibodies, in (a) 'leaky' eight month old SCID mice, (b) relatively non-leaky 2 month old SCID mice, and (c) non-leaky Rag-1\(^{-/}\) mice.

2. The aim of chapters four and five was to investigate further the \textit{in vitro} and \textit{in vivo} functional effects, of alterations to the amino acid sequence of human anti-dsDNA monoclonal antibody B3. Amongst the relatively small number of human anti-dsDNA monoclonal antibodies, B3 has already been well characterised and was derived from a patient with active SLE.

3. The aim of chapter six was to investigate whether \(\alpha\)-actinin is also a target for the binding of pathogenic human anti-dsDNA antibodies.
CHAPTER TWO

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MATERIALS AND METHODS

2.1 ELISA protocols
These are the protocols for standard ELISAs used throughout the work for this thesis. The ELISAs measuring murine immunoglobulins (for experiments in Chapter three) are described in section 2.3.4. The specialised ELISA measuring immunoglobulin binding to α-actinin (for experiments in Chapter six) is described in section 2.6.2.

2.1.1 Buffers
All chemicals used in the buffers are from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.1.1.1 Phosphate Buffered Saline (PBS), pH 7.4
Sodium chloride (NaCl) 0.14 M, disodium hydrogen phosphate (Na₂HPO₄) 0.008 M and potassium dihydrogen phosphate (KH₂PO₄) 0.0015 M freshly made up in distilled water. Alternatively, for small volumes PBS tablets (Sigma P-4417, 0.01 M phosphate buffer, 0.0027 M potassium chloride [KCl] and 0.137 M NaCl) were used, 1 tablet was dissolved in 200ml of distilled water.

2.1.1.2 Phosphate Buffered Saline containing tween (PBS/tween)
PBS/tween contains 0.05% polyoxyethylene sorbitan monolaurate (tween 20) (Sigma P-1379).

2.1.1.3 Tris Buffered Saline (TBS), pH 7.6
TBS tablets (Sigma T-5030, 50 mM tris-HCl and 150 mM NaCl) were used to provide fresh buffer as required, 1 tablet was dissolved in 15ml of distilled water.

2.1.1.4 Tris Buffered Saline containing tween (TBS/tween)
TBS/tween contains 0.05% polyoxyethylene sorbitan monolaurate (tween 20).
2.1.1.5 Bicarbonate Buffer (BIC), pH 9.6
Sodium bicarbonate (NaHCO₃) 35mM (1.6g/L), disodium carbonate (Na₂CO₃) 15mM (2.9g/L) made up to 1.0L with distilled water.

2.1.2 ELISA measuring human IgG
Maxisorp 96 well ELISA plates (Nunc) were coated on one side with 100µl/well of 2.5µg/ml of γ-chain specific goat anti-human IgG (Sigma 1-9885) diluted in BIC buffer pH 9.6 and ‘sham’ coated on the other side with BIC buffer. The plates were incubated overnight at +4°C and then any unbound antigen was removed by washing 3 times with PBS. Non-specific binding was reduced by blocking with 200µl/well of 2% BSA (Sigma) dissolved in PBS/tween, for 1 hour at 37°C. The blocking buffer was tipped out and the plates blotted by banging upside down on tissue. 100µl/well of the samples and positive control were added diluted in PBS/tween, in duplicate wells, to both the anti-human IgG and ‘sham’ coated sides of the plate. The positive control / standard curve was prepared by fresh dilution of an aliquot of human IgG (Sigma I-2511, prepared from human sera, stock was 5.3mg/ml, which was aliquoted and stored at -20°C), the standard curve started at 1µg/ml. As a negative control, 100µl/well of the sample diluent (usually PBS/tween), was also added to duplicate wells on the anti-human IgG and ‘sham’ coated sides of the plate. The samples were incubated at 37°C for 1 hour. The plates were washed 5 times with TBS/tween. Any bound antibodies were detected using 50µl/well of gamma chain specific goat anti-human IgG conjugated to alkaline phosphatase (Sigma A-3150), which was diluted 1:1000 in TBS/tween and incubated for 1 hour at 37°C. The plates were thoroughly washed 5 times with TBS/tween and once with BIC buffer. The plates were then developed using alkaline phosphatase substrate, p-nitrophenol phosphate (Sigma 104-105, 1 tablet dissolved in 5ml of BIC buffer). The plates were read at 405nm using a reference filter of 490nm. When calculating the final optical density (OD) values, first the OD values of the diluent alone were deducted from the other OD values on the anti-human IgG coated and ‘sham’ coated sides. Then for each positive and sample, the duplicate OD values obtained on the ‘sham’ coated side were deducted from those obtained on the anti-human IgG coated side. The concentration of human IgG in the samples was calculated by reference to the linear portion of the standard curve of human IgG.
2.1.3 ELISA measuring human IgM

This ELISA was carried out using the same protocol as that described in section 2.1.2, except the coating antibody was goat anti-human IgM (Sigma I-0759) used at 5μg/ml and the detection antibody was goat anti-human IgM conjugated to alkaline phosphatase (Sigma A-3275) used at 1/1000 dilution. The positive control was 3-5, a human anti-dsDNA IgM monoclonal antibody (mAb), which was supplied as a supernatant containing 16μg/ml of human IgM (a kind gift from Anne Davidson, Albert Einstein College of Medicine, USA). Since this was not a calibrated control, I used the OD values of samples (which fell within the linear part of the 3-5 mAb standard curve) from this assay rather than converting them to μg/ml based on this standard. This assay was only used for the results reported in section 6.3.3.

2.1.4 Optimisation and standardisation of the Anti-DNA ELISA

2.1.4.1 Purification of dsDNA from calf thymus DNA

Since antibodies which bind DNA, are renowned for their poly-reactive binding, it was important to use dsDNA which was as pure as possible, to coat the ELISA plates. The calf-thymus DNA purchased from Sigma (D-1501) contains approximately 3% contaminating protein. In order to remove the protein impurities from the calf thymus DNA, the DNA was phenol chloroform extracted. I used dsDNA, which Arti Sharma a previous PhD student in our laboratory had prepared (and tested in anti-DNA ELISAs) according to the following method: 30mg of calf thymus DNA (Sigma D-1501) was dissolved overnight on a rotor at 4°C in 50ml tris-EDTA (TE) buffer (10mM tris, 1mM EDTA, pH 7.8). The dissolved DNA was mixed with an equal volume of phenol:sevac (25ml phenol: 24ml chloroform: 1ml isoamyl alcohol), and vortexed for 1 minute before centrifugation at 1000rpm for 5 minutes. The top layer containing the DNA was carefully removed, leaving both the interface and the bottom layer which contained protein contaminants. The DNA was precipitated by the addition of 1/10th of the volume of 3M sodium acetate and then an equal volume of ice-cold 100% ethanol. This solution was mixed gently to precipitate the DNA and then centrifuged at 1500 rpm for 10 minutes. The DNA pellet was washed/desalted with 10ml of ice-cold 100% ethanol and respun at 1500 for 10 minutes, this step was repeated. The DNA pellet was air dried for 30 minutes before being re-dissolved overnight in TE buffer. In order to increase the
reproducibility of the assay, by having DNA strands of similar sizes, the DNA was sonicated on ice at high frequency for 2 minutes, in 30 second bursts. Any single-stranded DNA fragments were removed by passing through a Millex-HA 0.45µm filter, (S1-nuclease can be used to remove single-stranded ends, but this was not done in this case). The DNA purity and concentration were measured using a spectrophotometer to measure the ratio of absorbance at 260:280nm. (as described in section 2.5.11) The dsDNA prepared by Arti Sharma was 500µg/ml and stored in small aliquots at −80°C. I compared the purified dsDNA with the ‘crude’ calf thymus DNA direct from Sigma as coating antigens in the anti-DNA ELISA. I found only a slight difference between the binding of my positive control antibody (anti-dsDNA human IgG monoclonal antibody, RH-14, described in section 2.2) to the two different DNAs (Figure 2.1). I suspect that the ‘crude’ calf thymus DNA was of sufficient purity for use in the assay after all. Based on this optimisation it should have been perfectly acceptable to use either DNA in the ELISA, but since I had a plentiful supply of the purified dsDNA, I used this one batch of DNA throughout all the work described in this thesis. Based on the optimisation assay shown in Figure 2.1, subsequent ELISAs were carried out using plates coated with 10µg/ml of purified dsDNA.

2.1.4.2 Protocol for ELISA measuring human immunoglobulin binding to DNA

Maxisorp 96 well ELISA plates¹ (Nunc, Roskilde, Denmark) were coated on one half (48 wells) with 50µl/well of 10µg/ml purified dsDNA (prepared as described in section 2.1.4.1) diluted in PBS and ‘sham’ coated on the other half with PBS. The plates were incubated overnight at +4°C and then any unbound antigen was removed by washing 3 times with PBS. Non-specific binding was reduced by blocking with 150µl/well of 2% casein (BDH, Poole, U.K) dissolved in PBS/tween, for 1 hour at 37°C. The blocking buffer was tipped out and the plates blotted by banging upside down on tissue. 50µl/well of the samples and positive control were added diluted in PBS/tween, in duplicate wells, to both the dsDNA and ‘sham’ coated sides of the

¹ I also tested a high binding plate (Costar) and ‘EIA II’ microplate (ICN/Flow laboratories) but dsDNA did not bind as well to these plates. I also tried coating the plates with methylated BSA or poly-L-lysine prior to coating with dsDNA but these both lead to an increased non-specific background binding.
Figure 2.1: Comparison of ‘purified’ and ‘crude’ DNA in the anti-dsDNA solid-phase ELISA

The ‘crude’ DNA as supplied by Sigma (D-1501) is actually pretty pure, containing approximately 3% protein. The ‘pure’ DNA is dsDNA after phenol/chloroform extraction, sonication and removal of ssDNA as described in section 2.1.2.1. This figure shows that there is little difference between the binding of positive control RH-14 to the ‘crude’ or ‘pure’ DNAs, coated at 4 different concentrations diluted in PBS. Also shown is the baseline binding of RH-14 to wells ‘sham’ coated with PBS.
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plate. The positive control\(^1\) was RH-14, an anti-dsDNA human IgG monoclonal antibody (described in section 2.2), which was titrated on every plate from a 1:100 dilution of the concentrated supernatant. As a negative control, 50μl/well of the sample diluent usually PBS/tween, was also added to duplicate wells on the dsDNA and 'sham' coated sides of the plate. The samples were incubated at 37°C for 1 hour. The plates were washed 5 times with TBS/tween. Any bound antibodies were detected using 50μl/well of gamma chain specific goat anti-human IgG conjugated to alkaline phosphatase (Sigma A-3150), which was diluted 1:1000 in TBS/tween and incubated for 1 hour at 37°C. The plates were thoroughly washed 5 times with TBS/tween and once with BIC buffer. The plates were then developed using alkaline phosphatase substrate, p-nitrophenol phosphate (Sigma 104-105, 1 tablet dissolved in 5ml of BIC buffer). The plates were read at 405nm using a reference filter of 490nm. When calculating the final OD values of the samples, the OD values of the diluent alone were first deducted from the other OD values on the dsDNA coated and 'sham' coated sides. Then for each sample, the duplicate OD values obtained on the 'sham' coated side were deducted from those obtained on the dsDNA coated side.

2.1.4.3. Standardisation of positive control using WHO reference serum

RH-14 was calibrated in terms of international units (IU) by comparison with the WHO reference serum Wo/80 (Central laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). Wo/80 was obtained from a patient with SLE and validated as an international standard for antibodies to dsDNA (Feltkamp et al. 1988). Wo/80 was supplied as a freeze-dried vial of serum, this was reconstituted with 500μl of sterile distilled water to give a solution which contained 200 IU/ml of anti-dsDNA antibodies. RH-14 was calibrated by titrating in triplicate on 4 different plates, coated with purified dsDNA, on different occasions alongside a titration of Wo/80. The concentration of RH-14 was calculated by reference to the linear portion of the Wo/80 curve. Using this method the concentrated positive control batch of RH-14 was calibrated to contain 715 IU/ml of anti-dsDNA antibodies.

\(^1\) The concentration of the positive control batch of RH-14 was measured at 50μg/ml using the human IgG ELISA, and was calibrated against the WHO reference serum as described in section 2.1.4.3. The positive control was used to check the assay was working and to standardise variation between plates, but not to quantify the results. The OD values obtained in the anti-dsDNA assay were interpreted with reference to the concentration of human IgG of the samples, measured using assay described in section 2.1.2, with reference to a standard curve of human IgG.
2.2 Human monoclonal antibodies.

The properties of the human anti-DNA and anti-phospholipid monoclonal antibodies used throughout the experiments described in this thesis are summarised in Tables 2.1 and 2.2. RH-14 (Ravirajan et al. 1998), B3 (Ehrenstein et al. 1993), DIL-6 (Ravirajan et al. 1998), 33.H11 (Winkler et al. 1991) and UK4 (Menon et al. 1997) are all human IgG monoclonal antibodies derived by the fusion of peripheral blood lymphocytes (PBL) from patients who had active SLE with cells of the mouse heteromyeloma line CB-F7.

RH-14 was derived from a female patient with a 3 year history of active SLE, which was clinically manifest as arthritis and glomerulonephritis. The patient’s renal biopsy showed segmental proliferative glomerulonephritis with crescents, she had high levels of serum anti-dsDNA IgG and rheumatoid factor (Ravirajan et al. 1998).

B3 was derived from a patient with active SLE (Ehrenstein et al. 1993), her disease was most evident in the musculoskeletal, cardiovascular/respiratory and haematological systems. DIL-6 was derived from a female patient with a 12 year history of active SLE, manifest as arthritis, renal disease (renal biopsy showed diffuse proliferative lupus nephritis), rash and cerebral disease. The patient had a high level of serum IgG, anti-Ro and anti-RNP (Ravirajan et al. 1998).

33.H11 was derived from a patient with active SLE who presented with severe nephritis with proteinuria. The patient had high serum anti-dsDNA antibody levels, low C4 and an increased blood sedimentation rate (Winkler et al. 1991). 33.H11 was a kind gift from Dr Thomas Winkler (Erlangen, Germany).

UK4 was derived from a 49 year old female with active SLE, her clinical symptoms included glomerulonephritis, arthritis, serositis, migranes and she had had an intracerebral haemorrhage. The patient’s serum was positive for anti-dsDNA, rheumatoid factor, IgG anti-cardiolipin and her ANA titre was 1:640 (Menon et al. 1997). The two anti-phospholipid mAbs, CL24 and IS4 (Zhu et al. 1999) were produced by Epstein-Barr virus transformation of peripheral blood mononuclear cells (PBMC) from patients with antiphospholipid syndrome (APS). CL24 (Zhu et al. 1999) was derived from a 17 year old male patient with a 5 year history of secondary antiphospholipid syndrome (APS). He had symptoms suggestive of SLE, fulfilling 3 of the ACR criteria for SLE (Hochberg 1997). The patient from whom IS4 was derived, was a 19 year old female with primary APS of 3 years duration. She had deep venous thrombosis, high titres of
### Table 2.1: Summary of the Properties of Human IgG Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>IgG Subclass</th>
<th>Light Chain</th>
<th>Patient</th>
<th>Known Binding Specificities</th>
<th>ANA</th>
<th>Pathogenicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH-14</td>
<td>IgG1</td>
<td>λ</td>
<td>SLE patient</td>
<td>dsDNA: √&lt;br&gt;ssDNA: √&lt;br&gt;H: √&lt;br&gt;N: x&lt;br&gt;HS: x&lt;br&gt;CL: h</td>
<td>When implanted in SCID mice, causes GN and proteinuria (3-4+)</td>
<td>Ravirajan et al. 1998</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>IgG1</td>
<td>λ</td>
<td>SLE patient</td>
<td>dsDNA: √&lt;br&gt;ssDNA: √&lt;br&gt;H: x&lt;br&gt;N: x&lt;br&gt;HS: x&lt;br&gt;CL: s</td>
<td>When implanted or injected i.v in SCID mice, binds nuclear structures in kidney and other organs, no GN but proteinuria (2-3+).</td>
<td>Ehrenstein et al. 1993&lt;br&gt;Ehrenstein et al. 1995</td>
<td></td>
</tr>
<tr>
<td>DIL-6</td>
<td>IgG3</td>
<td>λ</td>
<td>SLE patient</td>
<td>dsDNA: √&lt;br&gt;ssDNA: x&lt;br&gt;H: x&lt;br&gt;N: x&lt;br&gt;HS: x&lt;br&gt;CL: h</td>
<td>When implanted in SCID mice, no deposition or proteinuria.</td>
<td>Ravirajan et al. 1998</td>
<td></td>
</tr>
<tr>
<td>33.H11</td>
<td>IgG1</td>
<td>λ</td>
<td>SLE patient</td>
<td>dsDNA: √&lt;br&gt;ssDNA: x&lt;br&gt;H: x&lt;br&gt;N: x&lt;br&gt;HS: x&lt;br&gt;CL: ?</td>
<td>Not tested in SCID mice</td>
<td>Winkler et al. 1991</td>
<td></td>
</tr>
<tr>
<td>1S4</td>
<td>IgG3</td>
<td>λ</td>
<td>1° APS</td>
<td>dsDNA: x&lt;br&gt;ssDNA: x&lt;br&gt;H: x&lt;br&gt;N: x&lt;br&gt;HS: x&lt;br&gt;CL: ?</td>
<td>Not tested in SCID mice</td>
<td>Zhu et al. 1999</td>
<td></td>
</tr>
<tr>
<td>CL24</td>
<td>IgG3</td>
<td>κ</td>
<td>2° APS</td>
<td>dsDNA: x&lt;br&gt;ssDNA: x&lt;br&gt;H: x&lt;br&gt;N: x&lt;br&gt;HS: x&lt;br&gt;CL: ?</td>
<td>Not tested in SCID mice</td>
<td>Zhu et al. 1999</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** APS, antiphospholipid syndrome; ANA, antinuclear antibody; H, histones; N, nucleosomes; HS, heparan sulphate; CL, cardiolipin; GN, glomerulonephritis; i.v, intravenous; √ = positive; x = negative; E, ELISA; i.i, inhibition index (measurement of functional affinity, <1.5=low affinity, >3.0=high affinity [Devey et al. 1988]); Kd, dissociation constant (measure of affinity, the lower Kd the stronger the binding [Winkler et al. 1991]); C, crithidia; F, Farr assay; β, β₂GPI dependent; h, homogeneous; s, speckled; ? = unknown.
### Table 2.2: Summary of the Properties of Human IgM Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Clone</th>
<th>Subclass</th>
<th>Light Chain</th>
<th>Known Binding specificities</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>dsDNA</td>
<td>ssDNA</td>
</tr>
<tr>
<td>RT16</td>
<td>IgM</td>
<td>λ</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>RT55</td>
<td>IgM</td>
<td>κ</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>RT72</td>
<td>IgM</td>
<td>κ</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>RT79</td>
<td>IgM</td>
<td>κ</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>RT84</td>
<td>IgM</td>
<td>κ</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>RT115</td>
<td>IgM</td>
<td>κ</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>RT129</td>
<td>IgM</td>
<td>κ</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

**Abbreviations:** ✓ = positive; x = negative; i.i, inhibition index (measurement of functional affinity, <1.5=low affinity, >3.0=high affinity [Devey et al. 1988]); ? = not tested.

All of the RT clones were derived from a 13 year old female patient with active SLE (Ravirajan et al. 1992).
IgG anti-cardiolipin and no clinical evidence of SLE (Zhu, Olee, Le, Rouhey, Hahn, Woods, Jr., and Chen 1999). CL24 and IS4 were kind gifts from Dr Pojen Chen. The human IgM anti-DNA mAbs (Table 2.2) were derived by fusion of CB-F7 with splenocytes from a 13 year old female patient with active SLE. The patient had high serum levels of anti-dsDNA antibodies and a positive ANA, she was negative for lupus anti-coagulant and IgG anti-cardiolipin. At the time of the fusion she had undergone a splenectomy for severe thrombocytopenia (Ravirajan, Kalsi, Wiloch, Barakat, Tuaillon, Irvine, Cockayne, Harris, Williams, Williams, and . 1992).
2.3 Implanting human hybridoma cell lines secreting human IgG monoclonal antibodies into SCID and Rag-1− immunodeficient mice

This section describes the methods used specifically in the experiments described in Chapter three of this thesis.

2.3.1 Culture of hybridoma cells

The human hybridomas RH-14, DIL-6, IS4 and CL24 (described in section 2.2) were cultured for implanting into SCID mice. Another human hybridoma TW (a kind gift from Thomas Winkler, Erlangen, Germany), producing human IgGκ of unknown specificity which does not bind to DNA and the mouse-human heteromyeloma fusion partner cell line CB-F7 (Grunow et al. 1988) were also cultured as controls for implantation. All cell culture was carried out under sterile conditions.

2.3.1.1 Growth medium for human hybridoma cell lines

The growth medium (500ml) was prepared from the following constituents: 84.8% v/v (424ml) RPMI (RPMI, Rothwell Park Memorial Institute) 1640 medium HEPES modification (Gibco 42401-018); 10% v/v (50ml) heat inactivated foetal calf serum (FCS, Sigma F-7524); 2% v/v (10ml) of 100x MEM non-essential amino acids (Gibco 11140-035); 1% v/v (5ml) 10000 IU/ml Penicillin / 10mg/ml Streptomycin solution (Gibco 15140-023); 1% v/v (5ml) L-glutamine [200mM] (Gibco 25030-024); 1% v/v (5ml) Sodium pyruvate [100mM] (Gibco 11360-039) and 0.2% v/v (1ml) Gentamicin [10mg/ml] (Gibco 15710-031).

2.3.1.2 Culture of the human hybridoma cell lines

The human hybridoma cells were grown in suspension in growth medium (section 2.3.1.1) in 175cm² tissue culture flasks (Nunc). The cells were maintained in continuous culture by replacing 90% of the media, with fresh media twice a week. To minimise the risk of infection, the flasks were replaced once a week. The supernatant level of human IgG was tested regularly, especially once the cells started to grow after thawing and on the day before implanting into the mice, using the ELISA (section 2.1.2).
23.1.3 Freezing and thawing of hybridoma cell lines
The hybridoma cells were thawed approximately one month before implantation into the mice, to allow time for recovery and growth of cells but to minimise the number of passages in vitro. The cells were thawed in a 37°C water bath and immediately added to pre-warmed growth medium containing 20% FCS. The cells were spun at 1200 rpm for 5 minutes and the cell pellet was re-suspended in 2-5ml (depending on the size of the pellet) of growth medium containing 15% FCS and added 1ml/well to a 24 well tissue culture plate. The plate was placed in a CO₂ incubator at 37°C and the medium was partially replaced once or twice a week as necessary, until the cells had proliferated enough to be transferred to a small tissue culture flask.

When the cells were growing well and producing high levels of human IgG, surplus cells were frozen down. The cells were pelleted at 1200 rpm and re-suspended at 5x10⁶-1x10⁷ cells/ml in freezing medium prepared with 50% RPMI 1640 medium with 30% FCS and 20% dimethyl sulphoxide (DMSO, Sigma), 1ml was added to each cryovial. The vials were placed in a freezing pot containing propan-2-ol (to regulate the freezing by 1°C/minute) at -80°C. The next day the vials were transferred for long-term storage in liquid nitrogen.

23.2 Implantation into immunodeficient mice
2.2.2.1 SCID mice
Female Balb/C SCID mice were obtained from Harlan UK (Bicester, U.K.) at either 6-8 weeks or 8 months of age (ex-breeders). The mice were housed in sterile conditions on vented racks.

2.2.2.2. Rag-1⁻/⁻ mice
C57BL/6J RAG-1⁻/⁻ mice were imported from Jackson Laboratories (Bar Harbor, Maine, U.S.A) at 6 weeks of age. The mice were housed in sterile conditions on vented racks

2.3.3. Experimental schedule
All procedures were carried out wearing clean gowns, gloves and overshoes and inside sterile cabinets to protect the mice from infection. The mice were acclimatised for 1 week and then tail bled prior to the start of the experiment. The
mice were primed with 500μl i.p. of pristane (2,6,10,14-tetramethylpentadecane, Sigma) which activates macrophages to produce growth factors and create optimal environment for hybridoma cell growth. Ten days later the mice were implanted with the hybridoma cells. Before implanting the cells were washed in RPMI 1640, pelleted and resuspended at 2×10^6 cells/ml in RPMI 1640 medium, the mice were injected i.p with 1×10^6 cells in 500μl of RPMI 1640. Three separate experiments were undertaken, implanting hybridoma cell lines into SCID and Rag-1^-^- mice, the numbers of mice implanted with each hybridoma cell line in each experiment are shown in the results, Table 3.1. Throughout the experiments proteinuria was assessed using Albustix (Bayer Diagnostics, Berks, U.K.), proteinuria was scored as negative or trace which is negligible, (+) 0.3g/L, (++) 1.0g/L, (+++) 3.0g/L and (++++) more than 20g/L. The mice were sacrificed either when the ascites had developed to a degree which had resulted in a 20% increase in body weight, or after 2 months if ascites had not developed. On sacrifice, sera, ascites fluid and organs were collected for further analysis.

2.3.4 Serology
The levels of human IgG and human anti-dsDNA IgG, secreted by the hybridoma cells implanted into the mice, were measured in the mouse sera using the ELISAs described in sections 2.1.2 and 2.1.4.2 respectively. In order to assess 'leakiness' (production of murine immunoglobulins), prior to hybridoma injection the levels of murine IgM and IgG, in the sera of the mice were measured by ELISA (sections 2.3.4.1 & 2.3.4.2).

2.3.4.1 ELISA measuring murine IgG
'Maxisorp' plates (Nunc) were coated overnight at 4°C with 100μl/well of goat anti-mouse IgG (Sigma M-0659) diluted to 1μg/ml in BIC buffer. Half of the plate was coated with the capture antibody and the other half of the plate was 'sham' coated with BIC buffer. The plates were washed 3 times with PBS and blocked with 200μl/well of 2% BSA in PBS/tween for 1 hour at 37°C. The murine serum samples were diluted to 1:50 and 1:500 in PBS/tween and for each sample 100μl/well was incubated in duplicate on the plate for 1 hour at 37°C. The positive standard was mouse IgG (Sigma I-8765) titrated from 1mg/ml in PBS/tween. As a negative
control, PBS/tween alone was added to duplicate wells on the coated and ‘sham’ coated sides. After washing 5 times with TBS/Tween, any bound antibodies were detected by incubation with 100μl/well of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma A-3438) diluted 1:1000 in TBS/tween for 1 hour at 37°C. The plates were developed using alkaline phosphatase substrate, p-nitrophenol phosphate (Sigma 104-105, 1 tablet dissolved in 5ml of BIC buffer). The plates were read at 405nm using a reference filter of 490nm. When calculating the final OD values, first the OD values of wells containing PBS/tween alone were deducted from the other OD values on the anti-murine IgG coated and ‘sham’ coated sides. Then for each positive control and sample, the duplicate OD values obtained on the ‘sham’ coated side were deducted from those obtained on the anti-murine IgG coated side. The concentration of murine IgG in the samples was calculated by reference to the linear portion of the standard curve of murine IgG.

2.3.4.2 ELISA measuring murine IgM
This assay was exactly the same as that for murine IgG, as described in section 2.3.4.1, except the capture antibody was goat anti-mouse IgM (Sigma M-8644) and the detecting antibody was goat anti-mouse IgM conjugated to alkaline phosphatase (Sigma A-9688), diluted 1:5000.

2.3.4.3 ELISA to detect complexes of human IgG and murine immunoglobulins
A modification of the human IgG and murine Ig ELISAs was developed in order to detect complexes of human IgG (from the hybridoma cells implanted in the SCID mice) and any murine immunoglobulins from the sera of the mice. The murine sera were incubated in blocked ELISA plates, which had been coated with anti-human IgG. Any human and mouse immunoglobulin complexes were detected using anti-murine IgM and IgG conjugated to alkaline phosphatase. The basic procedure for this assay was as described as in 2.3.4.1.

2.3.5. Histology
On dissection of the mice, portions of the kidney, spleen, liver, heart, lungs and skin were immediately fixed in formal saline (BDH) and another portion of each organ was placed on a cork disc (Raymond A Lamb, London, UK) covered in O.C.T compound (Tissue-Tek, Raymond A Lamb) and snap-frozen immediately in hexane.
(BDH) pre-cooled in liquid nitrogen. Small pieces of kidney, approximately $1\text{mm}^2$ were fixed immediately in Karnovsky's fixative (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1M phosphate buffer, from EM laboratory) and these were then embedded and processed for electron microscopy (EM). The embedding and sectioning was carried out by a specialist EM technician, and the EM sections were analysed and photographed by an experienced EM pathologist. The organs, fixed in formal saline, were subsequently paraffin embedded and sectioned (by the UCL hospitals routine histopathology laboratory).

2.3.5.1. Haematoxylin and Eosin morphological stain

Formalin fixed, paraffin embedded sections were dewaxed by placing in two changes of histoclear (National Diagnostics) and brought to water through a series of alcohols ($1\times100\%, 1\times90\%, 1\times70\%$ and $1\times30\%$ ethanol). The sections were stained with haematoxylin (Sigma) for 2 minutes, blued in running tap water before staining with eosin (BDH) for 1 minute. The section was rinsed under running tap water, and dehydrated back through the alcohols from 30% to 100%. The slides were rinsed in two changes of histoclear before mounting with Depex mounting medium (Raymond A Lamb). The slides were then examined with the expert guidance of a histopathologist for morphological evidence of disease.

2.3.5.2. Immunohistochemical staining for human IgG in murine sections

Formalin fixed, paraffin embedded, kidney sections were dewaxed and endogenous peroxidase was blocked using 0.5% hydrogen peroxide, $\text{H}_2\text{O}_2$ (Sigma) in methanol for 10-15 minutes. The sections were washed in water and in order to expose the antigen after formalin fixation, the sections were digested in 0.1% protease XXIV (Sigma) in distilled water, adjusted to pH 7.8 with 0.1M NaOH, for 40 minutes at $37^\circ\text{C}$. The kidney sections were then washed in running water and transferred to TBS. Non-specific binding was blocked with 5% normal swine serum in TBS for 10 minutes. The swine serum was tipped off and the sections incubated with rabbit polyclonal anti-human IgG (Dako A0424) diluted in TBS for 1 hour at $37^\circ\text{C}$. Binding was detected using peroxidase-anti-peroxidase (PAP, Dako, Denmark) for 1 hour at $37^\circ\text{C}$ and developed with 3,3'-diaminobenzidine (DAB, Sigma). The sections were counterstained with Mayer's haematoxylin (Sigma).
2.3.5.3. Staining for fibrin deposition in murine kidneys

Formalin fixed, paraffin embedded, kidney sections were dewaxed and stained for fibrin using two standard histological stains, MSB (martius yellow, brilliant crystal scarlet and soluble blue) and phosphotungstic acid haematoxylin (PTAH). This procedure was carried out in the routine histopathology laboratory at UCL.

2.3.5.4 Staining for murine IgM and IgG in kidney of SCID mice

This method was used to try and stain any murine immunoglobulins that might be present in the thrombi found in the kidneys of the SCID mice implanted with RH-14. The paraffin embedded sections were dewaxed as described in section 2.5.3.1. The sections were digested in 0.1% protease XXIV (Sigma) in distilled water, adjusted to pH 7.8 with 0.1M NaOH, for 40 minutes at 37°C. The sections were washed in running water and transferred to PBS. Non-specific binding was blocked by incubation with 2% BSA in TBS for 30 minutes. To detect murine immunoglobulins, goat anti-mouse IgM (Sigma M-8644) or IgG (Sigma M-0659) diluted in to 10μg/ml in 3%BSA/PBS was added for 1 hour at room temperature. After washing in PBS, endogenous peroxidase was blocked by incubating the sections in 1.0% hydrogen peroxide in methanol for 30 minutes. The detecting antibody was murine anti-goat immunoglobulins conjugated to horse-radish peroxidase, diluted 1/200 in 3% BSA/PBS and incubated on the sections for 30 minutes. After washing in PBS the staining was developed with DAB (Sigma) and counterstained with Mayer’s haematoxylin. As a negative control the primary antibody was replaced with 3%BSA/PBS. As a positive tissue control, sections cut from the spleen and lymph node of an MRL-lpr mouse were used.
2.4 The human IgG heavy and light chain expression constructs

This section describes the expression constructs used in the experiments described in Chapter four of this thesis. The original heavy chain expression construct, B3Vh/pG1D1 (Figure 2.2) and the light chain expression constructs, B3V_\lambda/pLN10, 33.H11V_\lambda/pLN10 and UK4V_\lambda/pLN10 were prepared by Dr Anisur Rahman (Figure 2.3). The assembly of these constructs is fully described in (Rahman et al. 1998). The expression vectors pG1D1 and pLN10 were both kind gifts from Dr C.A. Kettleborough and Dr T. Jones at AERES Biomedical, MRC Collaborative Unit, Mill Hill, London. Due to commercial sensitivity, no published references are available for these vectors.

Each expression vector contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and either the bacterial neo gene (in pLN10) or mouse dhfr gene (in pG1D1 or pG1D210) coding sequences driven by the SV40 early promoter to act as dominant selectable markers during stable transformation. However the SV40 promoter that drives the dhfr is crippled by the presence of a defective SV40 promoter-enhancer sequence so that expression is poor thus allowing for the selection of high expression level clones using comparatively low levels of methotrexate. Each vector also contains an ampicillin resistance gene driven by an internal promoter to enable it to be selected in E.coli. pG1D1 is a heavy chain expression vector as it also contains a cloned PCR fragment that encodes the human \( \gamma_1 \) constant region (\( C_\gamma 1 \)) whilst pLN10 is a \( \lambda \) light chain vector as it contains a cloned PCR fragment of DNA encoding the human \( \lambda_2 \) constant region (\( C_\lambda \)).

The \( V_\text{H} \) region sequence of B3 was cloned into the expression vector pG1D1 5' to the \( C_\gamma 1 \) human DNA sequence to produce the construct B3Vh/pG1D1 (see Figure 2.2). Each of the \( V_\lambda \) region sequences were ligated into expression vector pLN10 5' to the \( C_\lambda \) human DNA sequence to produce the constructs, B3V_\lambda/pLN10, 33.H11V_\lambda/pLN10 and UK4V_\lambda/pLN10 (see Figure 2.3) (Rahman et al. 1998).
Figure 2.2  Vector map of recombinant heavy chain expression vector B3Vh/pG1D1

pG1D1 contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and the mouse dhfr gene coding sequence driven by the SV40 early promoter to act as a dominant selectable marker during stable transformation. The SV40 promoter that drives the dhfr is crippled by the presence of a defective SV40 promoter-enhancer sequence so that expression is poor thus allowing for the selection of high expression level clones using comparatively low levels of methotrexate. pG1D1 also contains an ampicillin resistance gene (AmpR) driven by an internal promoter to enable it to be selected in E.coli and a cloned PCR fragment that encodes the human γ1 constant region (Cγ1). The Vh region sequences of both B3 and 33.H11 were each cloned separately into expression vector pG1D1 5’ to the Cγ1 human DNA sequence to produce the constructs, B3Vh/pG1D1 and 33.H11Vh/pG1D1. The immunoglobulin variable (V) region sequence is immediately followed by a splice donor (SD) site whilst the immunoglobulin constant (C) region sequence is immediately preceded by a splice acceptor (SA) site. The DNA between these SA and SD sites is treated like an intron by mammalian cells and is not represented in the expressed heavy or light chain peptide. The purpose of these splice sites was to facilitate the transfer of the V region cassette (V region sequence and immunoglobulin leader sequence) into the expression vectors.
Materials and Methods

<table>
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<tr>
<th>Gene</th>
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<th>Additional Information</th>
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**KEY**

- **Promoter**
- **Gene**
- **Antibody DNA sequence**
- **Intron between SA and SD not represented in final expression product**
- **Immunoglobulin leader sequence**
- **SA** Splice acceptor site
- **SD** Splice donor site
- **AmpR** Ampicillin resistance gene
- **dhfr** Dihydrofolate reductase gene
- **HCMV** Human cytomegalovirus

Most restriction sites are shown in black. Those written in red indicate that they were used for cloning described in this thesis.
Chapter Two

Figure 2.3 Vector map of recombinant light chain expression vector pLN10 containing $V_\lambda$ cDNA

The $V_\lambda$ region sequences of B3, UK4 and 33.H11 were each cloned separately into expression vector pLN10 5' to the $C_\lambda$ human DNA sequences to produce the constructs, B3$V_\lambda$/pLN10, UK-4$V_\lambda$/pLN10 and 33.H11$V_\lambda$/pLN10. The enzyme restriction sites are shown in black type for B3$V_\lambda$/pLN10 and 33.H11$V_\lambda$/pLN10, the sequence of UK4 $V_\lambda$ is 6 bp shorter than B3$V_\lambda$/pLN10 and 33.H11$V_\lambda$/pLN10, thus the total vector size of UK4$V_\lambda$/pLN10 and the position of all the sites after BamHI are 6 bp less, as shown in red type.

pLN10 contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and the bacterial neo gene coding sequence driven by the SV40 early promoter to act as a dominant selectable marker during stable transformation. pLN10 also contains an ampicillin resistance gene (AmpR) driven by an internal promoter to enable it to be selected in E.coli and a cloned PCR fragment that encodes the human $\lambda$2 constant region ($C_\lambda$). The immunoglobulin variable (V) region sequence is immediately followed by a splice donor (SD) site whilst the immunoglobulin constant (C) region sequence is immediately preceded by a splice acceptor (SA) site. The DNA between these SA and SD sites is treated like an intron by mammalian cells and is not represented in the expressed heavy or light chain peptide. The purpose of these splice sites was to facilitate the transfer of the V region cassette (V region sequence and immunoglobulin leader sequence) into the expression vectors.
Materials and Methods

Key

- Promoter
- Gene
- Antibody DNA sequence
- Intron between SA and SD not represented in final expression product
- Immunoglobulin leader sequence
- SA: Splice acceptor site
- SD: Splice donor site
- AmpR: Ampicillin resistance gene
- NeoR: Neomycin resistance gene
- HCMV: Human cytomegalovirus
In both of the vectors, pLN10 and pG1D1, the immunoglobulin variable (V) region sequence is immediately followed by a splice donor (SD) site, whilst the immunoglobulin constant (C) region sequence is immediately preceded by a splice acceptor (SA) site. The DNA between these SA and SD sites is treated like an intron by mammalian cells and is not represented in the expressed heavy or light chain peptide. The purpose of these splice sites was to facilitate the transfer of the V region cassette (V region sequence and immunoglobulin leader sequence) into the expression vectors. The SA site was from the original vector whilst the SD was not. The SD site and a BamHI site were added to the 3’ end of the V region DNA by PCR (as detailed previously in Rahman et al. 1998) prior to being incorporated into the final expression vector as part of a HindIII/BamHI insert with the V region cassette. Without the SA and SD sites the V region and C region DNA would need to be immediately adjacent to each other in the vector and therefore the restriction site for the V region cassette would need to be within the V region sequence. As a consequence, for some sequences this may not be possible without changing the expressed amino acid sequence of the V region, which is undesirable.
2.5 **Cloning of light chain expression vector CDR hybrids**

This section describes the cloning of the expression vectors containing the light chain CDR hybrids, the results of which are described in Chapter four. Previous work in our laboratory, had targeted single amino acids within the CDRs of the variable region of the light chain of B3 using site-directed mutagenesis (Rahman et al. 2001). The light chains of IgG monclonal antibodies B3, 33.H11 and UK4 are all derived from the same germline gene, 2a2, and therefore only differ at sites of somatic mutation, most of which are within the CDRs. In order to highlight those regions of B3, 33.H11 and UK4, most heavily involved in the interaction with dsDNA, it was decided to investigate the effect of swapping entire CDRs between the various light chains. Six new constructs were made by switching the CDR3 regions between the three different light chain sequences to create six new light chain sequences (Figure 2.4) which could later be combined with the heavy chain of B3 and expressed as new antibody molecules.

The following cloning steps were used to produce the expression vector hybrids of B3 and 33.H11, B3 and UK4, and 33.H11 and UK4 λ chain. The following method describes in detail the steps that I used to produce the four hybrids 33B (33.H11Vλ,
CDR1, B3V\textsubscript{\lambda} CDR2 and CDR3), B33 (B3V\textsubscript{\lambda} CDR1, 33.H11V\textsubscript{\lambda} CDR2 and CDR3), 33U (33.H11V\textsubscript{\lambda} CDR1, UK-4V\textsubscript{\lambda} CDR2 and CDR3) and U33 (UK-4V\textsubscript{\lambda} CDR1, 33.H11V\textsubscript{\lambda} CDR2 and CDR3). The other two hybrids BU (B3V\textsubscript{\lambda} CDR1, UK-4V\textsubscript{\lambda} CDR2 and CDR3) and UB (UK-4V\textsubscript{\lambda} CDR1, B3V\textsubscript{\lambda} CDR2 and CDR3) were produced using the same method, but by my colleague Joanna Haley.

2.5.1 **Reagents**

2.5.1.1 **Luria-Bertani(LB)bacterial growth medium**
5g tryptone (Difco), 2.5g yeast extract (Difco) and 5g NaCl (BDH) made up to 500ml with double distilled water and adjusted to pH 7.0 with NaOH. The growth medium was autoclaved before use.

2.5.1.2 **Ampicillin**
The stock solution was prepared from 50mg/ml of ampicillin (Sigma) dissolved in sterile water and was stored at -20\textdegree C wrapped in foil (fresh stock was made monthly). The ampicillin was used at 50\mu g/ml in agar plates and 100\mu g/ml in LB cultures.

2.5.1.3 **Agar plates**
Added 7.5g of micro agar to 500ml of LB (2.5.1.1) and autoclaved. In order to make plates for the selection of ampicillin resistant bacteria, the LB was cooled to hand heat before adding 50\mu g/ml ampicillin. The agar was poured into petri dishes, under a bunsen flame and allowed to set before use. The ampicillin was omitted from plates used to grow untransformed DH5\alpha cells. The plates were stored upside down at 4\textdegree C.

2.5.1.4 **Tris Acetate EDTA (TAE) buffer, pH 8.3**
Contained 40mM tris acetate and 1mM ethylenediaminetetraacetic acid (EDTA). Prepared a 50x stock solution by addition of 242g Tris HCl (BDH), 57.1ml glacial acetic acid (BDH) and 39.2g EDTA (BDH) made up to 1L with double distilled water. Freshly diluted to 1x as required.
2.5.1.5 Tris-EDTA (TE) buffer, pH 7.5
10mM Tris-HCl, 1mM EDTA made up in double distilled water.

2.5.1.6 Electrophoresis agarose gel
The 1% gel was prepared by adding 1.5g agarose (Boehringer) in 150ml TAE buffer. The solution was heated in a microwave until all the agarose had dissolved and was cooled to hand heat before the addition of 3μl ethidium bromide (ethidium bromide at a final concentration of 0.2μg/ml of agarose). The 0.7% gel was prepared by the addition of 3.5g agarose to 500ml of TAE buffer and 10μl of ethidium bromide, spare gel solution was kept in a 55°C water-bath until needed.

2.5.1.7 Sample loading dye
5mg bromophenol blue, 5mg xylene cyanole and 300mg Ficoll 400 dissolved in 1.7ml of double distilled water.

2.5.1.8 Molecular weight markers
The DNA molecular weight markers used were the 1Kb DNA ladder (Invitrogen, Paisley, U.K.) and the Lambda DNA/Hind III marker (Promega, Southampton, U.K.).

2.5.2 Extraction of plasmid DNA from Escherichia coli (E.coli)
DH5α strain E.coli containing the pLN10 vector plasmid DNA, encoding B3Vλ, 33.H11Vλ or UK4Vλ as prepared by Anisur Rahman (see section 2.4) were stored in media containing 15% (v/v) glycerol at −80°C. Under sterile conditions (under a bunsen burner flame), a sterilised wire loop was used to streak the glycerol-stored cultures onto an LB/ampicillin agar plate (section 2.5.1.3). The plate was incubated upside down at 37°C in a dry incubator overnight. The next morning the plates were transferred to the refrigerator and kept at 4°C until needed later that evening. A colony from the plate was picked using a sterile wire loop and used to inoculate 5ml of LB/ampicillin medium (2.5.1.1 & 2.5.1.2), in a 50ml polypropylene tube, which was then incubated at 37°C in a shaking incubator overnight. An additional control tube, containing 5ml of LB/ampicillin medium that had not been inoculated, was
incubated under the same conditions to ensure that irrelevant bacteria did not contaminate the LB medium.

Recombinant vector was extracted from overnight *E. coli* bacterial cultures using the QIAprep® Miniprep kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. This method was based on the rapid alkaline lysis procedure (Birnboim and Doly 1979). The DNA obtained from these ‘minipreps’ was re-suspended in TE buffer and stored at -20°C.

### 2.5.3 Restriction digest of pLN10/light chain expression vectors

The cloning of the four hybrids was carried out in pairs (B33 and 33B, 33U and U33) on separate occasions to avoid any contamination, but the method was the same. The pLN10 expression vector containing the light chain DNA was digested using *PvuI* and *KpnI* (Promega) as recommended in the manufacturers instructions (1U of enzyme/μg of DNA for 1 hour at 37°C). A test digest was carried out to ascertain which buffer to use, fortunately both enzymes cut well in buffer J, giving sticky ends which can easily be re-ligated.

The *PvuI* and *KpnI* restriction sites and resulting fragments are shown in Figure 2.5. *KpnI* was chosen because it cut only once and at the same place in each of B3V\(\lambda\), UK-4V\(\lambda\) and 33.H11V\(\lambda\) but did not cut pLN10. *KpnI* cuts the vectors at position 106 bp in FR2 of the V\(\lambda\) variable region sequence. *PvuI* was chosen because it cuts the vector pLN10, downstream of the constant region, but not any of the three inserts. Therefore each vector was digested into two linear bands, one of approximately 1.5 kb (containing V\(\lambda\) CDR2 and 3) and the other of approximately 5.9 kb (containing V\(\lambda\) CDR1).

### 2.5.4 Separation of the DNA fragments by agarose gel electrophoresis

After restriction enzyme digestion the resulting DNA fragments were separated by electrophoresis in a 1% agarose gel. The gel was prepared as described in section 2.5.1.6 and placed in an electrophoresis tank containing TAE running buffer (section 2.5.1.4). 10μl of the restriction digests were mixed with 2μl of loading dye (2.5.1.7) and added into wells in the gel and 5μl of the molecular weight markers (1kb and
Figure 2.5  
Restriction enzyme digest method used to exchange \(\kappa\)CDR cloned DNA sequences

Vector map of recombinant light chain expression vector, pLN10 showing the positions of the two restriction sites used to exchange \(\kappa\)CDR cloned DNA sequences, \(KpnI\) and \(PvuI\) highlighted in green type. The enzyme restriction sites are shown in black type for \(B3V_\kappa/pLN10\) and \(33.H11V_\kappa/pLN10\), the sequence of UK4 \(V_\kappa\) is 6 bp shorter than \(B3V_\kappa/pLN10\) and \(33.H11V_\kappa/pLN10\), thus the total vector size of UK4V_\kappa/pLN10 and the position of all the sites after BamHI are 6 bp less, as shown in red type.

a)  
KEY  
Promoter  
Gene  
Antibody DNA sequence  
Intron between SA and SD not represented in final expression product  
Immunoglobulin leader sequence  
SA  Splice acceptor site  
SD  Splice donor site  
AmpR  Ampicillin resistance gene  
NeoR  Neomycin resistance gene  
HCMV  Human cytomegalovirus  

b)  Products of \(KpnI/PvuI\) restriction digest of recombinant pLN10 containing B3, 33.H11 or UK4 \(V_\kappa\) cDNA sequences.
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a)

Recombinant pLN10
7496 bp
7490 bp

HCMV promoter

Light chain variable region

Intron between SA and SD

Light chain constant region

NeoR

NeoR prom

AmpR prom

SV40 origin

Pvu I 2939 2933

Light chain variable region

V_{\lambda} FR1, CDR1

5' Pvu I 3'

Kpn I

5.9 Kb
(5971 bp)

V_{\lambda} FR2, CDR2, FR3, CDR3

5' Kpn I 3' Pvu I

1.5 Kb

(1525 bp in B3V_{\lambda}/pLN10 and 33.H11V_{\lambda}/pLN10, 1519 bp in UK4V_{\lambda}/pLN10)
Lambda DNA/Hind III, section 2.5.1.8) were also loaded. The gel was run at 100 Volts. The DNA bands present in the gel were visualised by the intercalation of ethidium bromide and viewed by U.V light, each gel was recorded using ‘Gene Snap’ software (Syngene).

2.5.5 Purification of DNA from the agarose gels

The DNA bands of interest were cut out from the gel using a scalpel. The DNA was purified from the agarose gel as per the manufacturers instructions using either the GeneClean® kit (Qbiogene) or in later experiments the QIAquick® spin kit (Qiagen). Briefly the method entailed dissolving the agarose gel-slice in 3 volumes of chaotropic agent (NaI in Gene clean kit) at 55°C for 5-10 minutes, the DNA was purified using either glassmilk (silica matrix, GeneClean kit) or using custom spin-column (Qiagen kit) the DNA remains bound to glassmilk or in the column. The glassmilk or column was then washed with 70% ethanol (to remove salt and impurities) and the DNA eluted in 50μl of water or elution buffer. In each case, once purified from the agarose, 1μl of the DNA was run on a 1% agarose gel with the 1Kb DNA molecular weight marker, in order to calculate from the relative band intensities the amount of DNA available for the subsequent reactions.

2.5.6 Ligation of the 5.9 kb and 1.5 kb DNA fragments.

The 1.5 kb fragment contains CDR2 and CDR3 of the IgG Vλ region and also part of the downstream expression vector containing the λ constant region sequence whilst the 5.9 kb fragment contains the VλCDR1 and the rest of the vector. Therefore the objective was to ligate the B3Vλ vector-5.9 kb fragment with the 33.H11Vλ vector-1.5 kb fragment to create B33 and vice versa to produce 33B. The same method was used to ligate fragments derived from the B3Vλ and UK4Vλ vectors, to produce the second pair of hybrids, 33U and U33. Thus four separate ligation reactions were carried out using the following method.

The ligation of the DNA fragments was carried out using T4 DNA ligase enzyme and buffers (Promega) according to the manufacturer’s instructions. The reaction mix contained a 1:1 molar ratio of vector : insert DNA (a typical reaction used 100-200ng of vector DNA), 1U of T4 DNA ligase in a total volume of 20μl with water
(supplemented with the appropriate ligase buffer as supplied by the manufacturer). Two vector only controls were set up for each ligation in which the insert DNA was replaced by the appropriate volume of sterile ddH2O. In order to ensure that the T4 DNA ligase was functional, one of the controls did not contain T4 DNA ligase. The ligation mixes were incubated overnight at 4°C or for 3 hours at room temperature. In later experiments incubating overnight at 14°C in a PCR machine gave optimal ligation.

2.5.7 Production of fresh competent DH5α-strain *E. coli* cells.

Bacterial cultures were prepared from glycerol stocks of DH5α-strain *E. coli* as described in section 2.5.2 except that no antibiotic was added to the agar plates or the LB medium. This culture was then grown overnight in a shaking incubator (250rpm) at 37°C. 100μl of this overnight culture was added to 100ml of LB medium in a 500ml sterile (autoclaved) conical flask and then returned to the shaking incubator (250rpm) for 2 to 3 hours.

After 2 hours and then every 30 minutes after that, the growth of the cell culture was checked. The 100ml culture was removed from the incubator when the culture was in the exponential log phase of the growth curve (i.e. clouds of bacteria swirling in the medium were just visible when held up to the light and when the absorbance reading of the culture, measured on the spectrophotometer at 600nm was between 0.3 and 0.8).

The culture was then divided between two 50ml sterile falcon tubes and spun down at 1750g for 10 minutes in a Sorvall RTH-750 rotor at 4°C. The supernatant was discarded and the pellet in each tube was re-suspended in 10ml of ice-cold 100mM calcium chloride solution. The preparations were spun down again at 1750g for 10 minutes in a Sorvall RTH-750 rotor at 4°C. The supernatant was discarded and each pellet was re-suspended in 2ml of ice-cold 100mM calcium chloride solution. The cell suspensions were put on ice and used immediately for transformation with recombinant vector. This protocol was derived from Sambrook and Russell (2001).
2.5.8 Transformation of competent E.coli cells with recombinant vector

For each separate ligation reaction, 200μl of the competent cells were transferred to ice cold 1.5ml microcentrifuge tubes and kept on ice. 5μl of each ligation mix were added to 200μl of these competent cells. Two control transformations were also set up, a negative containing DH5α cells only (no DNA) and a positive containing circular plasmid DNA (containing the ampicillin resistance gene), which had previously been successfully transformed. The DNA/cell preparations were gently mixed, left on ice for 30 minutes and then subjected to a heat shock by incubating the samples for 90 seconds at 42°C in a water bath. The DNA/cell preparations were then returned to ice for a further two minutes in order to recover from the heat shock. 800μl of LB medium (no antibiotic) was added to each tube and then the samples were incubated in a shaking incubator (250rpm) at 37°C for one hour. The cell suspensions were centrifuged for 10 minutes at 3500g at room temperature in a microcentrifuge and the pellets then re-suspended in 100μl of LB medium. These samples were spread on an LB agar plate containing 50μg/ml ampicillin. Once dry, the plates were left upside down overnight at 37°C in a dry incubator.

The DH5α-strain of E. coli is unable to grow in the presence of the antibiotic ampicillin, however the vector pLN10 contains an ampicillin resistance gene. Therefore only those bacterial cells that incorporated the vector should have survived to produce a colony on the plate. The next day the plates were removed from the incubator and the number of colonies counted. A number of colonies were “picked” using a sterile wire loop and transferred into 1ml LB cultures containing 50μg/ml ampicillin. These cultures were transferred to a shaking incubator (250rpm) at 37°C overnight. From the overnight cultures, glycerol stocks were made by adding 0.5ml of sterile autoclaved 100% glycerol to 0.5ml of the overnight culture in a sterile 1.5ml microcentrifuge tube. The tube was vortexed to mix the contents and then immediately transferred to the -80°C freezer for storage. This protocol was derived from Sambrook and Russell (2001). DNA was extracted from the remainder of the overnight culture using the QIAprep® Miniprep kit (as described in section 2.5.2)
2.5.9 Restriction digest to identify those clones containing the correct hybrid $V_\lambda$ CDR regions.

To ensure that each construct (B33 and 33B) contained the correct CDRs, two different restriction digests were carried out; AatII digest and a HindIII/AvaI digest. The restriction digests were performed using Promega enzymes and buffers as recommended in the manufacturer’s instructions (1U of enzyme/μg of DNA for 1 hour at 37°C) and the digestion products were separated on an agarose gel as described in section 2.5.4. A KpnI/PvuI or a BamHI/HindIII digest was not sufficient as the 33.H11 and B3 $V_\lambda$ fragments were identical in size. Due to the different pattern of somatic mutations in the $V_\lambda$ of B3 and 33.H11, the bands produced from an AatII restriction digest and a double digest with HindIII and AvaI were sufficiently different to distinguish which CDRs each hybrid possessed (this is shown clearly in the results section 4). Figures 2.6, 2.7 and 2.8 show the AatII, AvaI and HindIII sites in B3$V_\lambda$/pLN10, 33.H11$V_\lambda$/pLN10 and UK4$V_\lambda$/pLN10 respectively.

2.5.10 Sequencing of pLN10/light chain expression vectors

Neither the original pLN10 constructs 33.H11$V_\lambda$ and UK4 $V_\lambda$, nor the new hybrid constructs 33U and U33, contained an AatII site within the $V_\lambda$ inserts, and although HindIII/AvaI digestion allowed U33 and 33U to be distinguished, it did not allow distinction of 33U from pLN10/UK4$V_\lambda$ or U33 from pLN10/33.H11$V_\lambda$. Therefore I sequenced the hybrid pLN10 constructs of U33 and 33U, to be sure I had produced the desired light chain hybrids.

2.5.10.1 Ethanol precipitation of DNA

Denatured template DNA was prepared from the pLN10 constructs of U33 and 33U using an ethanol precipitation protocol derived from Sambrook and Russell (2001). In the presence of salt (monovalent cations such as Na+) and at a temperature of -20°C or less, absolute ethanol efficiently precipitates nucleic acids. Therefore 0.1 volume of 3M Sodium Acetate, pH 5.2 and 2.5 volumes of 100% ethanol were added to the DNA preparation in a 1.5ml microcentrifuge tube. This was placed in a –80°C freezer for one hour to precipitate the DNA. To collect the precipitate, the preparation was spun in a microcentrifuge for 10 minutes at 10000g at 4°C. The
Figure 2.6  Restriction digest map of B3V\(\lambda\)/pLN10 recombinant light chain expression vector

The restriction digest map opposite of B3V\(\lambda\)/pLN10, shows the positions of the *AatII*, *Aval* and *HindIII* restriction sites used to determine which cloned DNA sequence V\(\lambda\) CDRs were present in each clone.

Two separate restriction digests were carried out, an *AatII* (sites shown in blue) digest and a *HindIII/Aval* (sites shown in red) restriction digest. Due to the different pattern of somatic mutations in the V\(\lambda\) of B3, 33.H11 and UK4, the bands produced from an *AatII* or a *HindIII/Aval* restriction digest were sufficiently different to distinguish which CDRs each new hybrid possessed.

**KEY**

- Promoter
- Gene
- Antibody DNA sequence
- Intron between SA and SD not represented in final expression product
- Immunoglobulin leader sequence
- Splice acceptor site
- Splice donor site
- Ampicillin resistance gene
- Neomycin resistance gene
- Human cytomegalovirus
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H pal 7144 E coR lI
Light chain variable region

V val 6255
NeoR

Light chain constant region

Nru I 6273
MCMV promoter

atll 2489
Prom

B3VL/pLN10
7496 bp

Hpa I 7144
EcoRI 1

BssH II 5815
AatII 689

AatII 742
Sae I 1133

AatII 825
Hind III 1224

AatII 1011
KpnI 1488

Sac I 1113

NeoR

BamHI 1630

Neor prom

SV40 origin

Nru I 6273

AatII 1392

Bgl II 5254

atll 689

AatII 1602

Bgl II 5254

AatII 742

Sfi I 14867

AatII 825

Eag I 5324

Sac I 1113

Bgl II 5254

AatII 1011

EcoRI 2309

Bgl II 5254

Xmn I 12708

AatII 2489

Sfi I 14867

EcoRI 2309

Sac I 1113

Sea I 2827

AatII 1392

Pvu II 2939

AatII 1602

Bgl II 5254

Sac I 1113

Bgl II 5254

Bgl II 5254

AatII 1011

EcoRI 2309

Sac I 1113

Pvu II 2939

Sfi I 14867

AatII 1392

Bgl II 5254

Sac I 1113

Bgl II 5254

Bgl II 5254

AatII 1011

EcoRI 2309

Sac I 1113

Pvu II 2939

Bgl II 5254

Sac I 1113

Bgl II 5254

Bgl II 5254

AatII 1011

EcoRI 2309

Sac I 1113

Pvu II 2939
Figure 2.7  Restriction digest map of 33.H11V3/pLN10 recombinant light chain expression vector

Figure legend and key as for figure 2.6
Figure 2.8  Restriction digest map of UK4Vκ/pLN10 recombinant light chain expression vector

Figure legend and key as for figure 2.6
supernatant was discarded and the pellet was gently washed with 1ml of 70% ice-cold ethanol. The solution was centrifuged again for 10 minutes in a microcentrifuge at 10000g at 4°C, and then air dried on the bench for 10-30 minutes. Care was taken to ensure that no ethanol remained as this could affect subsequent procedures such as sequencing, ligations, restriction digests or transfections. The DNA pellet was re-suspended in 10µl of double distilled water.

2.5.10.2 Manual sequencing method

DNA sequencing of the expression vector (pLN10) containing the hybrid light chain sequences of 33U and U33 was carried out using the Sanger method utilising chain-terminating inhibitors (Sanger et al. 1977) using the T7 Sequenase (de-aza) kit version 2.0 (Amersham), according to the manufacturers instructions. The primer used for sequencing was Neo A.

\[
\text{Neo A} \quad 5' \text{CTCCATAGAAGACACCG} 3' \\
\text{binds to pLN10 30bp 5' of insert}
\]

The reactions were run on a polyacrylamide gel (60ml acrylamide:bis-acrylamide, 19:1 (w/v) gel solution (Amresco, Anachem, Luton, Bedfordshire, UK), 300µl 10% ammonium persulphate (w/v) and 30µl TEMED (N, N, N', N'-tetramethylethylenediamine solution) (Sigma, Poole, UK) in a sequencing tank containing Tris Borate EDTA (TBE) buffer (45mM Tris-borate, 1mM EDTA). The gel was dried and exposed to X-ray film (Kodak, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) in a film cassette for 24 – 48 hours and developed using an automatic developer (Xograph Imaging System X4, Bedfordshire, UK). Using manual sequencing, I could read up to the end of the CDR1 but the sequence was too compressed to easily read past the Kpn I site where the hybrid sequences switched, the sequences were therefore confirmed by automated sequencing by MWG-Biotech, Milton Keynes, U.K.

2.5.11 Large scale extraction of plasmid DNA from \textit{E. coli}.

For transient eukaryotic expression in COS-7 cells, a larger quantity of DNA was required than was provided by the miniprep extraction described in 2.5.2. A large-
scale extraction of plasmid DNA, was carried out from a 500ml culture of *E. coli*, using a plasmid maxi kit (Qiagen) and the protocol recommended by the manufacturer. The DNA was re-suspended in TE buffer and stored at -20°C.

2.5.12 Quantification of DNA concentration
The dsDNA concentration of DNA preparations were quantified in two ways (as recommended in Sambrook and Russell 2001); by running the linearised DNA samples along side molecular weight markers of known DNA concentration on an agarose gel and comparing the intensity of the bands and by using ultraviolet absorbance spectrophotometry. The amount of ultraviolet light absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Absorbance was measured at 260nm, at which wavelength an absorbance ($A_{260}$) of 1.0 corresponds to 50μg of double-stranded DNA per ml. Ultraviolet absorbance was also measured at the wavelength 280nm in order to check the purity of the DNA preparation. The ratio of the absorbance at 260nm and 280 nm ($A_{260}/A_{280}$) of a pure sample of DNA would be 1.8. A ratio of less than 1.8 indicated that the preparation was contaminated with protein.

2.5.13 Preparation of DNA for transfection
To prepare the DNA for transfection into eukaryotic cells (described in section 2.6), the DNA was ethanol precipitated as described in section 2.5.10. After air drying the DNA was re-suspended in sterile autoclaved ddH$_2$O at a concentration of 2μg/μl, under sterile conditions in a tissue culture hood. The DNA was stored at -20°C.
Chapter Two  
Materials and Methods

2.6 Transient expression of recombinant human IgG in COS-7 cells

This section describes the maintenance and transfection of the COS-7 cell line, carried out for the experiments described in Chapter four of this thesis. The COS-7 cell line was a gift from Mrs Alison Levy, AERES Biomedical, MRC Collaborative Unit, Mill Hill, London and was originally derived from American Type Culture Collection, Ref No. CRL 1651. All protocols used for the culture and transfection of COS-7 cells, were obtained from AERES Biomedical.

2.6.1 COS-7 cell culture media

2.6.1.1 Pre-Electroporation COS-7 Growth Medium

This medium, used to culture the COS-7 cells prior to electroporation, consisted of Dulbecco’s Modified Eagle Medium (DMEM, 41966-029 Invitrogen, Paisley, UK), supplemented with 10% (v/v) foetal calf serum (FCS, 10099-133, Invitrogen), 580μg/ml L-glutamine (25030-024, Invitrogen) and 50 units/ml penicillin/50 μg/ml streptomycin (15140-122, Invitrogen).

2.6.1.2 Post-Electroporation COS-7 Growth Medium

This medium, used to culture the COS-7 cells after electroporation, consisted of Dulbecco’s Modified Eagle Medium (DMEM, 41966-029 Invitrogen, Paisley, UK), supplemented with 10% (v/v) ultra low IgG foetal calf serum (16250-078, Invitrogen), 580μg/ml L-glutamine (25030-024, Invitrogen) and 50 units/ml penicillin/50 μg/ml streptomycin (15140-122, Invitrogen).

2.6.2 Freezing and thawing of COS-7 cells

When using COS-7 cells in a transient expression system, efficiency is optimised by transfecting the cells at as low a passage number as possible. Therefore a master cell bank of the COS-7 cell line stored in liquid nitrogen was created. The cells were frozen down at as low a passage number as possible and at a concentration of 1 x 10⁶ to 1 x 10⁷ cells/ml when they were growing well, preferably in the exponential phase. The cells were trypsinised (as detailed in section 2.6.3, spun down at 250g for 10 minutes at room temperature in a Sorvall RTH-750 rotor and then re-suspended in an appropriate volume of COS-7 freezing medium, which consisted of pre-
electroporation growth medium (2.6.1.1) supplemented with 10% (v/v) dimethyl sulphoxide (DMSO, D-5879, Sigma).

Although during freezing DMSO is cryoprotective, after thawing the DMSO is toxic to the cells. Therefore the frozen cells were quickly defrosted in a waterbath at 37°C and the cell aliquot was immediately added to 10ml of pre-warmed COS-7 pre-electroporation growth medium. After spinning down at 250g for 10 minutes at room temperature in a Sorvall RTH-750 rotor. The supernatant was discarded whilst the pellet was re-suspended in 25ml of fresh pre-electroporation COS-7 growth medium and transferred to a 75cm² culture flask.

2.6.3 Maintenance of COS-7 cells in culture
COS-7 cells were grown in pre-electroporation COS-7 Growth Medium in either 80cm² or 175cm² tissue culture flasks. All work carried out with these cells was performed under sterile conditions in a sterile tissue culture hood. Furthermore, all buffers and solutions used for this work were kept in the tissue culture laboratory and only used for this purpose.

The COS-7 cells were maintained in continuous culture by splitting the cells every three to four days, when the cells were confluent. After checking the cells were confluent, the old medium was removed by aspiration and discarded. The cells were then washed with 8ml of sterile Hank’s Buffer Solution (HBS) containing no magnesium and no calcium (14175-053, Invitrogen, Paisley, UK). The wash buffer was removed by aspiration and 3ml of filter-sterilised trypsin/versene solution (at a final concentration of 0.25% trypsin [15090-046 Invitrogen] (v/v) in Versene (0.2g/L EDTA in PBS) [15040, Invitrogen ]) was added. The cells were incubated with the Trypsin/Versene solution at 37°C for two minutes. The cells were dislodged from the culture surface of the flask by firm tapping and a light microscope was used to check that the majority of cells were detached. 9ml of pre-electroporation COS-7 growth medium was added to the flask to halt the trypsin digestion. The trypsin reaction was not allowed to proceed for more than two minutes since this could damage the cells. The cell solution was split between three new flasks and fresh pre-warmed pre-electroporation growth medium was added to a final volume of either 25ml for a 175cm² flask or 20ml for a 80cm² flask. The flasks were then placed
(with the caps slightly open) in a 37°C incubator in which the concentration of CO$_2$ was 5%.

2.6.4 Preparation of COS-7 cells for electroporation

The COS-7 cell line was maintained as detailed in section 2.6.3 and transfected at as low a passage number as possible. To ensure the cells were growing in log phase when transfectected, the cells were split (as described in section 2.6.3) 24 hours prior to transfection.

Following overnight growth, the COS-7 cells were harvested by trypsinisation (as described in section 2.6.3) and pelleted at 250g at room temperature in a RTH-750 Sorvall rotor. The cells were then washed by re-suspending the pellets in 20ml of sterile autoclaved 0.15M phosphate-buffered saline, pH 7.4 (PBS, Invitrogen) and then re-pelleted at 250g for 5 minutes in a RTH-750 Sorvall rotor at room temperature. An aliquot of the cells was counted (twice to ensure accuracy) using 0.1% trypan blue exclusion dye (Sigma) and a haemocytometer to determine the number of viable cells, subsequently the remaining cells were re-suspended in sufficient PBS to produce a cell concentration of 1x10^7 cells/ml.

2.6.5 Transfection of COS-7 cells with the B3V$_h$/pG1D1 heavy chain vector and the pLN10 vector containing the light chain constructs

The DNA prepared as described in section 2.5.12 was thawed at room temperature. 5µl of the heavy chain expression vector DNA, B3V$_h$/pG1D1 (described in section 2.4), and 5µl of light chain expression vector DNA (each DNA was at 2µg/µl) were added to a sterile autoclaved 1.5ml microcentrifuge tube. A separate tube was used for each combination of heavy and light chain vectors. 7 x 10^6 washed COS-7 cells (700µl of COS-7 cells prepared as in section 2.6.4) were then added to each microcentrifuge tube and gently mixed with the DNA by slowly pipetting the contents of the tube up and down three times. The contents of each tube were then carefully pipetted into a Gene Pulser® (0.4cm gap) cuvette (Bio-Rad). To ensure that no arcing could occur during the electroporation, it was ensured that there was neither liquid on the side of the cuvette nor air bubbles nor clumps of cells in the cuvette. Using the Bio-Rad Gene Pulser® apparatus a 1900 Volt, 25µFarad
capacitance pulse was delivered to each cuvette. A "no DNA" negative control was also carried out by electroporating COS-7 cells in the absence of any DNA. After recovery at room temperature for 10 minutes, the transfected COS-7 cells from each cuvette were gently pipetted into a 100mm diameter tissue culture dish containing 8ml of fresh pre-warmed post-electroporation COS-7 growth medium (section 2.6.1.2). The cells were incubated in 5% CO$_2$ at 37°C for 72 hours.

### 2.6.6 Treatment of COS-7 cell supernatants with DNase I (RNase-free).

72 hours post-electroporation, the COS-7 supernatants were removed from the tissue culture dishes by aspiration and transferred to sterile 15ml falcon tubes. The supernatants were spun for 10 minutes at 700g in a Sorvall RTH-750 rotor at room temperature in order to remove the cell debris.

The treatment of the supernatants with DNase I was essential, since during electroporation up to 80% of the cells die, releasing their contents (including DNA) into the supernatant. If the whole IgG antibody molecules produced by the COS-7 cells following transfection bound to this DNA, the IgG detected using an ELISA might be reduced or undetectable. This was demonstrated previously by (Rahman et al. 1998). Therefore the DNA was removed using DNase I.

The COS-7 cell supernatants were treated with DNase I (RNase-free DNase I, 776 785, Roche, Lewes, East Sussex, UK) at a final concentration of 7.5U per ml of supernatant, at 37°C. After one-hour, the action of the DNase I was halted by the addition of EDTA, pH 8.0 to the supernatant at a final concentration of 15mM. The EDTA chelates the magnesium ions required by the DNase I to function. Finally sodium azide was added to the supernatants (final concentration 0.5mM) and the supernatants were stored at 4°C until required.

### 2.6.7 Concentration of the COS-7 cell supernatants.

Due to the relatively low concentrations of IgG produced by the transient expression system, the IgG in the COS-7 supernatants was concentrated prior to ELISA analysis. The supernatants were concentrated using Centricon-YM30 (30 000 MW
cut-off) centrifugal concentrators (Amicon Bioseparations, Millipore, UK) according to the manufacturer's instructions.

2.6.8 Measurement of whole human IgG molecules in COS-7 cell supernatant

Since transfection of the COS-7 cells was carried out using two separate vectors, one encoding the heavy chain and the other the light chain, the COS-7 cells may express either whole IgGλ or only the heavy chain or only the light chain. In order to detect only the whole IgGλ molecules expressed by the COS-7 cells, an ELISA was used which relied on the presence of both the heavy and light chain for a positive signal. The capture antibody bound specifically to the Fc region and the conjugated detection antibody only recognised the λ light chain.

Measurement of the IgG production in the COS-7 cell supernatants, was carried out by Joanna Haley using the following method: A 96 well Maxisorp ELISA plate (Nunc) was marked vertically into two halves, the test half and the control half. The test half of the plate was coated with 50μl of Fc fragment specific goat anti-human IgG (I8885, Sigma) at 400ng/ml in BIG buffer (as described in section 2.1.1.5). The control half of the plate was coated with BIG buffer only. The plate was incubated overnight at 4°C. The plate was washed three times with 0.15M phosphate-buffered saline (PBS), pH 7.4 (Invitrogen) supplemented with 0.1% Tween 20 (PBS/0.1% tween). 100μl of PBS containing 2% BSA (Sigma) was then added to the wells to block non-specific binding of the IgG to the plastic. The plate was incubated for 1 hour at 37°C and then washed three times with PBS/0.1% tween. Concentrated COS-7 supernatants were serially diluted in sample/enzyme/conjugate-dilution (SEC) buffer (100mM tris-HCl, 100mM, 0.02% tween 20 and 0.2% BSA) and 50μl added to wells on both the test half and the control half. Purified human IgGλ (I4014, Sigma) of known concentration (usually 100ng/ml or 25ng/ml) was diluted serially to provide a standard curve relating optical density to IgG concentration. The plate was incubated for one hour at 37°C and then washed three times with PBS/0.1% tween. To detect bound antibodies, 50μl of goat anti-human λ chain specific alkaline phosphatase conjugate (A2904, Sigma) diluted 1 in 2500 in SEC dilution buffer, was added to the wells and incubated for one hour at 37°C. After one hour, the plates were washed three times with PBS/0.1% tween and once with BIC.
buffer. One p-nitrophenyl phosphate substrate tablet (104-105, Sigma) was dissolved in 5ml BIC buffer supplemented with a final concentration of 2mM MgCl\textsubscript{2}. 50\mu l of the substrate solution were added to each of the wells on the plate. The plate was then developed at 37\degree C and the optical density of the reaction was read after 30 minutes and 60 minutes at 405nm (reference 490nm) using an ELISA plate reader (Labsystems, Cheshire, UK). The final reading was calculated by subtracting the OD value of each control well from the OD value of the same sample in the corresponding test well. This ensures that only conjugate bound directly to IgG in the supernatant that had been captured by the anti-Fc IgG, would contribute to the result.

2.6.9 Detection of anti-dsDNA activity in the COS-7 supernatants by ELISA.
I measured the anti-dsDNA binding of the concentrated COS-7 cell culture supernatants using the ELISA described in detail in section 2.1.4.2.
2.7 Production of supervectors

This section describes the production of the supervectors, used in the experiments described in Chapters four and five of this thesis. As described in the COS-7 transient expression system (section 2.6.5) the expression vectors containing the heavy chain and light chain constructs can be transfected separately into eukaryotic cells. The heavy chain expression vector pG1D1 contains the dihydrofolate reductase gene (dhfr) whilst the light chain expression vector pLN10 contains the neomycin resistance gene (neo). Therefore in selective CHOdhfr\(^{-}\) growth medium (without ribonucleosides and deoxyribonucleosides, described in section 2.8.4.2) with the addition of neomycin, only those CHO cells which were transfected with both vectors and therefore possessed both a functional dhfr gene and a functional neo gene would survive. Consequently, the surviving cells would also have the ability to produce IgG heavy chains and light chains. However, although this system had been shown to work in the past (AERES Biomedical, Mill Hill, London UK), there is a risk of excess production of heavy chain compared to light chain since co-transfection with dhfr may be comparatively more productive than co-transfection with neo. Therefore, it was decided that the two vectors should be combined to produce “supervectors”. An EcoRI fragment containing the HCMV promoter, the \(\lambda\) constant region gene and the \(\lambda\) variable region gene (of the proposed autoantibody) was excised from the plasmid vector pLN10 and transferred into the vector pG1D1/B3V\(_H\).

In the experiments described in Chapters 4 and 5, IgG molecules consisting of B3V\(_H\) combined with four different \(V_\lambda\) chains are studied. These were produced by stable expression in CHO cell lines transfected with supervector plasmid DNA. Two of these supervectors and stable CHO cell lines were produced by Joanna Haley. The first designated SVBL encoded the ‘wild type’ B3V\(_H\) and B3V\(_\lambda\); the second designated SVBLX encoded B3V\(_H\) and B3V\(_\lambda\) except that a single point mutation, arginine to serine, had been introduced at position 27a in the CDR1 of the light chain variable region. I produced two further supervectors for subsequent stable expression in CHO cell lines. These combined B3V\(_H\) with one of two light chain hybrids, either B33 (B3V\(_\lambda\) CDR1, 33.H11V\(_\lambda\) CDR2 and CDR3) or BU (B3V\(_\lambda\) CDR1, UK4V\(_\lambda\) CDR2 and CDR3), whose production was described in section 2.5. These
two new supervectors were designated SVB33 and SVBU respectively. The supervectors were produced as described in the following section.

2.7.1 Production of the supervectors.

From the light chain hybrid vectors B33 and BU in pLN10 (produced as described in section 2.5) a cassette containing the HCMV promoter, the C\(_\lambda\) region sequence and the B33 and BU V\(_\lambda\) region sequences was cut out of the vector using an *EcoRI* restriction digest, whilst the recombinant vector B3V\(_h\)/pG1D1 (Figure 2.2) was linearised using an *EcoRI* restriction digest. The cloning method is shown in Figure 2.9. The restriction digests were performed using Promega (Southampton, UK) enzymes and buffers as recommended in the manufacturer’s instructions (1U of enzyme / \(\mu g\) of DNA for 1 hour at 37\(^\circ\)C).

The heavy and light chain DNA digestion products were run on a 0.7% agarose gel as detailed in section 2.5.4. The linearised heavy chain and the light chain expression cassette (2309bp for B33 and 2303bp for BU) were excised and extracted from the gel as detailed in section 2.5.5.

Initially, prior to gel purification, the linearised pG1D1/B3V\(_h\) was treated with calf intestinal alkaline phosphatase (CIAP) using Promega enzymes and buffers as recommended by the manufacturer’s instructions (0.01U enzyme per \(\mu g\) of 6917bp DNA for 30 minutes at 37\(^\circ\)C and then 10 minutes at 90\(^\circ\)C) to reduce the likelihood of it re-ligating to itself. CIAP catalyses the dephosphorylation or hydrolysis of 5’-phosphate groups from DNA. Therefore by removing the phosphate groups from both of the 5’ termini of the linearised heavy chain vector, the vector is unlikely to re-ligate. This change significantly increases the chance of obtaining colonies that contain the heavy chain vector and the insert (light chain sequence), following the ligation and transformation of this DNA into *E.coli*. However, I found that CIAP treatment substantially reduced the amount of pG1D1/heavy chain construct DNA obtained, possibly due to the heat inactivation step. Therefore I omitted this step and later had to screen for the correct plasmid (as described in section 2.7.2), among many colonies containing the re-ligated heavy chain.
Figure 2.9 Cloning method used to construct the supervectors by combining the light chain and heavy chain expression vectors

- **EcoRI** restriction sites in recombinant light chain expression vector, pLN10 containing V_\(\lambda\) cloned DNA sequences

- **EcoRI**-digested light chain cassette containing HCMV promoter, immunoglobulin leader sequence, light chain variable region DNA sequence and constant region DNA sequence

- Ligation of light chain cassette into **EcoRI**-linearised B3V_\(\lambda\)/pG1D1 heavy chain vector (as shown in figure 2.2) to produce the final supervector, containing all components required to produce whole IgG1.

**NB:** This figure depicts the enzyme site maps of pLN10 containing B3V_\(\lambda\) or V_\(\lambda\)-hybrid B33 and the supervector maps of SVB3 and SVB33. The V_\(\lambda\)-hybrid BU in pLN10 and supervector SVBU are essentially the same but since UK-4 V_\(\lambda\) is 6 bp shorter than the other light chains, hence the hybrid light chain BUV_\(\lambda\) is 6 bp shorter and thus SVBU is also 6 bp shorter at every site after and including BamHI (1624 bp instead of 1630 bp in pLN10 and 1625 bp instead of 1631 bp in the supervector).

**KEY**
- **Promoter**
- **Gene**
- Light chain variable region DNA sequence
- Light (\(\lambda\)) chain constant region DNA sequence
- Heavy chain variable region DNA sequence
- Heavy chain (\(\gamma1\)) constant region DNA sequence
- Introns between SA and SD not represented in final expression product
- Immunoglobulin leader sequence

SA Splice acceptor site
SD Splice donor site
AmpR Ampicillin resistance gene
dhfr Dihydrofolate reductase gene
HCMV Human cytomegalovirus
Materials and Methods

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### a) Materials

- **Recombinant pLN10**
  - 7496 bp

- **HCMV promoter**

- **EcoRI digest**

- **BssHII 5815**
- **NruI 6273**
- **SacI 1133**
- **HindIII 1224**
- **BamHI 1630**
- **SD 1635**
- **SA 1755**

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### b) Ligation of EcoRI digested lambda fragment into EcoRI linearised recombinant pG1D1 / B3 V_H

- **EcoRI 1**
- **EcoRI 2309**

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### c) SUPERVECTOR

- **9226 bp**

- **SV40 origin**
- **SV40 early promoter**
- **HCMV promoter**
- **HpaI 5284**
- **BglII 5996**
- **dhfr**

- **Light chain**
  - **Variable region**
  - **HindIII 1225**
  - **BamHI 1631**

- **Light chain**
  - **Constant region**
  - **HindIII 3538**

- **Heavy chain**
  - **Variable region**
  - **SD 3975**
  - **BamHI 3977**
  - **SA 4039**
  - **AgeI 4146**

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The light chain cassette was ligated into the linearised pG1D1/B3VH using T4 DNA ligase enzyme and buffers (Promega) according to the manufacturer’s instructions as detailed in section 2.5.6. After overnight incubation at 14°C, the ligation mixes were transformed into competent *E. coli* cells as before (see sections 2.5.7 and 2.5.8).

### 2.7.2 Screening the *E. coli* colonies for the supervector plasmid.

Since the CIAP treatment step was omitted, the majority of colonies produced by the transformed *E. coli* cells were likely to contain the re-ligated pG1D1/heavy chain vector. In order to find the colonies containing supervector a large number of colonies were picked and screened using the following ‘bug-cracking’ protocol.

For each supervector, 40 colonies were picked from the agar plates and individually added to 1ml of LB/ampicillin that was then incubated for 4-6 hours in a shaking incubator at 37°C. When the bacteria were just visible in the tubes, 50μl of culture was removed from each tube and stored at +4°C, the remaining culture was spun at 10000g in a microcentrifuge for 1 minute. The supernatant was removed and 50μl of cell lysis solution (1g of sucrose (Sigma), 250μl of 10%SDS (Sigma) and 80μl of 10M NaOH (Sigma) made up to 5ml in sterile double-distilled water) was added to each tube and the pellet re-suspended by vortexing. The tubes were left at 70°C in a waterbath for 10 minutes, vortexing halfway through the incubation time. After cooling the samples were loaded into a large (400ml) 0.8% agarose gel (containing ethidium bromide at 0.2μg/ml), TAE buffer was carefully added and the gel was run overnight at 40V. The gel was visualised under ultra-violet light and any lanes that contained a larger band noted (since the majority of bands were religated vector, a larger band on the gel could contain the desired insert). The previously saved tubes containing 50μl of culture corresponding to the larger band or bands were mini-prepped as described in section 2.5.2 and an *EcoRI* restriction digest was used (using Promega enzyme and buffers as recommended in the manufacturer’s instructions) to determine if these were indeed the desired supervector, or a dimer of heavy chain vector.
2.7.3 **Restriction digests to verify the identity of the supervectors.**

In order to verify the correct supervectors had been produced, a double restriction digest with *Hind III* and *Ava I* were carried out on SVB33 and SVBU alongside SVBL. The restriction digests were carried out using Promega enzymes and buffers according to the manufacturer’s instructions. The digestion products were separated on a 0.7% agarose gel as described in 2.5.4. The maps of the supervectors showing the restriction sites are shown in Figures 2.10, 2.11 and 2.12. After cutting with *Hind III* and *Ava I*, each of the supervectors gave different fragments distinguishable on an agarose gel. The sizes of the fragments also showed the orientation of the insert within the vector. The sizes of the fragments are detailed in the results section 4.6.
Figure 2.10  Hind III and Ava I restriction digest sites in supervector SVBL

Restriction digest map of supervector SVBL, the Hind III and Ava I restriction sites are shown in red.

KEY

- Promoter
- Gene
- Light chain variable region DNA sequence
- Light (\(\lambda\)) chain constant region DNA sequence
- Heavy chain variable region DNA sequence
- Heavy chain (\(\gamma\)) constant region DNA sequence
- Intron between SA and SD not represented in final expression product
- Immunoglobulin leader sequence

SA  Splice acceptor site
SD  Splice donor site
AmpR  Ampicillin resistance gene
dhfr  Dihydrofolate reductase gene
HCMV  Human cytomegalovirus
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SVBL SUPERVECTOR
9226 bp

Nhe I 6729
Pvu I 8605
AmpR promoter
EcoRI 2
Hind III 1225
Light chain variable region
Light chain constant region
Ava I 1858
EcoRI 2310
Mlu I 2360
SV 40 origin
SV40 early promoter
HCMV promoter
Hind III 3538
SD 3975
BamHI 1397
SA 4039
Age 14146
Heavy chain variable region
Heavy chain constant region

Ava I
5113
5058
4750
&
4715

Bgl II 5996
dhfr

HCMV promoter

Materials and Methods
Figure 2.11  Hind III and Ava I restriction digest sites in supervector SVB33

Restriction digest map of supervector SVB33, the Hind III and Ava I restriction sites are shown in red. SVB33 has an extra Ava I site at 1591 bp, derived from 33.H111Vλ, which is not present in SVBL.

KEY

Promoter
Gene
Light chain variable region DNA sequence
Light (λ) chain constant region DNA sequence
Heavy chain variable region DNA sequence
Heavy chain (γ1) constant region DNA sequence
Intron between SA and SD not represented in final expression product
Immunoglobulin leader sequence

SA  Splice acceptor site
SD  Splice donor site
AmpR  Ampicillin resistance gene
dhfr  Dihydrofolate reductase gene
HCMV  Human cytomegalovirus
Materials and Methods

SVB33  
SUPERVECTOR  
9226 bp
Figure 2.12  Hind III and Ava I restriction digest sites in supervector SVBU

Restriction digest map of supervector SVBU, the Hind III and Ava I restriction sites are shown in red. SVBU has an extra Ava I site at 1421 bp, derived from UK4Vλ, which is not present in SVBL.

KEY
- Promoter
- Gene
- Light chain variable region DNA sequence
- Light (λ) chain constant region DNA sequence
- Heavy chain variable region DNA sequence
- Heavy chain (γ1) constant region DNA sequence
- Intron between SA and SD not represented in final expression product
- Immunoglobulin leader sequence
- Splice acceptor site
- Splice donor site
- Ampicillin resistance gene
- Dihydrofolate reductase gene
- Human cytomegalovirus
Materials and Methods

SVBU SUPERVECTOR
9220 bp

Light chain variable region

Light chain constant region

Heavy chain constant region

Heavy chain variable region

SV40 early promoter

HCMV promoter

Nhe I 6723
Pvu I 8599
AmpR promoter
EcoRI 2
Hind III 1225

Ava I 1421

MCMV promoter

SV40 origin

HCMV promoter

Bgl II 5990

Ava I 1852

Mlu I 2354

SgII 3532

Hind III 3969

BamHI 3971

Sa 4033

Age I 14140

Heavy chain constant region

Ava I 1852

Light chain constant region

Ava I 1421

Light chain variable region

Hind III 1225

AmpR promoter

EcoRI 2

Hind III 1225

AmpR promoter

EcoRI 2

Hind III 1225

AmpR promoter

EcoRI 2
Chapter Two
Materials and Methods

2.8 Stable expression system in Chinese Hamster Ovary (CHO) cells
This section describes the methods for transfection of the supervector DNA into CHO cells to allow stable expression of recombinant human IgG molecules, as utilised in the experiments described in Chapters four and five of this thesis.

2.8.1 Preparation of supervector DNA for transfection
Large scale preparation of the supervector DNA was carried out using the plasmid maxi kit (Qiagen) as described in section 2.5.11. The DNA was quantified as described in section 2.5.12 and ethanol precipitated as in section 2.5.13.

2.8.2 Transient expression of supervectors in COS-7 cells.
Since production of CHO cell clones producing human IgG can take several weeks using the stable expression system, expression from the supervectors was first checked in COS-7 cells using the method described in section 2.6.5, except 10μl (2μg/μl) of supervector (single expression vector containing both the heavy and light chain sequence) DNA was added to a sterile autoclaved 1.5ml microcentrifuge tube. A separate tube was used for each supervector and a no DNA control transfection carried out as before. After 72 hours the supernatants were harvested and treated with DNase I (as described in 2.6.6) and human IgG concentration and anti-dsDNA binding of the supernatants were measured using the assays described in sections 2.6.8 and 2.1.4.2 respectively.

2.8.3 The CHOdhfr^- cell line.
The CHOdhfr^- mutant line used was the DXB11 line, which contains a single point mutated allele, the other dhfr allele having been deleted and the cells therefore lack a functional dihydrofolate reductase (dhfr) gene. The enzyme dihydrofolate reductase (DHFR) is responsible for the intracellular reduction of dihydrofolic acid to tetrahydrofolic acid, which is an important co-factor in the synthesis of nucleic acid precursors. To survive in culture, the CHOdhfr^- cell line required exogenous adenine, glycine, proline and thymidine for survival (Urlaub and Chasin 1980). Prior to transfection of the CHOdhfr^- cells, all of these nutritional requirements were provided via growth in non-selective medium. However, if these CHOdhfr^- cells were successfully transfected with supervectors that contained a functional dhfr gene (see Figure 2.9), the cells were transformed to a dhfr^+ phenotype. This
transformation allowed them to grow in selective medium that was depleted of ribonucleosides and deoxyribonucleosides. (The selective medium still contained proline though, as all CHO cells are proline-dependent even in the presence of dhfr gene). Consequently, CHOdhfr cells were used to provide an expression system in which the genes required to produce IgG could be co-transfected with the dhfr gene.

Methotrexate is a competitive inhibitor of the intracellular activity of the DHFR enzyme. Progressive selection of cells that are resistant to increasing concentrations of methotrexate leads to amplification of the dhfr gene, with concomitant amplification of IgG-encoding sequences that flank the dhfr sequences (Schimke 1984). In those resistant cells where amplification has occurred, there is not only sufficient free DHFR enzyme to generate intracellular tetrahydrofolate required for cell biosynthesis but also these resulting cell lines express high levels of immunoglobulin. All protocols used for the culture and transfection of the CHOdhfr cells were obtained from AERES Biomedical, Mill Hill, UK.

2.8.4 CHOdhfr cell culture media.

2.8.4.1 Non-selective CHOdhfr growth medium.
MEM α-Medium with ribonucleosides and deoxyribonucleosides (32571-028, Invitrogen), supplemented with 10% (v/v) foetal calf serum (10099-133, Invitrogen), and 50 units/ml penicillin/50 µg/ml streptomycin (15140-122, Invitrogen)

2.8.4.2 Selective CHOdhfr growth medium.
MEM α-Medium without ribonucleosides and deoxyribonucleosides (32571-029, Invitrogen), supplemented with 10% (v/v) dialysed foetal bovine serum (Hyclone SH0079.03, from Perbio Science, Tattenhall, UK), this fetal bovine serum had been tested for the absence of ribonucleosides, deoxyribonucleosides and human IgG and 50 units/ml penicillin/50 µg/ml streptomycin (15140-122, Invitrogen)

2.8.4.3 CHOdhfr freezing medium.
When creating a cell bank of CHOdhfr cells, the growth medium and selective pressure used (for example the methotrexate concentration) was identical to the
medium that the cells were cultured in immediately prior to freezing, except that the freezing medium contained 10% DMSO (D-5879, Sigma).

2.8.5 Maintenance of the CHOdhfr cells in culture.
The CHOdhfr cell line was grown in non-selective CHOdhfr growth medium in 175cm² flasks. All work carried out with these cells was performed under sterile conditions in a sterile tissue culture hood. Furthermore, all buffers and solutions used for this work were kept in the tissue culture laboratory and only used for this purpose. The CHOdhfr cells were maintained in continuous culture by changing the growth medium every three to four days, when the cells were almost confluent, using the trypsin method that was used for the COS-7 cells (see section 2.6.3).

2.8.6 Preparation of the CHOdhfr cells for electroporation.
The CHOdhfr cell line was transfected at as low a passage number as possible. Therefore a master cell bank of the CHOdhfr cell line stored in liquid nitrogen was essential. The freezing and thawing of the CHOdhfr cells was carried out using the same protocols as for the COS-7 cells, see section 2.6.2. As with the COS-7 cells (section 2.6.4), the cells were split 24 hours prior to transfection to ensure exponential growth. The next day whilst still growing exponentially, the CHOdhfr cells were harvested and re-suspended in sufficient PBS to produce a cell concentration of 1x10⁷ cells/ml, this followed the same protocol as for the COS-7 cells (see section 2.6.4).

2.8.7 Transfection of CHOdhfr cells by electroporation.
The CHOdhfr cells were electroporated (using the same method as that used for the COS-7 cells, see section 2.6.5) with 10μl (2μg/μl) of the supervector (expression vector containing both the heavy and light chain sequence together). A "no DNA" negative control was also carried out by electroporating CHOdhfr cells in the absence of any DNA. The transfected CHOdhfr cells from each cuvette were pipetted into a 100mm diameter tissue culture dish containing 8ml of pre-warmed non-selective CHOdhfr growth medium and were incubated in 5% CO₂ at 37°C overnight.
2.8.8 Selection of transfected CHO cells following electroporation.

Following overnight growth of the transfected CHO<sub>dhfr<sup>-</sup></sub> cells, the cells from each dish were trypsinized and pelleted in a bench top centrifuge. The cells were re-suspended in 100ml of pre-warmed selective CHO<sub>dhfr<sup>-</sup></sub> growth medium. The cells were divided equally between ten 100mm-diameter tissue culture dishes and incubated in 5% CO<sub>2</sub> at 37°C for 10-14 days. The selective CHO<sub>dhfr<sup>-</sup></sub> growth medium was changed every 3-4 days. After 10-14 days, all the cells in the ‘no DNA’ negative control dish were dead and foci of transfected cells were clearly visible in the other dishes. The cell foci could be seen by the naked eye without the aid of a microscope.

The foci were ‘picked’ in a sterile tissue culture cabinet, by viewing the cell foci under a low power light microscope and drawing up 200μl of medium immediately above a cell focus using sterile pipette tips. The cells from each focus were transferred into a well of a 24-well tissue culture plate, containing 1ml of pre-warmed selective CHO<sub>dhfr<sup>-</sup></sub> growth medium. 48 foci were picked for each transfection (approximately 4 or 5 foci from each of the 10 petri dishes). A fresh pipette tip was used for each focus, to prevent any cross-contamination.

The ‘picked’ cells were allowed to grow in selective CHO<sub>dhfr<sup>-</sup></sub> growth medium (in the 24 well plates) until almost confluent (usually after 7-14 days), whilst changing the medium every 7 days or as required. Great variation in the cell growth rates was seen with individual clones of cells, an observation that was accommodated in this and the following steps.

Once almost confluent, the medium from the individual wells was tested for antibody production using the whole IgG ELISA (see section 2.6.8). Those clones producing the highest levels of antibody were selected for expansion (into 6 well tissue culture flasks and then in small, medium and large tissue culture flasks) in selective CHO<sub>dhfr<sup>-</sup></sub> growth medium until the cells were growing in 175cm<sup>2</sup> tissue culture flasks. In order to identify those clones worthy of further analysis and development, the antibody production rates of individual clones were then determined as described in section 2.8.9.
2.8.9 Assay of human IgG production of the transfected CHO cells.

In order to compare the human IgG production of the expanded clones of CHO cells, the following method was used: The CHO cells were grown to just below confluence in selective CHODhfr\textsuperscript{-} growth medium, the cells were trypsinised and spun down in a bench top centrifuge (250g for 5 minutes at room temperature). The cells were then re-suspended in 20ml of pre-warmed selective CHODhfr\textsuperscript{-} growth medium and a viable cell count was carried out using trypan blue exclusion dye. The cells were pelleted again as before and re-suspended in sufficient pre-warmed selective CHODhfr\textsuperscript{-} growth medium at 1 x 10\textsuperscript{6} cells/ml. 1ml (i.e. 1 x 10\textsuperscript{6} cells) was added to a 100mm diameter tissue culture dish, containing a further 9ml of pre-warmed selective CHODhfr\textsuperscript{-} growth medium. The cells were grown to near confluency, by incubating the cells for 3 days in 5% CO\textsubscript{2} at 37°C. After three days, the supernatant of these cells was decanted and the concentration of intact whole IgG antibody assayed (after treatment with DNase I, section 2.6.6) using an ELISA, as in section 2.6.8. The cells from each tissue culture dish were trypsinised and counted using trypan blue.

From the new viable cell count and the antibody concentration in the decanted supernatants, it was then possible to calculate the level of antibody production in ng/10\textsuperscript{6} cells/day. A cell bank was created of those cell lines producing the highest amounts of whole IgG and further selection was carried out using methotrexate amplification.

2.8.10 Methotrexate amplification of the transfected CHO cells.

The CHO cell lines producing the highest levels of IgG were expanded in selective CHODhfr\textsuperscript{-} growth medium until they were growing in 175cm\textsuperscript{2} flasks. When the cells were nearly confluent they were trypsinised and spun down at 250g for two minutes at room temperature in a RTH-750 Sorvall rotor. The cells were re-suspended in 20ml of selective CHODhfr\textsuperscript{-} growth medium to produce a final viable cell count of 1 x 10\textsuperscript{6} cells/ml. 0.5ml (i.e. 5 x 10\textsuperscript{5} cells) of this solution was diluted in a further 49.5ml of pre-warmed 1 x 10\textsuperscript{-9}M methotrexate amplification medium (i.e. selective CHODhfr\textsuperscript{-} growth medium supplemented with 1 x 10\textsuperscript{-9} M methotrexate [Amethopterin A6770, Sigma]). The resulting culture was then divided equally between five 100mm-diameter tissue culture dishes, (i.e. 1 x 10\textsuperscript{5} cells/culture dish).
This dilution was required in order to produce pure foci that could be easily identified from one another on the plate. In addition a second set of 10 dishes were set up using amplification medium supplemented with $1 \times 10^{-8}\text{ M}$ methotrexate. This second higher concentration of methotrexate was used in case the initial $1 \times 10^{-9}\text{ M}$ concentration was too low for the cell line to produce single discrete foci. There was also the added advantage, that if cell foci developed in amplification medium supplemented with $1 \times 10^{-8}\text{ M}$ methotrexate, a greater amplification of the human IgG production could be achieved in a single step. The cells were incubated at $37^\circ\text{C}$ in $5\% \text{ CO}_2$ for 10-14 days. The amplification medium (freshly supplemented with the appropriate concentration of methotrexate, since the methotrexate was only stable for 1 week at this dilution) was changed every 3-4 days. After 10-14 days, foci of transfected cells were easily visible in most, but not all of the culture dishes. The foci in the plates containing media supplemented with $1 \times 10^{-9}\text{ M}$ methotrexate were very close together and there were discrete foci in the dishes containing media supplemented with $1 \times 10^{-8}\text{ M}$ methotrexate, therefore these dishes were kept and the former discarded.

24 foci were picked for each of the supervector clones (see results section 4.8.2) using the same method as before (see section 2.8.8) and were transferred into individual wells of a 24-well tissue culture plate containing 1ml of pre-warmed CHOdhdr$^-$ amplification medium (supplemented with $1 \times 10^{-8}\text{ M}$ methotrexate, the same level of methotrexate as the foci were selected with). The ‘picked’ cells were allowed to grow in the amplification medium until almost confluent (usually after 7-14 days), whilst the medium was changed every 7 days or as required. Once almost confluent, the medium from the individual wells was tested for antibody production as before, using the whole IgG ELISA (see section 2.6.8).

Those clones producing the highest levels of antibody were selected for expansion in amplification medium, until the cells were growing in 175cm$^2$ tissue culture flasks. The antibody production rates of individual clones were determined as described in section 2.8.9. A cell bank was created of the selected amplified cell lines. When freezing the amplified cells, it was important that the selective pressure was maintained i.e. the concentration of methotrexate was kept the same on freezing as that used in culture.
2.8.11 Large-scale production of affinity purified human IgG from the CHO cells. Many of the experiments described in Chapters 4 and 5 were carried out using the transfected CHO cells (and their supernatant) grown in 175 cm² tissue culture flasks. However, for future experiments and indeed for the intravenous injection of the recombinant human IgG into SCID mice (section 5.5), it was necessary to produce larger quantities of purified human IgG. This was achieved by culturing the CHO cells (in the same selective amplification medium they were already growing in) in a hollow-fibre tissue culture system. The human IgG was then affinity purified from the supernatant using a protein A column. The large-scale cell culture and affinity purification of recombinant human IgG, was carried out by Chemicon Europe Ltd, Hampshire, UK.

2.8.12 Investigation of recombinant human IgG binding to dsDNA and α-actinin. The binding to dsDNA and α-actinin, of the recombinant human IgG produced by the transfected CHO cells, was assessed using the ELISA assays described in sections 2.1.4.2 and 2.10.2 respectively. When measuring the binding of the recombinant human IgG to dsDNA and α-actinin, the CHO supernatants were first treated with DNase I (as described in section 2.6.6). The concentration of human IgG in the CHO supernatants was measured using the assay described in section 2.6.8.

When measuring the binding of the affinity purified recombinant human IgG to dsDNA, the antibodies were tested diluted in SEC buffer (section 2.6.8), both with and without DNase I treatment. In order to test the requirement for a ‘cofactor’ (which might be present in supernatant) to enable binding of the affinity purified antibodies to dsDNA (as discussed in Chapter four), the affinity purified antibodies were also tested in the ELISA after dilution in supernatant from cultured COS-7 cells, and treated with DNase I prior to assaying.

2.8.13 In vitro anti-nuclear antibody (ANA) binding of recombinant human IgG. The binding of the affinity purified recombinant human IgG, produced by the transfected CHO cells, to cell nuclei in vitro was assessed using slides containing a preparation of Hep2 cells (INOVA Diagnostics, San Diego, CA, USA) and also
using in-house produced rat, liver and kidney cryosections (routine immunology laboratory, UCL). The affinity purified antibodies were diluted to 25μg/ml and 12.5μg/ml in PBS (and for comparison, dilutions were also made in COS-7 cell supernatant, and pre-treated with DNase I, before adding to the Hep2 cells). The antibodies were incubated on the Hep2 cells (and rat liver and kidney sections) at room temperature for 20 minutes before washing with PBS for 3 x 5 minutes. Human IgG bound to nuclear antigens were detected by incubation at room temperature for 20 minutes with polyclonal rabbit anti-human fluorescein isothiocyanate (FITC) conjugate (Dako). After washing in PBS for 3 x 5 minutes the slides were mounted in Citifluor (AF1) anti-fadent mounting medium (Citifluor, London, UK) and viewed under a microscope using U.V light.
2.9 Testing pathogenicity of recombinant anti-DNA IgG in SCID mice

This section describes the procedures used for the experiments described in Chapter five. In order to investigate the pathogenicity of the recombinant human IgG molecules produced in section 2.8, four experiments were carried out implanting CHO cell lines producing recombinant human IgG (cell lines summarised in Table 5.1) into immunodeficient SCID mice. Once sufficient quantities of recombinant IgG had been purified, an additional experiment was conducted by direct intravenous injection (i.v.) of SCID mice with the recombinant human IgG produced by the CHO cell lines. The numbers of mice implanted with each cell line or injected directly with each recombinant human IgG antibody, are described in detail in Chapter 5.

2.9.1 SCID mice

As described in section 2.3.2.1, female Balb/C SCID mice were obtained from Harlan UK at 6-8 weeks of age and housed in sterile conditions. In the experiments described in sections 5.3 and 5.4, Balb/C SCID mice were not available due to supply problems at Harlan UK. Therefore, female CB-17 SCID mice at 6-8 weeks old were obtained from Charles River Laboratories, UK.

2.9.2 Implanting CHO cells into SCID mice

The mice were acclimatised and primed as described in section 2.3.3. Ten days after priming, the mice were each injected with $1 \times 10^6$ CHO cells re-suspended in 0.5ml of plain MEM $\alpha$-Medium, without ribonucleosides and deoxyribonucleosides (not supplemented with foetal bovine serum or antibiotics). In the experiment described in 5.3, some additional mice were injected with 10 times as many ($1 \times 10^7$) CHO cells in 0.5ml of media. In the experiments described in sections 5.3 and 5.4, the cells were implanted in the media in which they were cultured, that is the selective level of methotrexate was maintained and the foetal bovine serum and antibiotics were included. In addition to pristane only controls, other control mice received non-transfected CHOdhfr cells (section 5.2.1, Table 5.2). In later experiments (sections 5.2.2, 5.3 and 5.4), CHO cells transfected with the empty vector pG1D210 (contained no DNA encoding human $V_H$) were used as controls, for reasons explained in section 5.2.1. The vector pG1D210 was essentially the same as the pG1D1 vector, which was used to prepare the supervectors (Figure 2.2), the only differences were the presence of a HMCV enhancement and the absence
of a splice donor (SD) site. The CHO cells were transfected with pG1D210 by Joanna Haley and underwent two rounds of methotrexate amplification and were subsequently maintained in $1 \times 10^7$ M methotrexate.

2.9.3 Assessing pathogenicity of CHO cells producing recombinant human IgG
Proteinuria was assessed frequently using Albustix as described in section 2.3.3. On sacrifice, sera and organs were collected for further analysis. The levels of recombinant human IgG was measured in the mouse serum using the ELISA described in section 2.6.8. The morphology of the kidneys, liver and spleen were examined by haematoxylin and eosin (H&E) staining of paraffin wax embedded sections as described in section 2.3.5.1. Deposition of human IgG was looked for using immunohistochemistry as described in section 2.3.5.2 and electron microscopy section 2.3.5. In addition, the sera from the mice in section 5.4 were assayed for binding to dsDNA and $\alpha$-actinin (using the assays described in sections 2.1.4.2 and 2.10.2 respectively), prior to adding to the blocked plates, the sera were first serially diluted in PBS containing 10mM MgCl$_2$ and treated with 20U/ml of DNase I for 1hour at 37°C before stopping with EDTA (final concentration of 15mM).

2.9.4 Staining of chloroacetate esterase (CAE) in neutrophils.
The rapid identification of neutrophils in the kidney and liver was aided by staining for the presence of chloroacetate esterase. Neutrophil lysozomes contain chloroacetate esterase and the staining procedure utilises the ability of this esterase to breakdown naphtol AS-D chloroacetate, this releases naphtol, which reacts with a diazonium salt (Fast Red Violet) to form red deposits which are visible by light microscopy. This method stains all granulocytes, but neutrophils can also be confirmed by their characteristic nucleus under H&E staining. (This specialised staining procedure was done by histopathologists in the routine histopathology department, UCL).

2.9.5 Intravenous (i.v.) injection of recombinant human IgG into SCID mice.
Female 8 week old CB-17 SCID mice (as 2.9.1), were injected i.v. in the tail vein with 100$\mu$g (100$\mu$l at 1mg/ml) of the affinity purified recombinant human IgG (as obtained from Chemicon, see section 2.8.11). The antibodies were first all diluted to 1mg/ml (the antibodies were supplied by Chemicon at a range of concentrations from
1mg/ml to 2mg/ml) in sterile saline and sterile filtered through 0.2μm disc filters. Two mice were injected with each recombinant antibody or IgG1λ control as described in section 5.5. The mice were sacrificed 6 hours after injection of the antibodies and sera, kidney, liver and spleen collected. The kidneys were divided up and part was snap-frozen, part fixed in formal saline and part in Karnovsky’s fixative (these techniques have already been described fully in section 2.3.5) in anticipation of future analysis.

2.9.6 Direct immunofluorescence to detect human IgG in kidney sections from mice injected i.v. with recombinant human IgG.

Frozen sections, 5μm thick, of mouse kidney were air-dried for 30 minutes, and fixed with 4% paraformaldehyde (4g paraformaldehyde in 100ml PBS, heated up to 60°C until solution clears) for 10 minutes before washing in TBS (section 2.1.1.3). Any human IgG deposited in the kidney was detected by incubation for 1 hour at room temperature, in a humid slide chamber, with F(ab’)_2 fragment of rabbit anti-human IgG-FITC (F-315, Dako) diluted at 1:10 and 1:20 (as recommended by the manufacturer for direct immunofluorescence, stock at 1 mg/ml) in TBS. The slides were then washed in TBS and mounted in ‘Citifluor’ mounting medium. As a positive tissue control, frozen sections of kidney from a SCID mouse implanted with RH-14, which was known to give strongly positive staining for human IgG deposited in hyaline thrombi of its kidney glomeruli (see section 3.3, Table 3.4 and Figure 3.2 [A]), were stained alongside the sections from the mice injected i.v with recombinant human IgG, the hyaline thrombi in the positive control stained very strongly using this immunofluorescence method.
2.10 Binding of human anti-DNA antibodies to α-actinin

This section describes the methods used in the experiments described in Chapter six. The binding of human anti-DNA antibodies to α-actinin was initially investigated using whole sera, and subsequently using antibodies affinity purified from the sera of patients with SLE. The human anti-DNA mAbs, RH-14, B3, 33.H11, DIL-6 and the IgM ‘RT’ monoclonal antibodies (properties shown in Tables 2.1 and 2.2 respectively) were also tested for their binding to α-actinin. As negative controls in the anti-α-actinin ELISA, and in western blotting experiments, purified non-specific immunoglobulins were used. These were human IgG1λ purified myeloma protein (Sigma I-5029), which was isotype matched to RH-14, B3 and 33.H11, and human IgM (Sigma I-8260).

2.10.1 Selection of sera from patients with SLE.

Sera were selected at times when the patients exhibited high binding to dsDNA, as measured by the hospital routine laboratory service using a diagnostic ELISA kit (Shield Diagnostics, Dundee). The sera studied were taken from patients when their anti-DNA level was over 200 IU/ml (i.e. at least 4x the upper limit of normal, 50 IU/ml).

Two groups of patients were selected, depending on their scores for the renal component of the British Isles Lupus Assessment Group (BILAG) assessment. The BILAG index is a validated measure of clinical disease activity in SLE (Hay et al. 1993). The criteria for each category of the renal assessment are shown in Table 1.3. The first group of patients, from hereon referred to as ‘renal’ patients, had highly active renal disease as determined by kidney biopsy and a renal BILAG score of A or B (note patient 377 was recorded as a C, this patient was newly diagnosed and had highly active disease, with early renal involvement indicated by proteinuria of [+] ).

The second group, referred to hereafter as ‘non-renal’ patients, had either never had renal disease at all (renal BILAG score of E), or they had no evidence of current renal disease when the sera were collected, having had some renal involvement in the past (renal BILAG score of D). Sera were also obtained from 12 healthy controls (Colindale, U.K).
Table 2.3  Summary of patients with renal disease whose whole sera was used in 'binding to α-actinin experiments' (section 6.4.2)

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<th>Patient's Clinic No.</th>
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<th>Disease Duration (yrs)</th>
<th>Age</th>
<th>Gender</th>
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<th>Prednisolone (mg)</th>
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Ethnicity: c=Caucasian; AC=Afro-carribean; ME=Mixed ethnicity

Treatment groups: (I) no immunosuppressive drugs, (II) prednisolone up to 10 mg/day, (III) another immunosuppressive (with or without prednisolone) OR over 10 mg/day prednisolone alone.
Table 2.4  Summary of patients without renal disease whose whole sera was used in 'binding to α-actinin experiments' (section 6.4.2)

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<th>Disease Duration (yrs)</th>
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Ethnicity: c=Caucasian; AC=Afro-carribean; A=Asian.

Treatment groups: (I) no immunosuppressive drugs, (II) prednisolone up to 10 mg/day, (III) another immunosuppressive (with or without prednisolone) OR over 10 mg/day prednisolone alone.
Table 2.5 Summary of patients with renal disease from which anti-DNA antibodies were affinity purified in 'binding to α-actinin experiments' (section 6.4.3)

<table>
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Ethnicity: c=Caucasian; AC=Afro-carribean; A=Asian; ME=Mixed ethnicity.

Treatment groups: (I) no immunosuppressive drugs, (II) prednisolone up to 10 mg/day, (III) another immunosuppressive (with or without prednisolone) OR over 10 mg/day prednisolone alone.
Table 2.6 Summary of patients without renal disease from which anti-DNA antibodies were affinity purified in 'binding to α-actinin experiments' (section 6.4.3)

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Ethnicity: c=Caucasian; O=Oriental

Treatment groups: (I) no immunosuppressive drugs, (II) prednisolone up to 10 mg/day, (III) another immunosuppressive (with or without prednisolone) OR over 10 mg/day prednisolone alone.
Tables 2.3 - 2.6 show a summary of the clinical data and drug regimen of the patients at the date the sera samples were taken. The patients whose sera were used in the initial experiments investigating the binding of IgG from whole sera to α-actinin are shown in Tables 2.3 and 2.4. The patients, whose sera from which the anti-dsDNA antibodies were affinity purified prior to testing for binding to α-actinin, are shown in Tables 2.5 and 2.6. The patients received a variety of different drug treatments, to simplify comparisons between the ‘renal’ and ‘non-renal’ groups, the patients were divided into three treatment regimens, (I) those whom received no immunosuppressive drugs, (II) those on prednisolone up to 10 mg/day and (III) those receiving another immunosuppressive, either with or without prednisolone, or those that received over 10 mg/day of prednisolone alone.

2.10.2 Establishing an ELISA to measure human IgG and IgM binding to α-actinin

A solid-phase ELISA was developed to measure immunoglobulin binding to α-actinin. One half of a polystyrene 96-well plate was coated with 50μl/well of 20μg/ml α-actinin purified from chicken gizzard (A-9776 from Sigma, Poole, U.K.) diluted in BIC buffer, the other control half of the plate was coated with 50μl/well BIC buffer alone. The plate was left uncovered to evaporate overnight at 37°C and then washed three times with PBS before being blocked with 2% (w/v) BSA in PBS/tween for 1 hour at 37°C. The samples were diluted in PBS/tween containing 1% (w/v) BSA. 50μl/well of the diluted samples and positive control were added to the plate and incubated for 1.5 hours at 37°C. The plates were washed five times in TBS/tween. The bound antibodies were detected by incubation for 1 hour at 37°C with 50μl/well of affinity purified goat anti-human IgG (or IgM, as

---

1 For assaying the binding of the antibodies purified from the patient sera and the hybridoma cell culture supernatants, Nunc ‘Maxisorp’ microplates (VWR International, Poole, UK) were used, whilst optimal results were found using Linbro ‘EIA II’ microplates (ICN/Flow laboratories, U.K) when assaying human sera.

2 Evaporation overnight was a more successful method of coating the plate with α-actinin than either incubation at 37°C for one hour or overnight incubation at +4°C.

3 In this ELISA, 2% BSA w/v in PBS/tween was found to be more effective at blocking non-specific binding than 2% casein w/v in PBS/tween.

4 The hybridoma cell culture supernatants were titrated from neat up to 1:64 (and resulting OD values for anti-α-actinin binding were plotted against human IgG concentration, measured using the human IgG ELISA). The optimal dilution for measuring the binding of the patient serum samples to α-actinin was determined (after titration from 1:100-1:800, see section 6.4.1 and figure 6.7) to be 1:200. The samples affinity purified from the patients (section 2.10.4) were diluted to the concentration of the least concentrated sample (2.4 μg/ml), as measured using the human IgG ELISA.
appropriate) conjugated to alkaline phosphatase (Sigma) diluted in TBS/tween. After washing five times in TBS/tween and one time in BIC buffer the plates were developed with 50 μl/well of alkaline phosphatase substrate p-nitrophenol phosphate (Sigma 104-105) diluted in BIC buffer. Optical density was measured at 405nm with a reference filter 490nm.

Two monoclonal antibodies shown in other laboratories to give strong binding to α-actinin, were used as positive controls in optimising our assay. The first of these antibodies 3-5 (a kind gift from Anne Davidson, Albert Einstein College of Medicine, USA), is a human monoclonal anti-dsDNA IgM antibody derived by EBV transformation of PBLs from an SLE patient with lupus nephritis. Antibody 3-5, is pathogenic in SCID mice and binds to 100KD (α-actinin) as well as 70KD and 40KD proteins on a western blot of human mesangial cell extract (Marambio et al. 2002). The second antibody A52, a murine monoclonal anti-DNA IgG2b antibody (Mostoslavsky et al. 2001, a kind gift from Dan Eilat, Hadassah University Hospital, Israel) was used initially to establish the ELISA conditions but was superseded by human monoclonal antibody 3-5. The human monoclonal 3-5 was run on every plate to standardise inter-assay variation (representative standard curve is shown in Chapter six, Figure 6.1). As a negative control, duplicate wells containing only the diluent used in the assay usually PBS/tween containing 1% (w/v) BSA (for supernatants used appropriate medium), were always included on both the α-actinin coated and uncoated sides of the plate. On each side of the plate, the mean optical density (OD) reading obtained for the diluent was deducted from the other OD readings. The uncoated/sham coated OD readings were then deducted from the corresponding OD readings obtained on the α-actinin coated side.

The concentration of IgG or IgM present in the supernatants and affinity purified antibodies was determined using ELISAs measuring human IgG or IgM, as described in sections 2.1.2 and 2.1.3 respectively, by reference to the linear portion of a standard curve of purified human IgG or IgM run on every plate.

2.10.3 Inhibition ELISA of IgG binding to α-actinin

The inhibition ELISA was carried out using the same protocol described in section 2.10.2, except before adding the samples to the blocked plates the supernatant
samples were first pre-incubated with increasing concentrations of purified calf thymus dsDNA (as described in section 2.1.4.1) added at final concentrations of 0 - 2.5mg/ml to tubes containing a fixed dilution of supernatant. The tubes containing the supernatant and dsDNA were pre-incubated for 1.5 hours at 37°C and then 50μl/well added to the blocked plates and the ELISA completed as described in section 2.10.2.

2.10.4 **Affinity purification of anti-DNA antibodies from the sera of patients with SLE.**

Antibodies specific for binding to dsDNA were isolated from the patient sera using a simplified affinity purification method. Purification was carried out using dsDNA-cellulose prepared from calf-thymus DNA (Sigma D-8515). In order to remove any free dsDNA 5g of dsDNA cellulose was thoroughly washed for a total of 2.5 hours on a roller at +4°C in 5 changes of 50ml of tris-EDTA buffer, pH 8.0 (1.0 M tris-HCl containing 0.1 M ethylenediaminetetraacetic acid, 100x concentrate diluted in distilled water, Sigma T-9285). All washing steps consisted of mixing in 50ml falcon tubes followed by centrifugation at 1500 rpm for 10 minutes. For each patient, 0.5ml of serum was added to a 15 ml falcon tube containing 0.5g of thoroughly pre-washed dsDNA-cellulose (Sigma) suspended in 4.5ml of Tris-EDTA buffer containing 0.1% sodium azide (Sigma). Following continuous mixing overnight at room temperature, the dsDNA cellulose in each tube was rigorously washed for 5 hours with 10 changes of 15mls of Tris-EDTA. After the final spin, the dsDNA cellulose was resuspended in 2ml of Tris-EDTA and devided between 3 spin modules (a spin module consisted of an insert with a membrane which retained the dsDNA-cellulose but allowed the free flow of buffer and antibodies, the insert was placed inside of a 1.5ml collection tube, Anachem 2080-400). The tubes were spun briefly at 14 000 rpm in a microfuge to remove the Tris-EDTA buffer, which was discarded from the collection tubes. The bound antibodies were eluted from the dsDNA-cellulose by the addition of 1ml of 6M urea / 2M NaCl, after 2 minutes incubation, the tubes were spun for 1 minute at 14 000 rpm. The collected supernatants containing any eluted antibodies were immediately dialysed against 5L PBS overnight at +4°C (as small volumes, used 'Slide-A-Lyzer' dialysis cassettes with a 10KB molecular weight cut off membrane, from Perbio, catalogue no. 66380). Using this method I obtained between 2.4 and 24.5 μg/ml of human IgG per patient.
The dialysed antibodies were stored at 4°C with sodium azide at a final concentration 0.5mM.

This method proved a rapid way of purifying the anti-dsDNA antibodies from 18 patients in two days. Obviously the yield of anti-dsDNA antibodies was probably limited due to some of the capacity of the dsDNA-cellulose being occupied by DNA binding proteins from the sera. The yield could probably have been substantially increased by initial purification of IgG using protein G or A, followed by the specific purification of the anti-dsDNA using dsDNA-cellulose. However, this method yielded sufficient material for my experiments during which I only detected IgG anti-dsDNA by the use of anti-human IgG conjugated to either alkaline phosphatase (ELISA) or horseradish peroxidase (western blotting).

2.10.5 Western blotting.

2.10.5.1. Buffers for western blotting

1.5M Tris-HCl, pH 8.8
Dissolved 54.45g of tris base (Sigma T-6066) in approximately 150ml of distilled water, adjusted to pH 8.8 with concentrated HCl and made volume up to 300ml with distilled water.

1.0M Tris-HCl, pH 6.8
Dissolved 6g of tris base in approximately 60ml of distilled water, adjusted to pH 6.8 with concentrated HCl and made up to 100ml with distilled water.

10% Sodium dodecyl sulphate (SDS)
Added 10g of SDS (Sigma L-4390) to distilled water and stirred gently, when dissolved, the solution was made up to 100ml with distilled water.

10% Ammonium Persulphate
100mg of ammonium persulphate (Sigma A-7460) in 1ml of distilled water, freshly prepared before use.
**Chapter Two Materials and Methods**

*Tris Glycine SDS-PAGE Running Buffer, pH 8.3 - 8.7*

0.025M tris base, 0.192 M glycine (Sigma G-8898), 0.017M SDS. Prepared 1L of 10x stock solution with 29g tris base, 144g glycine and 10g of SDS, made up to 1L with distilled water.

*Immunoblot Transfer Buffer, pH 8.3 - 8.7*

0.012M tris buffer, 0.096M glycine, 20% methanol added fresh immediately prior to use. Prepared 500ml of a 25x stock solution (without methanol) with 18.1g tris base and 90g glycine, and made up to 500ml with distilled water.

*Washing Buffer pH 7.4*

PBS (made from tablets, Sigma p-4417) with 0.1% tween 20.

*Blocking Buffer*

5% non-fat milk (skimmed milk powder, Tesco) dissolved in washing buffer.

*2.10.5.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Purified α-actinin (Chicken gizzard, Sigma) was run on an 8% SDS-PAGE resolving gel. The α-actinin was diluted in Laemmli sample buffer (Bio-Rad 161-0737) and loaded on the gel at 10μg α-actinin / sample well.

*Preparation and running of 8% SDS-PAGE gel*

Prepared 15ml of 8% SDS-PAGE gel solution (enough for two 8cm² gels) by mixing 6.9ml of distilled water, 4ml of 30% acrylamide/Bis solution (40% acrylamide/Bis solution 37.5:1, Bio-Rad 161-0148, diluted to 30% with distilled water), 3.8ml 1.5M tris-HCl (pH 8.8), 150μl 10% SDS, 150μl 10% ammonium persulphate and 9μl of TEMED (N,N,N,N’- tetra-methyl-ethylenediamine, Bio-Rad 161-0800). For each gel, the gel mixture was pipetted between two glass plates that had been assembled in a Sci-plas mini-gel system (Sci-Plas). The gel was topped with a thin layer of 1-butanol (Sigma, BT-105), to prevent drying and allowed to polymerise for 90 minutes. 10ml of 5% stacking gel was prepared by mixing 6.8ml distilled water, 1.7ml 30% acrylamide, 1.25ml 1.0M tris-HCl (pH 6.8), 100μl 10% SDS, 100μl 10% ammonium persulphate and 10μl TEMED. When the gel had set, washed off the 1-
butanol with distilled water and added the stacking gel, before inserting a gel comb
to prepare the sample wells. After the stacking gel had polymerised for 30 minutes,
the comb was removed and the sample wells were flushed with running buffer. 10μl
of molecular weight markers containing 10 standard proteins of 10-250KD
(Precision Plus Protein Standards - All Blue, Bio-Rad) were added to every other
well to allow accurate determination of the sample proteins. 20μl of sample buffer
(1:1 with distilled water) containing 10μg α-actinin was added to each sample well.
The tank was topped up with cold running buffer and the gel was run at 125V, 50mA
for approximately 2 hours, until the 10KD marker was near the bottom of the gel.

*Electrotransfer to polyvinylidene fluoride (PVDF) membrane.*
The proteins separated on the SDS-PAGE gel were transferred to a PVDF membrane
(Immobilon-P transfer membrane, Millipore, IPVH00010) using the Sci-plas
electrotransfer system (Sci-Plas). The PVDF membrane was cut to the size of the gel
and activated in methanol for 15 seconds, before rinsing in distilled water for 2
minutes and left in transfer buffer until required. The gel was placed on top of the
PVDF membrane and sandwiched between filter papers and sponges soaked in
transfer buffer and held together in a plastic cassette. The two cassettes holding each
gel were placed in the Sci-plas tank full of transfer buffer and the proteins were
transferred from the gel to the membrane (from anode/+ve to cathode/-ve) for 2
hours at 100V, 200mA.

2.10.5.3 Immunoblotting (western blotting)
After electro-transfer the PVDF membrane was blocked, either overnight at +4°C or
at room temperature for 1 hour, with 5% non-fat milk in PBS/tween. Strips of the
membrane were cut which contained a lane of molecular weight markers and a lane
of resolved α-actinin. The strips were placed in a universal tube and incubated on a
roller overnight at +4°C with the primary antibody. The primary antibodies were
either the positive control rabbit anti-α-actinin (diluted 1:500, the antibody was
raised against chicken gizzard α-actinin, Sigma A-2543) or anti-dsDNA antibodies
purified from the patient’s sera (patients as described in Table 2.5 & 2.6, purified as
section 2.10.4) or the supernatants from the CHO cells producing recombinant anti-
DNA antibodies (section 2.8). The primary antibodies were diluted to 0.5μg/ml in
5% non-fat milk/PBS tween. In the absorption experiments described in section 6.4.4 (Figure 6.13), the primary antibodies at 0.5μg/ml were first incubated overnight at +4°C with calf thymus dsDNA at a final concentration of 50μg/ml, and then incubated with the blocked membrane for 1 hour at room temperature. After incubation with the primary antibody the strips were washed 1x quickly then 3x5 minutes in PBS/tween. The strips were then incubated for 1 hour at room temperature with the secondary antibody, either sheep anti-rabbit-HRP (Sigma) or goat anti-human IgG-HRP (Sigma A-8667) diluted 1:20,000 in 5% non-fat milk/PBS tween. The membrane strips were developed using an enhanced chemiluminescence system (Amersham, UK), equal volumes of two solutions were freshly mixed and the strips developed for 1 minute. The strips were then placed between two strips of acetate film and taped into a hypercassette (Amersham). In a dark room chemiluminescence film (Hyperfilm ECL, Amersham) was placed in the cassette for timed exposures and developed using a X-ograph automatic film processor.

2.11 Statistical analysis of data

Appropriate statistical tests were used to analyse the data throughout this thesis. The statistical tests which were employed are stated in the relevant results sections. The statistical analyses were performed using a statistics software package (Prism from Graphpad Software Inc.).
RESULTS CHAPTER THREE

Immunodeficient mice as a tool for assessing the pathogenicity of IgG produced by human hybridoma cell lines
CHAPTER THREE

IMMUNODEFICIENT MICE AS A TOOL FOR ASSESSING THE PATHOGENICITY OF IgG PRODUCED BY HUMAN HYBRIDOMA CELL LINES

3.1 Introduction and aims of this chapter

As described in the introduction (section 1.4.8.3), SCID mice were previously used to show for the first time that a human IgG anti-dsDNA monoclonal antibody, RH-14, was nephritogenic and that deposition of this antibody was sufficient to induce renal damage (Ravirajan et al. 1998). SCID mice implanted with hybridoma cells secreting RH-14, subsequently developed proteinuria and fluorescence staining showed the deposition of human IgG in the kidney. Electron microscopy (EM) of kidney sections from the SCID mice showed prominent subendothelial deposits, thickening of the glomerular basement membrane and patchy effacement of the podocyte foot processes. These changes, seen in the glomerular structures of the SCID mice, resemble the pathological changes in patients with lupus nephritis. However, light microscopy of the kidneys from mice implanted with RH-14 showed no evidence of leukocyte infiltration or fibrotic change. These results might have been due to the short duration (4-5 weeks) of antibody exposure, to the lack of functional T and B cells in SCID mice, or the failure of RH-14 to activate murine complement.

The SCID mice used in our laboratory's previous experiments (Ravirajan et al. 1998), were approximately 2 months of age, at which time they possess virtually no functional T or B cells. It is known that by 10-14 months of age virtually all SCID mice become 'leaky', that is they possess some mature lymphocyte clones. The aim of the initial study reported in this thesis, was to compare the pathology resulting from implanting hybridoma cells producing anti-DNA antibodies into older (eight month old) 'leaky' SCID mice, with that observed in relatively non-leaky younger (6-8 week old) SCID mice (Ravirajan et al. 1998). As a further comparison, the
effect of implanting the hybridoma cells into ‘non-leaky’ immunodeficient homozygous Rag-1 gene knockout mice (Rag-1<sup>-/-</sup>) was also investigated.

### 3.2 Implanting the SCID and Rag-1<sup>-/-</sup> mice with human hybridoma cells

Initially, the aim was to investigate the effect of implanting cells producing RH-14 into eight month old ‘leaky’ SCID mice and Rag-1<sup>-/-</sup> ‘non-leaky’ immunodeficient mice, and to compare any resulting pathological changes with those that had been observed in the two month old SCID mice in previous experiments. Three experiments were performed, because interesting results were observed in the older SCID mice implanted with RH-14, and also due to unreliable human IgG production by the human hybridoma cells. In addition to RH-14, a non-pathogenic (in SCID mice) human anti-DNA antibody DIL-6, and the mouse-human heteromyeloma fusion partner cell line CB-F7 were included as controls. The subsequent experiments also included human hybridoma cell lines producing two anti-phospholipid antibodies (CL24 and IS4) and a non-specific human IgGlκ antibody (TW, section 2.3.1) that was known not to bind to DNA. The human hybridoma cell lines are fully described in sections 2.2 (properties are shown in Table 2.1) and the control cell lines are described in 2.3.1. The numbers of mice receiving each hybridoma cell line in each experiment are shown in Table 3.1.

#### 3.2.1 Assessing the murine immunoglobulin of the SCID and Rag-1<sup>-/-</sup> mice

It is reported that ‘leakiness’ increases with age, this can easily be detected by assaying serum for murine immunoglobulin (Vladutiu 1993). Tail bleeding the mice was risky due to the increased risk of infection in these immunodeficient mice. However, in order to verify the levels of murine immunoglobulins before pristane priming, bleeding was carried out (day -11) in all of the eight month old SCID mice in experiments 1 and 2. Rag-1<sup>-/-</sup> mice are known to be non-leaky, to verify the absence of murine immunoglobulins, three of these mice were bled in experiment 1. The murine immunoglobulin levels were also measured in the sera of all mice on termination of each experiment. The serum murine IgM and IgG were measured using the solid-phase ELISAs as described in sections 2.3.4.1 and 2.3.4.2. Table 3.2 shows the numbers of mice in each experiment with murine immunoglobulin in their sera.
<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Mouse strain</th>
<th>Age at start of experiment</th>
<th>Number of mice/group</th>
<th>Hybridoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SCID</td>
<td>8 months</td>
<td>5</td>
<td>RH-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>DIL-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>CB-F7</td>
</tr>
<tr>
<td></td>
<td>Rag-1⁻/⁻</td>
<td>6 weeks</td>
<td>5</td>
<td>RH-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>DIL-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>CB-F7</td>
</tr>
<tr>
<td>2</td>
<td>SCID</td>
<td>8 months</td>
<td>5</td>
<td>RH-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>DIL-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>TW</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>CL24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>CB-F7</td>
</tr>
<tr>
<td>3</td>
<td>SCID</td>
<td>8 months</td>
<td>5</td>
<td>RH-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>IS4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>CB-F7</td>
</tr>
</tbody>
</table>

Table 3.1: **Summary of the hybridomas implanted into immunodeficient mice**

The table summarises the numbers of mice of each strain and age, which were implanted with each human IgG producing hybridoma cell line, in each of three experiments. Pristane primed only negative controls were also included in every experiment. The hybridomas were all implanted on day 0, 10 days after pristane priming.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mice and age</th>
<th>Time point of bleed (day)</th>
<th>Murine immunoglobulin</th>
<th>Number of mice With Ig in sera</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SCID 8 months</td>
<td>-11</td>
<td>IgM</td>
<td>14/15</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgM</td>
<td>14/15</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-11</td>
<td>IgG</td>
<td>3/15</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgG</td>
<td>3/15</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rag-1&lt;sup&gt;−−&lt;/sup&gt;</td>
<td>-11</td>
<td>IgM</td>
<td>0/3*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgM</td>
<td>0/15</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-11</td>
<td>IgG</td>
<td>0/3*</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgG</td>
<td>0/15</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 SCID 8 months</td>
<td>-11</td>
<td>IgM</td>
<td>20/20</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgM</td>
<td>19/20</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-11</td>
<td>IgG</td>
<td>7/20</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgG</td>
<td>11/20</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCID 2 months</td>
<td>-11</td>
<td>IgM</td>
<td>n/m</td>
<td>n/m</td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgM</td>
<td>8/27</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-11</td>
<td>IgG</td>
<td>n/m</td>
<td>n/m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>IgG</td>
<td>4/27</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Murine IgM and IgG in the sera of immunodeficient mice
Murine IgM and IgG were measured in the sera prior to pristane priming on day -11, and on termination of the mice at the end of the experiment. * In experiment 1, only 3 representative Rag-1<sup>−−</sup> mice were tail bled to minimise the infection risk to the mice. For the same reason, in experiment 2, the 2 month old SCID mice were not bled prior to pristane priming (n/m = not measured), just on termination. Murine immunoglobulins were not measured in experiment 3, since no significant human IgG was present in these mice and therefore no further information was to be gained from this experiment.
It was confirmed that the eight month old SCID mice were extremely 'leaky', with 93-100% of mice having murine IgM in their sera before the experiment and 93-95% of mice on termination. 20-35% of the eight month old SCID mice, also had murine IgG before the experiment and 20-55% on termination. In contrast, as expected the Rag-1\(^{-/}\) mice had no murine immunoglobulins present in their sera at any time. The 2 month old SCID mice, in experiment 2, were not bled before implanting the hybridoma cells. However, the terminal bleeds from the 2 month old mice, show that they were much less 'leaky' than the eight month old SCID mice, with only 30% and 15% of two month old mice having serum murine IgM and IgG respectively (Table 3.2).

### 3.2.2 Production of human IgG by hybridoma cells implanted in the SCID and Rag-1\(^{-/}\) mice

The main difficulty encountered throughout these experiments was the unreliable secretion of human IgG by the hybridomas. The \textit{in vitro} production of human IgG by the hybridoma cell lines was always measured regularly using the human IgG ELISA described in section 2.1.2. All of the hybridoma cell lines were secreting human IgG, at high OD values, when implanted into the SCID mice. However, on termination of the experiments many of the mice had no detectable human IgG present in either their sera or ascites fluid. Table 3.3 summarises the presence of human IgG, in the sera of each group of mice, at the end of the three experiments. Due to the inconsistent secretion of human IgG by the hybridomas, comparison of the effects of different human IgG in the different strains and ages of mice was made more difficult. Table 3.3 also summarises, for each group of mice, the mean day of termination. Termination was necessary when a 20\% increase in body weight occurred, due to the growth of the hybridoma cells leading to development of ascites (excess fluid in the peritoneal cavity).

### 3.2.3 Failure of RH-14 to grow in the Rag-1\(^{-/}\) mice

Unfortunately, the hybridoma cells did not grow very well in the Rag-1\(^{-/}\) mice. As shown in the summary Table 3.3, only 2/5 mice implanted with RH-14 developed ascites. 4/5 of the Rag-1\(^{-/}\) mice implanted with DIL-6 developed ascites, but none of the mice in this group had detectable human IgG in their sera. Table 3.4 shows a summary of the results for the Rag-1\(^{-/}\) mice implanted with RH-14 and the control
Chapter Three Immunodeficient mice as a tool for assessing the pathogenicity of human IgG

Table 3.3 Summary of human IgG production in three experiments implanting human hybridomas into immunodeficient mice

This table summarises the numbers of mice in each group that had human IgG present in the sera on termination of the experiments (expressed in terms of the number of mice with human IgG in the sera, detectable using ELISA / total number of mice implanted in that group).

It can be seen, that only the 8 month old SCID mice implanted with RH-14, in experiment 1 and CL24 or TW in experiment 2, had significant levels of human IgG in their sera (groups highlighted in yellow). The results for these three groups of SCID mice are discussed in more detail in section 3.3 and table 3.5.

Only 2/5 Rag-1^− mice implanted with RH-14 had any human IgG in the sera and ascites fluid (highlighted in turquoise), these results are discussed in section 3.2.3 and table 3.4.

Also shown in the table, are the mean day at which the mice in each group were terminated, usually due to a 20% increase in weight through development of ascites. In the Rag-1^− mice the hybridoma cells did not grow very well and ascites did not develop in *3/5 and **1/5 mice in these groups, hence these mice were killed on day 68 at the end of the experiment (which skews the mean data for these two cases).
<table>
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<tr>
<th>Experiment number</th>
<th>Immunodeficient mouse strain and age</th>
<th>Hybridoma cells implanted in this group of mice</th>
<th>Mean day of termination of mice in this group</th>
<th>Number of mice with human IgG in sera on termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SCID 8 months</td>
<td>RH-14</td>
<td>37</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIL-6</td>
<td>28</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CB-F7</td>
<td>20</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Rag-1 deficient</td>
<td>RH-14</td>
<td>60*</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIL-6</td>
<td>37**</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CB-F7</td>
<td>28</td>
<td>0/4</td>
</tr>
<tr>
<td>2</td>
<td>SCID 8 months</td>
<td>RH-14</td>
<td>21</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIL-6</td>
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<td>4/5</td>
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<td></td>
<td></td>
<td>CB-F7</td>
<td>22</td>
<td>0/2</td>
</tr>
<tr>
<td>3</td>
<td>SCID 2 months</td>
<td>RH-14</td>
<td>23</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
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<td>CB-F7</td>
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<td>0/3</td>
</tr>
<tr>
<td></td>
<td>SCID 8 months</td>
<td>RH-14</td>
<td>24</td>
<td>0/5</td>
</tr>
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<td>1/5</td>
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<tr>
<td></td>
<td></td>
<td>CB-F7</td>
<td>19</td>
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</tbody>
</table>

Table 3.3
Table 3.4 Rag-1<sup>−/−</sup> mice implanted with hybridoma cells producing human anti-dsDNA monoclonal antibody, RH-14

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Ascites (day)</th>
<th>HuIgG (μg/ml in Ascites)</th>
<th>Anti-dsDNA IgG (O.D at 405 nm)</th>
<th>Proteinuria (plus score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH14</td>
<td>*68</td>
<td>0</td>
<td>0.011</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>26</td>
<td>30</td>
<td>++</td>
</tr>
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<td></td>
<td>42</td>
<td>57</td>
<td>57</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>*68</td>
<td>0</td>
<td>0.001</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>*68</td>
<td>0</td>
<td>0.002</td>
<td>+</td>
</tr>
<tr>
<td>CBF7</td>
<td>28</td>
<td>0</td>
<td>0.0165</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>0.0025</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>0.0025</td>
<td>trace/+</td>
</tr>
</tbody>
</table>

The data shown, is from sera collected the day that the experiment was terminated, when ascites had developed. Mice indicated (*) were terminated at day 68 when ascites had failed to develop. Only 2/5 mice implanted with RH-14, had human IgG in their sera or ascites fluid, as measured using the human IgG ELISA (section 2.1.2). This was confirmed by measuring serum human IgG binding to dsDNA by ELISA (2.1.4.2). Proteinuria was only slightly elevated in one mouse and this did not reflect the mouse with the highest level of human IgG anti-dsDNA antibodies. Proteinuria was measured with Albustix (Bayer diagnostics), plus score: + = 0.3, ++ = 1.0 g/L.
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CB-F7 cells. As seen in Table 3.4, only two mice had low levels of RH-14 in sera and ascites fluid, they had no significant proteinuria and their kidney morphology was normal and showed negative staining for human IgG.

3.3 Formation of hyaline thrombi in the kidneys of eight month old SCID mice implanted with RH-14.

As described in section 3.2.2 and Table 3.3 significant levels of human IgG were only detected in a small number of the eight month old SCID mice, namely after implantation with either RH-14 (in experiment 1), or CL24 and TW (experiment 2). Further details of the findings in these mice are shown in Table 3.5. The human IgG present in the sera or ascites fluid of individual mice, within groups implanted with the same hybridoma, varied widely from over 5mg/ml to under 2 µg/ml. Only the mice implanted with RH-14 were positive, when their sera were measured for human IgG binding to dsDNA by ELISA. The SCID mice implanted with RH-14 had significant proteinuria, which was greatest in the mice with high levels of human IgG in the serum and ascites fluid (Table 3.5). The level of proteinuria ++ - +++ (1.0-3.0 g/L) was higher than that found in the control groups, but was not as marked as that which one would find in a murine model of lupus such as the MRL Ipr/lpr mouse, (which commonly have between 3.0 and 20 g/L). The mouse that had the lowest amount (23.8µg/ml) of human IgG in its serum, also had the lowest proteinuria.

Histological examination of the organs from the mice was carried out as described in the methods (section 2.3.5). Haematoxylin and eosin (H&E) staining of the kidneys showed that 4/5 mice implanted with RH-14 had hyaline thrombi in the glomeruli and in some peritubular capillaries (the mouse which had ++ for histology in Table 3.5, is shown stained with H&E in Figure 3.1), these thrombi were positive when stained for the presence of human IgG (Figure 3.2, A) and fibrin (Figure 3.2, B & C). These thrombi were most numerous in the mouse with the highest levels of RH-14, being present in all the glomeruli of the sections that were stained. The mice that had been implanted with CL24, TW and CB-F7, all had normal kidney morphology (H&E staining of kidneys from mice implanted with CL24 and TW are shown in Figure 3.1) and had no deposition of human IgG. All liver, spleen, and skin sections from RH-14 treated SCID mice showed normal morphology and were negative for human IgG staining. Experiment 2 was conducted in order to directly compare these
Table 3.5  ‘Leaky’ eight month old SCID mice implanted with hybridoma cells producing human monoclonal antibodies

This table summarises the data from the eight month old SCID mice, implanted with the human hybridomas which did produce human IgG after implantation. Definitions: Ascites = the day when the mice were terminated due to development of ascites, causing a 20% increase in body weight. Mouse IgM and IgG were measured by ELISA before pristane priming and on termination of the experiment to assess the ‘leakiness’ of the mice. Human IgG was measured by ELISA in the sera and ascites fluid on termination of the experiment (section 2.1.2). IgG binding of the sera to dsDNA was measured by ELISA on termination of the experiment (2.1.4.2). Proteinuria was measured throughout the experiment, the values shown here are for the day the mice were killed (+ = 0.3, ++ = 1.0, +++ = 3.0 g/L of protein). The histology refers to staining of the kidneys from the mice, and shows the presence or absence of hyaline thrombi by haematoxylin and eosin (H&E) staining; positive or negative immunohistochemical (IC) staining for human IgG (Hu IgG) deposition, and for fibrin (MSB, martius yellow, brilliant crystal scarlet and soluble blue and PTAH, phosphotungstic acid haematoxylin). ‘+’ represents positive staining present in >50% of glomeruli, ‘++’ represents positive staining in all of the glomeruli in the kidney section.
<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Ascites (day)</th>
<th>Mouse IgM Optical Density values at 410nm (O.D.)</th>
<th>Mouse IgG Optical Density values at 410nm (O.D.)</th>
<th>Human IgG µg/ml</th>
<th>Anti-dsDNA (O.D.)</th>
<th>Proteinuria (terminal)</th>
<th>Histology</th>
<th>Thrombi</th>
<th>Hu IgG</th>
<th>Fibrin</th>
</tr>
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<tr>
<td></td>
<td>day -11</td>
<td>terminal</td>
<td>day -11</td>
<td>terminal</td>
<td>ascites</td>
<td>serum</td>
<td>serum</td>
<td>plus score</td>
<td>H&amp;E</td>
<td>IC</td>
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<td>280</td>
<td>1254</td>
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<td>+</td>
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<td>5760</td>
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<td>++/+++</td>
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<td>0.0</td>
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<td>352</td>
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<td>0.3</td>
<td>0.0</td>
<td>0.1</td>
<td>2</td>
<td>4.5</td>
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<td>0.4</td>
<td>0.0</td>
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<td>270</td>
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<td>0.0</td>
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<td>0.003</td>
<td>+</td>
<td>-</td>
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<td>0.0</td>
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<td>0.9</td>
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<td>0.003</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.5
Figure 3.1  Haematoxylin and eosin (H&E) staining of kidney sections from eight month old SCID mice implanted with human hybridoma cells

H&E staining of paraffin wax sections of kidney from eight month old SCID mice implanted with either RH-14, CL24 or TW. The presence of hyaline thrombi in a representative glomerulus, can clearly be seen (as indicated by the arrow), in the section from a mouse implanted with human hybridoma cells producing RH-14, the pathogenic anti-dsDNA IgG. Kidney sections from the mice implanted with hybridoma cells producing either CL24, the anti-phospholipid IgG, or TW an irrelevant human IgG, show normal morphology.

(Magnification is x 400)
Immunodeficient mice as a tool for assessing the pathogenicity of human IgG
Figure 3.2  Staining of hyaline thrombi in the kidney of an eight month old SCID mouse implanted with RH-14

These three sections are all from the kidney of an eight month old SCID mouse, implanted with RH-14. Figure (A) shows positive staining of human IgG deposited in hyaline thrombi both in the glomerulus (as indicated by the solid arrow) and peritubular capillaries (as shown by the dotted arrow). The human IgG is stained brown by rabbit anti-human IgG-HRP, PAP system and DAB (as described in section 2.3.5.2). Figure (B) shows positive staining of fibrin in the hyaline thrombi, in both the glomerulus and peritubular capillaries. The fibrin is stained red by the MSB stain (as described in section 2.3.5.3). Figure (C) shows the fibrin present in the hyaline thrombi, stained purple using another stain PTAH (as described in 2.3.5.3).

Magnification is x 400
Immunodeficient mice as a tool for assessing the pathogenicity of human IgG

A

B

C
findings in the eight month old SCID mice, in parallel with two month old mice, using the same batch of RH-14 cells. Unfortunately, as shown in Table 3.3, the hybridomas failed to secrete RH-14 in experiment 2, so I can only compare my results in eight month old mice (experiment 1) with our group's previous published experiments in 2 month old SCID mice (Ravirajan et al. 1998).

When the kidneys from the mice implanted with RH-14 were examined using EM, the presence of the hyaline thrombi with fibrin deposition was very clear. Electron microscopy revealed that RH-14 deposition resulted in a lesser degree of pathological change in the eight month old SCID mice, than was previously reported in two month old SCID mice (Ravirajan et al. 1998). In the eight month old SCID mice implanted with RH-14, there was no effacement of the foot processes or thickening of the basement membrane. However, there was occasional ischaemic-type wrinkling in paramesangial area, electron-dense fibrils, possibly fibrin within the mesangium and in one of the observed loops a degree of interposition of the glomerular basement membrane was noted.

3.4 Were murine immunoglobulins involved in hyaline thrombi formation?
The observation of hyaline thrombi in the eight month old leaky SCED mice, which had not been seen in the two month old SCID mice (Ravirajan et al. 1998) was intriguing. This finding posed the question, were the thrombi found in the older 'leaky' SCID mice, due to the additional presence of murine immunoglobulins, changing the type of glomerular lesion caused by RH-14? These thrombi were not observed in the SCID mice implanted with CL24 or TW, and these mice also had murine IgM or IgG antibodies present in their sera. Might the thrombi result from deposition of complexes of human and murine immunoglobulin, but only bind to the kidney when the human immunoglobulin can bind specifically as in the case of RH-14?

In order to address this question I carried out two further experiments. Since hyaline thrombi are intraluminal aggregates of IgG and IgM complexes, which cause occlusion of the capillary lumen, I wondered whether murine anti-human IgG antibodies (IgM or IgG) were forming complexes in these 'leaky' SCID mice. I attempted to look for complexes of murine and human antibodies in the sera, by
incubating the sera in ELISA plates coated with anti-human IgG and subsequently
detecting any complexes using anti-murine IgM and IgG conjugated to alkaline
phosphatase (as described in section 2.3.4.3). Using this method it was determined
that there were no detectable complexes of human and murine immunoglobulins
present in the sera of the SCID mice. I also looked directly at the thrombi for the
presence of murine IgM and IgG by immunohistochemistry in kidney sections
(section 2.3.5.4). The positive control tissue, MRL-\textit{lpr}/\textit{lpr} spleen, was positive for
murine immunoglobulin but the vast majority of the renal thrombi were negative
when stained for murine IgM or IgG. Therefore these antibodies do not appear to be
involved in the thrombi formation. There were occasional thrombi with very limited
staining, however the murine antibodies probably adhered to the already formed
thrombi.
3.5 Discussion of Results from Chapter Three

Conducting experiments with antibodies produced by human anti-DNA hybridomas is fraught with difficulty, due to the unstable nature of such human hybridoma cell lines, and their inconsistent secretion of anti-DNA antibodies. The lack of human hybridoma cell growth in the Rag-1\(^{-/-}\) mice was disappointing, since these mice have been successfully been used to grow murine anti-DNA antibody producing hybridoma cells by other authors (Mostoslavsky et al. 2001). However, interesting results were found in the eight month old SCID mice implanted with RH-14.

The primary conclusion is that, as was found in the younger SCID mice (Ravirajan et al. 1998), RH-14 deposits in the kidneys of the eight month old ‘leaky’ SCID mice, and the mice develop proteinuria. The binding of RH-14 is probably enhanced by its ability to bind nucleosomes and histones as well as single and double-stranded DNA (see Table 2.1). As discussed in the introduction, there is considerable evidence to suggest that some anti-DNA antibodies bind to the kidney via nucleosomes or histones (Berden et al. 1999). The other anti-DNA monoclonal antibody, DIL-6 does not bind either nucleosomes or histones, and does not deposit in the kidneys of SCID mice of either age group. The kidneys of the eight month old SCID mice implanted with RH-14, showed no evidence of greater pathological changes, reminiscent of those seen in patients with lupus, than did the younger SCID mice (Ravirajan et al. 1998). In fact, EM examination of the kidney sections from these mice, found less pathology in terms of a lack of podocyte foot process effacement and no thickening of the GBM.

However, interestingly in these older ‘leaky’ SCID mice it appears that RH-14 deposition in the kidney caused the development of hyaline thrombi. Hyaline thrombi are intraluminal homogeneous deposits that often occlude the capillary lumen, they are acellular and electron-dense, they do not usually contain significant amounts of fibrin. Hyaline thrombi are seen less frequently than other signs of inflammation in lupus nephritis, and are sometimes related to the presence of cryoglobulins in the serum. Hyaline thrombi have been observed previously in SCID mice, Rag-1\(^{-/-}\) mice, and also (MRL/lpr x BALB/c) F1 mice, implanted with murine anti-DNA antibodies (Vlahakos et al. 1992b; Mostoslavsky et al. 2001; Itoh et al. 1993).
As discussed in section 1.4.8, some but not all human anti-dsDNA antibodies produced by hybridoma cells bind to the kidney when implanted into SCID mice, and these antibodies may exhibit different localisation of binding (Ravirajan et al. 1998; Ehrenstein et al. 1995). This variability in antibody pathogenicity or pattern of kidney localisation has also been demonstrated for murine monoclonal anti-DNA antibodies (Vlahakos et al. 1992b; Mostoslavsky et al. 2001; Itoh et al. 1993) with some anti-DNA antibodies exhibiting mesangial deposition, others resulting in cell-proliferative lesions associated with macrophage infiltrates or in subendothelial and intraluminal hyaline deposits. It is likely that specific features of the antigen binding site of individual antibodies, influences the site of antibody deposition. However, the interesting observation herein, was that the human monoclonal antibody RH-14, showed a different localisation of binding in two sets of experiments. The most apparent difference between these experiments being the age of the SCID mice, that is two months in the original experiments (Ravirajan et al. 1998) and eight months of age in this case.

The hyaline thrombi occurred in the most glomeruli, in the kidney of the mouse that had the highest level of RH-14 in its serum. Some mice, shown in Table 3.4, had human IgG levels in the sera and ascites that were ten times greater than those observed in the previous experiment (Ravirajan et al. 1998), in which human IgG levels were between 320-390 μg/ml. However, the presence of hyaline thrombi may not simply be dependant upon the quantity of human IgG in the sera of the mice, since the thrombi were also present in a mouse that only had 352μg/ml of RH-14 in its serum (shown in Table 3.5). This was equivalent to the level of RH-14 in the previous experiment, in which no hyaline thrombi occurred (Ravirajan et al. 1998). The formation of hyaline thrombi was likely to have been dependent on the binding specificity of RH-14, since mice implanted with CL24 or TW, some of which had human IgG of over 2mg/ml in their ascites fluid, showed no hyaline thrombi or deposition of antibody. However, the level of human IgG measured in the serum at the end of the experiments (on sacrifice) may not accurately reflect the levels which were present at the time of the hyaline thrombi formation. It is possible that the RH-14 production in this experiment involving the eight month old SCID mice was initially or cumulatively higher than that which occurred in the two month old SCID mice.
Chapter Three Immunodeficient mice as a tool for assessing the pathogenicity of human IgG

The limited period of antibody exposure (due to ascites formation) in mice implanted with hybridoma cells has always been a problem with this type of experiment, since the time period may not be sufficient to allow development of pathology, as a result of antibody deposition. The two month old mice showed greater pathology, in terms of foot process effacement and thickening of the GBM, than the eight month old mice. However, the number of days for which the mice were exposed to RH-14 prior to termination, due to ascites formation, was not significantly different in this experiment (mean 37 days, table 3.3), as compared to the previous experiment (mean 30 ± 3 days, [Ravirajan et al. 1998]). It is possible that rapid deposition of RH-14 as hyaline thrombi in the capillary lumen of the eight month old mice, rather than as the subendothelial deposits seen in the two month old mice, meant there was less visible secondary damage, such as thickening of the GBM or foot process effacement, within the same time frame.

SCID mice are known to possess a restricted number of mature B cell clones, which increase with age and are usually specific for antigens that they may have encountered such as self-antigens or pathogens (Hinkley et al. 2002). Indeed, the eight month old SCID mice did possess significant quantities of endogenous IgM antibodies, and some mice even had murine IgG antibodies (table 3.5). The presence of more murine immunoglobulins in the eight month old mice, posed the question of whether these antibodies played a role in altering the pathology observed in these 'leaky' SCID mice. Possible support for this hypotheses was provided by Ito et al., who showed remodelling of glomerular lesions in SCID mice, by non-nephritogenic bystander IgM antibodies (Ito et al. 2000). The glomerular lesions, in this case, were caused by injection of a nephritogenic murine monoclonal anti-DNA antibody derived from an MRL-lpr mouse (Itoh et al. 1993). The lesions were remodelled from a wire-loop type glomerular lesion to a cell-proliferative type lesion. The authors suggested that the bystander IgM (raised against trinitrophenyl), which caused no nephritis by itself, might modify or accelerate the lupus nephritis by entrapment in the glomerular lesions.

In my experiments, the lesions were not of the cell-proliferative type, but were changed from the simple immune deposition, which was previously observed in the two month old mice (Ravirajan et al. 1998), to the thrombi seen in the 'leaky' eight
Chapter Three Immunodeficient mice as a tool for assessing the pathogenicity of human IgG

month old mice. I examined the possibility that the increased presence of murine immunoglobulins in the older ‘leaky’ SCID mice, in combination with the specific binding of RH-14, could be causing the formation of hyaline thrombi (as described in section 3.4). However, after not finding any evidence of complexes of human and mouse immunoglobulins in the sera, and finding negligible positive staining for murine immunoglobulins in the hyaline thrombi, it seems unlikely that the hyaline thrombi were formed due to the presence of ‘bystander’ murine IgM or indeed murine rheumatoid factors or anti-idiotypic antibodies in the eight month old mice.

It was interesting to note that in the study of Vlahakos et al, the murine anti-DNA antibody H221 caused hyaline thrombi when injected in SCID mice as a hybridoma cell line, but not when injected i.v. in the form of purified antibody (Vlahakos et al. 1992b). This suggested either a quantitative difference in the level of circulating antibody or an effect due to a factor present in the hybridoma cells or the culture supernatant. In the case of RH-14, the same hybridoma cells are present in the studies of both the eight and two month old mice, and the culture supernatants should contain the same components. It is possible, that the hyaline thrombi could occur due to specific deposition of large complexes of RH-14 and nucleosomes in the capillary lumen. There is evidence to suggest that apoptosis is the most prevalent mode of cell death in hybridoma cell cultures (Franek et al. 1992) and this would generate nucleosomes which could form complexes with anti-DNA antibodies. However, there is no evidence that the RH-14 culture used in this experiment may have contained higher levels of such nucleosomes than those used in the previous experiment. There is some evidence that the rate of apoptosis may increase with aging (Sainz et al. 2003), so is it possible that the eight month old SCID mice contained higher levels of apoptotic debris, such as nucleosomes? However apoptotic fragments, are usually rapidly cleared, and there is no reason why this clearance should be impaired in SCID mice of any age.

The presence of fibrin in hyaline thrombi is unusual, there is no mention of fibrin in the hyaline deposits reported by authors in studies using murine monoclonal anti-DNA antibodies (Vlahakos et al. 1992b; Mostoslavsky et al. 2001; Itoh et al. 1993). However equally, these studies do not say there was no fibrin in the hyaline thrombi of their mice, and they may not have looked for fibrin. The thrombi that occur in
antiphospholipid syndrome (APS) are the conventional clots containing fibrin. Antiphospholipid antibodies are the causative agents in APS, however RH-14 does not bind cardiolipin. Previous work by Emma Radway-Bright (a PhD student in our laboratory), implanting anti-phospholipid antibodies including CL24 and IS4 in two month old SCID mice, had not shown any pathology in any of the organs examined (personal communication). Also as shown in table 3.5, despite milligram quantities of anti-phospholipid antibody in the sera of 2/3 mice, no thrombi were found in the kidneys of the eight month old SCID mice.

The difference in the pathology seen in the eight month and two month old SCID mice may be due to age related factors, such as changes in the endothelium, vascular tone or the involvement of oxidative stress (J. Alves, personal communication). The expression level of RH-14’s target antigen could also be different in the older mice. If ‘leakiness’ can cause a difference in the pattern of immunodeposition of anti-DNA antibodies this is an important finding since although two month old mice are used in the majority of published reports, there is great variability in the ‘leakiness’ of individual mice and leakiness may increase in response to inoculated antigens (either the antibodies, hybridoma cells or their culture medium). Equally if the different pathology in the two experiments, is simply due to quantitative differences between levels of antibody in the serum at crucial time points or indeed age related factors, this would have implications for the design and interpretation of future studies in mice.
RESULTS CHAPTER FOUR

The importance of sequence features of the light chain variable region in human IgG binding to DNA
CHAPTER FOUR

THE IMPORTANCE OF SEQUENCE FEATURES OF THE LIGHT CHAIN VARIABLE REGION IN HUMAN IgG BINDING TO DNA

4.1 Introduction and aims of this chapter

Sequence analysis of both murine and human monoclonal anti-DNA antibodies, suggests that high affinity for dsDNA is associated with the presence of certain amino acid residues such as arginine, asparagine and lysine within the CDRs (section 1.4.4). In IgG antibodies these amino acids frequently arise at sites of somatic mutation. Expression and modification of murine and human anti-DNA antibodies in vitro has shown that substitution of arginine residues, especially those in V\text{H}CDR3, often leads to a decrease in affinity for dsDNA.

Previous studies (described in section 1.4.7.3), implied that the critical arginine residues for DNA binding in the 2a2 derived light chains of human anti-DNA antibodies B3 and 33.H11, were those in B3V\text{\lambda} CDR1 and 33.H11V\text{\lambda} CDR3, whilst an arginine in UK4V\text{\lambda} CDR3 appears to block DNA binding of this anti-phospholipid antibody (Rahman et al. 2001). These findings lead to new questions: If the critical arginines in B3V\text{\lambda} CDR1 and 33.H11V\text{\lambda} CDR3 were combined in one molecule, would they exhibit an additive effect on binding to DNA and if the arginine in UK4V\text{\lambda} CDR3 was added to IgG molecules which did originally bind to DNA, would this hinder or block DNA binding? In order to attempt to answer these questions and to further highlight those regions of B3, 33.H11 and UK4 most heavily involved in the interaction with dsDNA, the effect of swapping entire CDRs between the various light chains was investigated.

Six new constructs were made, by switching the CDR3 regions between the three different light chain sequences, to create six new light chain sequences. The new light chains were combined with the heavy chain of B3 and expressed as new antibody molecules. The new constructs were expressed in the COS-7 transient
Chapter Four

Sequence features of $V_{\lambda}$ affect human IgG binding to DNA

eexpression system and their binding to DNA assessed. Two of the $V_{\lambda}$ hybrids B33 and BU were selected for subsequent cloning into pG1D1/B3V$_H$ to produce supervectors, which were stably expressed in CHO cells. The stable long-term production of IgG in CHO cells, allowed for more extensive in vitro and in vivo characterisation of the binding of the recombinant human IgG.

4.2 Production of the light chain expression vector CDR hybrids

The method used to produce the four hybrids 33B (33.H11V$_{\lambda}$ CDR1, B3V$_{\lambda}$ CDR2 and CDR3), B33 (B3V$_{\lambda}$ CDR1, 33.H11V$_{\lambda}$ CDR2 and CDR3), 33U (33.H11V$_{\lambda}$ CDR1, UK-4V$_{\lambda}$ CDR2 and CDR3) and U33 (UK-4V$_{\lambda}$ CDR1, 33.H11V$_{\lambda}$ CDR2 and CDR3) is described in detail in section 2.5 (Figure 2.4). The other two hybrids BU (B3V$_{\lambda}$ CDR1, UK-4V$_{\lambda}$ CDR2 and CDR3) and UB (UK-4V$_{\lambda}$ CDR1, B3V$_{\lambda}$ CDR2 and CDR3) were produced using the same method, but by my colleague Joanna Haley. The amino acid sequences of the six new constructs are shown in alignment with the sequences of B3V$_{\lambda}$, 33.H11V$_{\lambda}$ and UK-4V$_{\lambda}$ in Figure 4.1.

4.2.1 Preparation of B33 and 33B $V_{\lambda}$ hybrids in pLN10

This section describes the cloning of the light chain expression vector CDR hybrids B33 (B3V$_{\lambda}$ CDR1, 33.H11V$_{\lambda}$ CDR2 and CDR3) and 33B (33.H11V$_{\lambda}$ CDR1, B3V$_{\lambda}$ CDR2 and CDR3) in pLN10. Initially the pLN10 expression vectors containing B3V$_{\lambda}$ and 33.H11V$_{\lambda}$ were digested with KpnI and PvuI as described in section 2.5.3 and Figure 2.5. The DNA fragments were separated on an agarose gel. Figure 4.2 shows the uncut vectors, singly cut vector and the products of the KpnI and PvuI digest. Each vector was cut into a 5.9 Kb (5971 bp) fragment containing the $V_{\lambda}$ CDR1 and a 1.5 Kb (1525 bp) fragment containing the $V_{\lambda}$ CDR2 and CDR3. The 5.9 Kb fragment of one recombinant vector was then ligated to the 1.5 Kb fragment of the other vector to produce the two new hybrids, B33 and 33B. To check the hybrid vectors were the same size as the original parent vectors, they were all re-digested with KpnI and PvuI and run on an agarose gel.

To ensure that each construct contained the correct $V_{\lambda}$ CDRs, both an AatII digest and a HindIII/Aval digest were carried out to distinguish the new constructs from the parent molecules. The restriction sites of the parent vectors are shown in Figure 2.6
Figure 4.1  Amino acid sequences of expressed $V_\lambda$ regions compared to their closest germline $\lambda$ gene, 2a2

The amino acid sequences of B3$V_\lambda$ (wild type), B33, BU, U33, UB, 33B, 33U, 33.H11$V_\lambda$ and UK-4$V_\lambda$ regions are numbered according to Wu and Kabat (1970).

Amino acids are indicated according to their one letter code as listed in Appendix A. Dots have been inserted to facilitate the alignment. A dash indicates sequence identity with that of 2a2. The complementarity-determining regions (CDR) and framework regions (FR) have been defined according to the Kabat system (Wu and Kabat 1970). Antigen contact sites, as defined by (MacCallum et al. 1996), are shown by red arrows.
Sequence features of V_{i} affect human IgG binding to DNA

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Figure 4.2  *Kpnl* and *PvuI* restriction digest of pLN10/B3V<sub>λ</sub> and pLN10/33.H11V<sub>λ</sub> on 1% agarose gel

Lane A  1Kb DNA molecular weight marker
Lane B  *HindIII* DNA molecular weight marker
Lane C  Uncut pLN10/B3V<sub>λ</sub>
Lane D  pLN10/B3V<sub>λ</sub> cut with *Kpnl*
Lane E  pLN10/B3V<sub>λ</sub> cut with *Kpnl* and *PvuI*
Lane F  Uncut pLN10/33.H11V<sub>λ</sub>
Lane G  pLN10/33.H11V<sub>λ</sub> cut with *Kpnl*
Lane H  pLN10/33.H11V<sub>λ</sub> cut with *Kpnl* and *PvuI*
and Figure 2.7. B3V, differed from 33.H11V, by the presence of an extra AatII site in CDR1 and by the loss of an Aval site in CDR3. Thus the pattern of bands in an AatII digest, showed which parent sequence matched the sequence of the new construct in CDR1. Likewise, the pattern of bands in the HindIII/Aval digest, showed which parent sequence matched the new construct in CDR3. As shown in Figure 4.3, B33 matched B3V, in the AatII digest but 33.H11V, in the HindIII/Aval digest. Conversely, Figure 4.4, shows that 33B matched 33.H11V, in the AatII digest and B3V, in the HindIII/Aval digest.

4.2.2 Preparation of 33U and U33 V, hybrids in pLN10

This section describes the cloning of the light chain expression vector CDR hybrids 33U (33.H11V, CDR1, UK4V, CDR2 and CDR3) and U33 (UK4V, CDR1, 33.H11V, CDR2 and CDR3). These two hybrids were cloned as detailed in the methods section 2.5. Briefly, each vector was cut into a 5.9Kb (5971 bp) fragment containing the V, CDR1 and a 1.5 Kb (1525 bp in 33.H11V, /pLN10 and 1519 bp in UK4V, /pLN10) fragment containing the V, CDR2 and CDR3. The 5.9Kb fragment of one recombinant vector was then ligated to the 1.5 Kb fragment of the other vector to produce the two new hybrids, 33U and U33. As described in the previous section, a HindIII/Aval digest was used to show that the CDR2/CDR3 segment of U33 was the same as that of 33.H11V,, whereas the CDR2/CDR3 segment of 33U was the same as that of UK4V,, (see Figure 4.5). This was possible because the positions of the Aval sites in UK4V, and 33.H11V, are different (the restriction sites of the parent vectors are shown in Figures 2.7 and 2.8).

However, there were no convenient restriction sites which could distinguish UK4V, and 33.H11V, in the segment proximal to the KpnI site used for cloning. Unlike B3V,, neither of these sequences has an AatII site in CDR1. 33U and U33 were therefore sequenced, as described in section 2.5.10, to distinguish them from the parent molecules. Using manual sequencing, I could read up to the end of the CDR1 but the sequence was too compressed to easily read past the Kpn I site where the hybrid sequences switched, the sequence was therefore confirmed by automated sequencing as shown in Figure 4.6.
Figure 4.3  *Aval/HindIII* and *AatII* restriction digest of pLN10 vector containing B33 hybrid construct, B3V\_\lambda and 33.H11V\_\lambda on 1% agarose gel

The actual 1% agarose gel is shown above a diagram indicating the expected band sizes. The new hybrid construct B33 has an additional *Aval* restriction site, which it acquired from the CDR2/CDR3 portion of 33.H11V\_\lambda, this site is not present in B3V\_\lambda, so this digest shows two smaller fragments (366 and 267 bp) in B33 and 33.H11V\_\lambda which were not in the digest of B3V\_\lambda (single band of 633 bp). There is also an extra *AatII* restriction site in B33 and B3V\_\lambda which is not present in 33.H11V\_\lambda, resulting in smaller fragments (1017 and 381 bp) in B33 and B3V\_\lambda after *AatII* digest, compared with a single larger fragment (1398 bp) in 33.H11V\_\lambda. Thus, the gel shows that the B33 hybrid V\_\lambda sequence gives the same pattern as B3V\_\lambda on digestion with *AatII*, but the same pattern as 33.H11V\_\lambda when digested with *HindIII/Aval*.

Lane A  1Kb DNA molecular weight marker  
Lane B  *HindIII* DNA molecular weight marker  
Lane C  pLN10/B3V\_\lambda  
Lane D  pLN10/33.H11V\_\lambda  
Lane E  B33  
Lane F  pLN10/B3V\_\lambda  
Lane G  pLN10/33.H11V\_\lambda  
Lane H  B33
Sequence features of V<sub>L</sub> affect human IgG binding to DNA.
Figure 4.4  *Aval/HindIII and AatII* restriction digest of pLN10 vector containing 33B hybrid construct, B3V\(\lambda\) and 33.H11V\(\lambda\) on 1% agarose gel

The actual 1% agarose gel is shown above a diagram indicating the expected band sizes. Some of the bands are depicted in pale grey in the diagram to indicate faint bands on the gel, other bands depicted by a dotted white line on the diagram are not visible on the photograph of the gel. It is possible to see that after digestion with *Aval/HindIII*, 33B gives the same fragments as pLN10/B3V\(\lambda\), in pLN10/33.H11V\(\lambda\) the 633 bp fragment is absent. After *AatII* digestion 33B gives the same pattern of fragments as pLN10/33.H11V\(\lambda\), whereas the 1398 bp band is absent having been cut into two fragments of 1017 bp and 381 bp (1017 is just visible but unfortunately 381 is not visible on the photograph of the gel).

Lane A  1Kb DNA molecular weight marker
Lane B  *HindIII* DNA molecular weight marker
Lane C  33B
Lane D  pLN10/B3V\(\lambda\)  \textit{Aval/HindIII}
Lane E  pLN10/33.H11V\(\lambda\)
Lane F  33B
Lane G  pLN10/33.H11V\(\lambda\)  \textit{AatII}
Lane H  pLN10/B3V\(\lambda\)
Sequence features of Vλ affect human IgG binding to DNA

A B C D E

6557 bp
4391 bp
3054 bp
2322 bp
2027 bp
1018 bp
564 bp
506, 517 bp
396 bp
220 bp

C D E F G H

4398 bp
2465 bp

633 bp
366 bp
267 bp

5776 bp

1398 bp
1017 bp

381 bp

186 bp
83 bp
53 bp
Figure 4.5  *AvaI/HindIII* restriction digest of pLN10 vector containing 33U and U33 hybrid constructs, UK4Vλ and 33.H11Vλ on 1% agarose gel

Lane A  1Kb DNA molecular weight marker
Lane B  *HindIII* DNA molecular weight marker
Lane C  33U
Lane D  pLN10/33.H11Vλ
Lane E  U33
Lane F  pLN10/UK4Vλ
Figure 4.6  Comparison of the sequences of V\(\lambda\) hybrids 33U and U33

The sequences of 33U and U33 were confirmed by automated sequencing. The chromatograph trace for each V\(\lambda\) hybrid is shown here from the beginning of the CDR1 alongside the sequences of the parent V\(\lambda\) chains of UK4 and 33.H11 (The full DNA and amino acid sequences of the parent and hybrid V\(\lambda\) chains are shown in figure 4.1).

The Kpn I restriction site (GGTACC) is indicated in red, the actual position of the cut is between C and C (indicated by the red arrows). The differences in the DNA sequence are shown in blue in the parent sequences and by black arrows in the hybrid sequences. The automated sequencing results clearly show that the hybrids switch sequence after the Kpn I site, thus confirming the correct production of the V\(\lambda\) hybrids 33U and U33.
4.3 Transient expression of human IgG in COS-7 cells

The following light chains were expressed in combination with the B3 heavy chain in COS-7 cells; B3Vλ, 33.H11Vλ, UK-4Vλ, B33Vλ hybrid, 33BVλ hybrid, BUVλ hybrid, UBVλ hybrid, U33Vλ hybrid and 33UVλ hybrid. The transfection of the COS-7 cells was carried out as described in section 2.6.5, and after 72 hours the supernatants from the cells were DNase I treated and concentrated (2.6.6 – 2.6.7). Three or more separate expression experiments were carried out for each combination.

The mean whole human IgG yields (± standard deviation) obtained in the expression experiments, measured as described in section 2.6.8 after concentration of the supernatant, are shown in Table 4.1. The negative control sample supernatant was derived from COS-7 cells that were electroporated without plasmid DNA and contained no detectable IgG1. For the majority of combinations, the whole human IgG yields for each B3VH/Vλ pairing were similar between each of the expression experiments. However, human IgG yields of particular light/heavy chain combinations differed considerably between electroporations. This variation could have occurred for a number of reasons, such as variable post-electroporation COS-7 cell growth, or transfection efficiency. There was also considerable variation, between the yields of different light/heavy chain combinations, from 4.8-102.4 ng/ml, as seen in table 4.1. All three combinations consisting of B3VH paired with light chain constructs containing UK-4Vλ CDR3, produced noticeably higher yields of whole human IgG than the other combinations.

4.4 Anti-DNA binding of expressed whole IgG molecules

The dsDNA and ssDNA binding ability of each of the B3VH/2a2-derived-Vλ combinations were tested by ELISA and are shown in Figures 4.7 and 4.8. Each expression experiment (i.e. electroporation and subsequent human IgG, anti-ssDNA and anti-dsDNA ELISAs) was carried out three times and in each case, similar results were seen in each. Figures 4.7 and 4.8 show the DNA binding results of a single, representative, experiment. However, the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve (shown in the figure legends). The negative control in each
Table 4.1  The mean whole human IgG yields produced by each B3 heavy/light chain combination in the COS-7 transient expression system

Three or more electroporations of COS-7 cells were carried out for each B3 heavy chain with each of the nine different light chain constructs. For each electroporation the whole human IgG production was measured by ELISA (as section 2.6.8), after DNase I treatment and concentration of the supernatant. The mean whole human IgG yields (± standard deviation) obtained in these expression experiments are shown above. The negative control supernatant (referred to as ‘no DNA’) was derived from COS-7 cells that were electroporated without any plasmid DNA.
Figure 4.7 Anti-dsDNA activity of B3 heavy chain/2a2-derived \( \lambda \) chain human IgG in COS-7 cell supernatants, detected by ELISA

The graph shows binding of human IgG in COS-7 cell supernatants containing each B3 heavy chain/\( \lambda \) gene 2a2-derived light chain combination to dsDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The graph shows the results of a representative experiment (after one-hour incubation with substrate). However, the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.2 OD units for all points on curve B3V\( \text{H} \)/B3V\( \lambda \)(wild type). Similarly, at all points, SD < 0.15 for B3V\( \text{H} \)/33.H11V\( \lambda \), SD < 0.033 for B3V\( \text{H} \)/UK-4V\( \lambda \), SD < 0.04 for B3V\( \text{H} \)/BU-hybrid V\( \lambda \), SD < 0.043 for B3V\( \text{H} \)/UB-hybrid V\( \lambda \), SD < 0.047 for B3V\( \text{H} \)/33B-hybrid V\( \lambda \), SD < 0.18 for B3V\( \text{H} \)/B33-hybrid V\( \lambda \), SD < 0.051 for B3V\( \text{H} \)/33U-hybrid V\( \lambda \) and SD < 0.045 for B3V\( \text{H} \)/U33-hybrid V\( \lambda \). The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG (RH-14). The same aliquot of RH-14 was used for all the ELISAs and the SD of the positive control between the OD readings of the three experiments was always less than 0.1. The positive control of the representative experiment is shown in the graph.
Sequence features of \( V_L \) affect human IgG binding to DNA

![Graph showing optical density (OD) at 490nm against whole IgG concentration (ng/ml)]

- B3VH + B3VL (wild type)
- B3VH + 33H11VL
- B3VH + UK-4VL
- B3VH + UB-hybrid VL
- B3VH + BU-hybrid VL
- B3VH + 33B-hybrid VL
- B3VH + B33-hybrid VL
- B3VH + U33-hybrid VL
- B3VH + 33U-hybrid VL
- Positive Control
Figure 4.8 Anti-ssDNA activity of B3 heavy chain/2a2-derived \( \lambda \) chain IgG in COS-7 cell supernatants, detected by ELISA

The graph shows binding of IgG in COS-7 cell supernatants containing each B3 heavy chain/\( \lambda \) gene 2a2-derived light chain combination to dsDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The graph shows the results of a representative experiment (after one-hour incubation with substrate). However, the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.23 OD units for all points on curve B3V\( H \)/B3V\( \lambda \)(wild type). Similarly, at all points, SD < 0.024 for B3V\( H \)/33.H11V\( \lambda \), SD < 0.0042 for B3V\( H \)/UK-4V\( \lambda \), SD < 0.05 for B3V\( H \)/BU-hybrid V\( \lambda \), SD < 0.11 for B3V\( H \)/UB-hybrid V\( \lambda \), SD < 0.05 for B3V\( H \)/33B-hybrid V\( \lambda \), SD < 0.038 for B3V\( H \)/B33-hybrid V\( \lambda \), SD < 0.004 for B3V\( H \)/33U-hybrid V\( \lambda \), and SD < 0.13 for B3V\( H \)/U33-hybrid V\( \lambda \). The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the OD readings of the three experiments was always less than 0.1. The positive control of the representative experiment is shown in the graph.
Sequence features of V\textsubscript{L} affect human IgG binding to DNA

![Graph showing optical density (OD) at 450nm against whole IgG concentration (ng/ml).]
case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither human IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was RH-14 hybridoma supernatant. The same aliquot of RH-14 hybridoma supernatant was used for all the ELISAs and the standard deviation of the positive control between the OD readings of the three experiments was always less than 0.1 in both the anti-dsDNA and anti-ssDNA ELISAs.

The B3V_{H}/B33 hybrid V_{\lambda} combination consistently showed the strongest binding to dsDNA (Figure 4.7). This combination was followed closely by the wild type B3V_{H}/B3V_{\lambda} (wild type), and the B3V_{H}/U33 hybrid V_{\lambda}. As previously reported, the presence of the 33.H11V_{\lambda} chain led to a slight reduction in binding to dsDNA (Rahman et al. 2001). The binding of dsDNA, was further reduced in those combinations lacking both the 33.H11V_{\lambda} CDR2/CDR3 and the B3V_{\lambda} CDR1, (i.e. B3V_{H}/33B and B3V_{H}/UB) such that approximately double the concentration of IgG1 was required to obtain the same OD reading as that of the highest dsDNA binding combination, B3V_{H}/B33. Figure 4.8 shows that binding to ssDNA gave similar results for these different combinations, although the overall OD readings were lower.

Despite being tested at a range of concentrations between four and fifty times higher than those which gave the highest binding to dsDNA for the other combinations, those combinations containing the UK-4V_{\lambda} CDR2 and CDR3 showed no binding to dsDNA regardless of the origin of the V_{\lambda} CDR1 present (i.e. B3V_{H}/BU V_{\lambda} and B3V_{H}/33U V_{\lambda}). As described previously, B3V_{H}/UK-4V_{\lambda} showed no binding to either dsDNA or ssDNA (Rahman et al. 2001).

### 4.5 Computer modelling of three-dimensional structures in the interaction of the heavy/light chain combinations with dsDNA

The strongest binding to dsDNA was obtained using the hybrid light chain B33. In the models (made by Dr Sylvia Nagl) reported previously in the introduction (sections 1.4.5 and 1.4.7.3), it was shown that arginine 27a (R27a) in B3 V_{\lambda} CDR1 (Figures 1.4 and 1.5) and arginine 92 (R92) in 33.H11 V_{\lambda} CDR3 (Figure 1.6), could
both make contacts with dsDNA. The strong binding found with the light chain B33, which unlike either of the parent light chains, contains both of these arginines, might therefore be explained if both R27a and R92 could be shown to contact DNA. To address this question, further computer modelling was carried out by Dr. Sylvia Nagl. Figures 4.9[a] and 4.9[b] show models of the complex of dsDNA with B3V_{ih}/B33 hybrid V_{\lambda}. These models do indeed predict that both of these arginines interact with the dsDNA molecule in this complex.

Figures 4.9[c] and 4.9[d] show models of B3V_{ih}/BU hybrid V_{\lambda} and B3V_{ih}/UB hybrid V_{\lambda} respectively. The first does not bind DNA due to the presence of the large blocking arginine group (shown in yellow) at position 94 in the CDR3 of UK-4 V_{\lambda}. When this group is removed from UK-4 V_{\lambda}, by replacing CDR2 and 3 with those of B3 V_{\lambda} (Figure 4.9[d]), the resultant light chain UB would be predicted by the model to support binding to DNA. The presence of the UK-4 V_{\lambda}CDR1 region would not prevent this binding. The results predicted by these models are exactly those found in the ELISA assays. UB hybrid V_{\lambda} supported binding to dsDNA whereas BU hybrid V_{\lambda} did not.
Figure 4.9  Computer models of B3V_{H}/hybrid germline gene 2a2-derived V_{\lambda} chain combinations

The molecular surfaces of the antibody chains are shown in dark blue (light chain) and cyan (B3V_{H}), dsDNA is depicted in stick mode and coloured by element. Key argine residues predicted to enhance DNA binding are shown in grey and red. The B3V_{H}/hybrid germline gene 2a2-derived V_{\lambda} chain combinations depicted are:

A  B3V_{H}/B33-hybrid V_{\lambda} (contains B3V_{\lambda} CDR1 and 33.H11V_{\lambda} CDR2 & CDR3).

B  Detailed view of the predicted stabilizing interactions, between the DNA backbone and the arginine residues at positions 27a (R27a) and 92 (R92) of B3V_{H}/B33-hybrid V_{\lambda}.

C  B3V_{H}/BU-hybrid V_{\lambda} (contains B3V_{\lambda} CDR1 and UK4V_{\lambda} CDR2 & CDR3). The predicted blocking residue Arg 94 is shown in yellow.

D  B3V_{H}/UB-hybrid V_{\lambda} (contains UK4V_{\lambda} CDR1 and B3V_{\lambda} CDR2 & CDR3).

Figures modeled by Dr Sylvia Nagl.
Sequence features of V₄ affect human IgG binding to DNA
4.6 Production of supervectors

In order to investigate the binding and pathogenic properties of the recombinant human IgG molecules further, it was necessary to produce cell lines which would stably produce recombinant IgG in larger quantities. As described in section 2.7 it was considered more efficient to transfect CHO cells with a single vector encoding both the heavy and light chains of the IgG, rather than two separate vectors, thus supervectors encoding the IgG molecules of interest were produced. In the experiments described in both this chapter and chapter 5, four B3VH/VL IgG molecules are studied. These were produced by stable expression in CHO cell lines transfected with supervector plasmid DNA.

Two of the supervectors and stable CHO cell lines were produced by Joanna Haley, the first designated SVBL encoded the ‘wild type’ B3VH and B3VL (there were two clones of SVBL used in this thesis: SVBL 5-40-24 amplified twice and maintained in 1 x 10^-7 M methotrexate and SVBL 3-26 amplified once and maintained in 1 x 10^-9 M methotrexate); the second designated SVBLX encoded the B3VH and B3VL except a single point mutation, arginine to serine had been introduced at position 27a in CDR1 of the light chain variable region (there were three clones of SVBLX used in this thesis: SVBLX 29-11-11 amplified twice and maintained in 1 x 10^-7 M methotrexate, SVBLX 10-47 and SVBLX 6-26 both amplified once and maintained in 1 x 10^-9 M methotrexate).

I produced two further supervectors for subsequent stable expression in CHO cell lines. The first of these combined B3VH with the light chain hybrid B33 (B3VL CDR1, 33.H11VL CDR2 and CDR3). This combination of heavy and light chain was of interest since it had shown the greatest binding to dsDNA, when transiently expressed in the COS-7 cells (section 4.4). The second supervector, combined B3VH with the light chain hybrid BU (B3VL CDR1, UK-4VL CDR2 and CDR3). This construct was developed as a control, since this combination of heavy and light chain had shown no binding to dsDNA in the transient expression system (section 4.4). These two new supervectors were designated SVB33 and SVBU respectively.
The supervectors were produced as described in section 2.7.1. To summarise, an *EcoRI* fragment containing the HCMV promoter, the λ constant region gene and the λ variable region gene (of the proposed autoantibody) was excised from the plasmid vector pLN10 and re-ligated into the vector pG1D1/B3Vh.

To verify the correct supervectors had been produced, SVB33 (PG1D1/B3Vh and B33Vλ) and SVBU (PG1D1/B3Vh and BU3Vλ) were digested with *AvaI* and *HindIII* alongside the original supervector SVBL (PG1D1/B3Vh and B3Vλ) and the resulting DNA fragments were separated on a 0.7% agarose gel (see Figure 4.10). It can be seen from the gel that the insert containing Vλ was ligated into all three vectors in the same orientation as B3Vh, since DNA bands of 5338 bp and 1680 bp are present (if the insert was placed in the opposite way, the bands would be 4566 bp and 2452 bp). It can also be seen that each supervector is correct since different sized DNA bands can be seen after the *AvaI* and *HindIII* digest, 633 bp in SVBL, 431 bp in SVBU and 366 bp in SVB33 (the smaller 267 bp band in SVB33 is hard to distinguish and the 196 bp band in SVBU is too faint to see, as are the small 55 and 35 bp bands, which with the 308 bp band are present in all three supervectors). The diagram in Figure 4.10 shows all the expected DNA band sizes, calculated by reference to the supervector maps, Figures 2.10 – 2.12, which show the restriction enzyme sites of SVBL, SVB33 and SVBU.

4.7 Transient expression of supervectors in COS-7 cells

To check that the supervectors would be expressed in eukaryotic cells and produce human IgG, the expression of SVB33 and SVBU was first carried out in COS-7 cells, since IgG production in these cells can be seen after 3 days (as opposed to several weeks in CHO cells). Two preparations of DNA were made for each supervector, one using the large scale extraction from *E. coli*, 'Maxi-prep' as section 2.5.11 and the other using multiple small scale extractions 'mini-prep' as section 2.5.2. The supervector DNA was prepared and 10μl (2μg/μl) transfected into COS-7 cells as described in section 2.8.2. Two electroporations were carried out with each supervector DNA, one using 'Maxi-prep' DNA and the other using 'Mini-prep' DNA, as a negative control a fifth electroporation was carried out without any DNA. After 72 hours the supernatant was collected, treated with DNase I and concentrated.
Figure 4.10  \textit{Aval/HindIII} restriction digest of the supervectors SVBL, SVB33 and SVBU on 0.7\% agarose gel

SVBL (PG1D1/B3V_{H} and B3V_{\lambda}), SVB33 (PG1D1/B3V_{H} and B33V_{\lambda}) or SVBU (PG1D1/B3V_{H} and BUV_{\lambda}) were digested with \textit{Aval} and \textit{HindIII} (as described in section 2.7.3) and the fragments separated on a 0.7\% agarose gel. The actual agarose gel is shown above a diagram indicating the expected band sizes. Some of the bands, depicted by a dotted white line on the diagram, are not visible on the photograph of the gel. It can be seen from the gel that the insert containing \( V_{\lambda} \) was ligated into all three vectors in the same orientation as DNA bands of 5338 bp and 1680 bp are present in each (if the insert was in the opposite orientation the bands would be 4566 bp and 2452 bp). It can also be seen that each supervector is correct since different sized DNA bands can be seen after the \textit{Aval} and \textit{HindIII} digest (633 bp in SVBL, 431 bp in SVBU and 366 bp in SVB33, the smaller bands are too faint to see, the 308 bp band is present in all three supervectors). Figures 2.10 – 2.12 show the restriction enzyme sites of SVBL, SVB33 and SVBU from which the expected band sizes were calculated.

Lane A  1Kb DNA molecular weight marker  
Lane B  SVB33  
Lane C  SVBU  
Lane D  SVBL  
Lane E  \textit{HindIII} DNA molecular weight marker
Sequence features of V_{i} affect human IgG binding to DNA.
as described in sections 2.6.6 and 2.6.7. The mean human IgG production shown in Table 4.2, was measured (by reference to a standard curve of the positive control human IgG) after titration of the supernatants in duplicate, in the human IgG ELISA (section 2.6.8). Table 4.2 shows the mean production of human IgG in the COS-7 cell supernatants, from the two electroporations using each supervector, after concentration of the supernatant.

The results seem consistent with the idea that transfection with a single supervector is more effective than transfection with separate vectors (encoding the heavy and light chains), since the human IgG production with the supervectors shown in Table 4.2 is higher than the equivalent heavy and light chain combinations in Table 4.1 (B3VH & B33, 5.2 ± 3.8 ng/ml; B3VH & BU, 102.4 ± 65.3 ng/ml). As seen during the transient expression of IgG from the separate heavy and light chain vectors, in this experiment the supervector (SVBU) coding for the combination containing the CDR2/3 from UK4Vx again expressed more human IgG than SVB33 in this transient system.

Table 4.2 Transient human IgG production of COS-7 cells transfected with supervector DNA

<table>
<thead>
<tr>
<th>Supervector DNA</th>
<th>Mean human IgG (ng/ml)</th>
<th>SD (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVB33</td>
<td>21</td>
<td>± 1.41</td>
</tr>
<tr>
<td>SVBU</td>
<td>245</td>
<td>± 63.6</td>
</tr>
<tr>
<td>No DNA control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The binding to dsDNA of the supernatants from the two electroporations using each supervector was assessed using the anti-dsDNA ELISA (section 2.1.4.2), and the results are shown in Figure 4.11. As in the transfection of the COS-7 cells with separate vectors, encoding the heavy and light chain, SVB33 bound to dsDNA whereas SVBU did not bind, despite being tested at much greater concentrations of
Chapter Four  
Sequence features of $V_L$ affect human IgG binding to DNA

Figure 4.11  Binding to dsDNA of human IgG transiently produced by COS-7 cells transfected with supervectors SVB33 and SVBU

Each point shown above, is the mean (± SD) of the anti-dsDNA binding of COS-7 supernatants from two electroporations of COS-7 cells with each supervector SVB33 and SVBU. Supernatant from each electroporation was DNase I treated prior to being titrated in triplicate on coated and sham coated sides of a anti-dsDNA ELISA. The same aliquot of RH-14 hybridoma supernatant was used as a positive control in all the anti-dsDNA ELISAs and the standard deviation of the OD readings for RH-14 between plates was always less than 0.1. The OD values on the sham-coated side were subtracted from the OD values on the coated side.
human IgG. The human IgG concentration of SVB33 produced by the COS-7 cells, was very low, thus the optical density values obtained in the anti-dsDNA binding ELISA were also pretty low. This illustrates the necessity for stable expression of these recombinant human IgG molecules.

4.8 Stable expression of supervectors in CHO cells

4.8.1 Transfection of CHOdhfr cells with SVB33 and SVBU

CHOdhfr cells were transfected with the supervectors SVB33 and SVBU by electroporation (as described in section 2.8.7), using the same two batches of plasmid DNA that were used to transfect the COS-7 cells in section 4.7 (ie 2 transfections with SVB33 and 2 with SVBU). Once again a fifth electroporation was carried out on CHOdhsfr cells without the addition of DNA (negative control). After 24 hours the cells were plated out in selective CHOdhfr growth medium as described in section 2.8.8. Following 10-14 days of culture, all the cells in the “no DNA” negative control dish were dead and foci of cells were clearly visible in the dishes containing cells transfected with SVB33 or SVBU.

Forty eight foci were picked from each of the four electroporations and cultured in 24 well tissue culture plates. When the cells were confluent the wells were tested for human IgG production (using the whole human IgG ELISA described in section 2.6.8). A total of 12 clones (from original 96 clones per supervector) were picked for each supervector based on their human IgG production. These clones were expanded in 6 well culture plates and eventually up into 75cm² culture flasks. When the cells were nearly confluent, the supernatants were tested for human IgG production, and the two highest producers for each supervector were selected for further expansion and methotrexate amplification. The other 10 clones for each supervector were frozen down as a backup. The two clones producing the highest amount of SVB33 human IgG were SVB33 2-16 and SVB33 3-15. Two clones (highest human IgG) producing SVBU were also selected SVBU 4-12 and SVBU 5-18. These four clones were expanded into 175cm² culture flasks and a cell bank was created. The human IgG production / 1x10⁶ cells / day of each clone, shown in Table 4.3, was measured as described in section 2.8.9.
Table 4.3  Human IgG production of CHO cells transfected with the supervectors SVB33 and SVBU

<table>
<thead>
<tr>
<th>CHO cell line</th>
<th>Human IgG production (ng/1x10^6 cells/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVB33 2-16</td>
<td>2.0</td>
</tr>
<tr>
<td>SVB33 3-15</td>
<td>83</td>
</tr>
<tr>
<td>SVBU 4-12</td>
<td>10</td>
</tr>
<tr>
<td>SVBU 5-18</td>
<td>71</td>
</tr>
</tbody>
</table>

Each value in the table was derived, by reference to a standard curve of human IgG of known concentration, using the mean of the OD values of the supernatant, incubated in duplicate wells of a human IgG ELISA plate after the background diluent and sham coated well OD values had been subtracted.

4.8.2 Methotrexate amplification of the transfected CHO cell lines.

Methotrexate is a competitive inhibitor of the intracellular activity of the DHFR enzyme. Progressive selection of cells that are resistant to increasing concentrations of methotrexate leads to amplification of the dhfr gene, with concomitant amplification of IgG-encoding sequences that flank the dhfr sequences (Schimke 1984). In those resistant cells where amplification has occurred, there is not only sufficient free DHFR enzyme to generate intracellular tetrahydrofolate required for cell biosynthesis but also these resulting cell lines express high levels of immunoglobulin.

The two clones of SVB33 (SVB33 2-16 and 3-15) and SVBU (SVBU 4-12 and 5-18) were amplified in both 1x10^-9 M and 1x10^-8 M methotrexate. As described in section 2.8.10, the foci in the plates containing media supplemented with 1 x 10^-9 M methotrexate were very close together and there were discrete foci in the dishes containing media supplemented with 1 x 10^-8 M methotrexate, therefore these were kept and the former discarded.

Twenty four foci were picked for each of the four (2 x 2 supervectors) clones, and when almost confluent the supernatant from each well was tested using the human
IgG ELISA, to determine which clones were to be further expanded. Six clones each of SVB33 2-16, SVB33 3-15, SVBU 4-12 and SVBU 5-18 were expanded into 75cm² culture flasks and the human IgG production / 1x10⁶ cells / day of each clone was measured as described in section 2.8.9. The human IgG production of the amplified clones can be seen in Table 4.4. There was a wide range in the human IgG production of different clones derived from the same original clone.

The clones with the highest production of human IgG for each supervector, SVB33 3-15-23 and SVBU 5-18-6 (shown in bold type in Table 4.4), were selected for expansion in amplification medium until the cells were eventually growing in 175cm² tissue culture flasks. A cell bank was created of these two clones, and as a backup, all of the other clones that were producing high levels of human IgG (SVB33 2-16-24, 2-16-22, all clones derived from SVB33 3-15, SVBU 4-12-8, SVBU 5-18-7 and 5-18-8) were also frozen.

4.9 Binding of recombinant human IgG produced by CHO cells to dsDNA

The supernatants from CHO cell cultures producing each of the recombinant human anti-DNA antibodies were tested for binding to dsDNA in the ELISA (2.1.4.2). Figure 4.12 demonstrates the necessity of DNase I treatment (2.6.6) of the supernatants prior to incubation in the ELISA. Without DNase I treatment there was no binding of SVBL or SVB33 to dsDNA, but after treatment with 7.5 U/ml of DNase I, both SVBL and SVB33 bound to dsDNA, SVBU did not bind to dsDNA at all. Increasing the DNase I concentration to 15 U/ml, did not further increase the binding to DNA. Figure 4.13, compares the DNA binding of the recombinant IgG molecules in the DNase I pretreated CHO cell supernatants. Due to the relatively low concentration of human IgG produced by the SVBL CHO clones, it was not possible to directly compare the binding of SVBL and SVB33 in this experiment. However, previous experiments in section 4.4 (Figure 4.7), co-expressing the B3V₇ and B33V₇ chains in COS-7 cells, suggested that the IgG produced by CHO cells transfected with the SVB33 supervector, should bind more strongly to dsDNA than SVBL.
Table 4.4 Human IgG production of methotrexate amplified CHO cell lines

<table>
<thead>
<tr>
<th>CHO cell line (amplified in media containing 1x10^{-8} M methotrexate)</th>
<th>Human IgG production (ng/1x10^6 cells/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVB33 2-16-24</td>
<td>62</td>
</tr>
<tr>
<td>SVB33 2-16-1</td>
<td>7.5</td>
</tr>
<tr>
<td>SVB33 2-16-22</td>
<td>34</td>
</tr>
<tr>
<td>SVB33 2-16-6</td>
<td>87</td>
</tr>
<tr>
<td>SVB33 2-16-16</td>
<td>120</td>
</tr>
<tr>
<td>SVB33 2-16-19</td>
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<tr>
<td>SVB33 3-15-5</td>
<td>1500</td>
</tr>
<tr>
<td>SVB33 3-15-13</td>
<td>1000</td>
</tr>
<tr>
<td>SVB33 3-15-24</td>
<td>533</td>
</tr>
<tr>
<td>SVB33 3-15-2</td>
<td>2700</td>
</tr>
<tr>
<td>SVB33 3-15-9</td>
<td>2700</td>
</tr>
<tr>
<td><strong>SVB33 3-15-23</strong></td>
<td><strong>6700</strong></td>
</tr>
<tr>
<td>SVBU 4-12-24</td>
<td>0</td>
</tr>
<tr>
<td>SVBU 4-12-14</td>
<td>0</td>
</tr>
<tr>
<td>SVBU 4-12-13</td>
<td>0</td>
</tr>
<tr>
<td>SVBU 4-12-3</td>
<td>0</td>
</tr>
<tr>
<td>SVBU 4-12-8</td>
<td>52</td>
</tr>
<tr>
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</tr>
<tr>
<td>SVBU 5-18-7</td>
<td>7.2</td>
</tr>
<tr>
<td><strong>SVBU 5-18-6</strong></td>
<td><strong>148</strong></td>
</tr>
<tr>
<td>SVBU 5-18-1</td>
<td>0</td>
</tr>
<tr>
<td>SVBU 5-18-8</td>
<td>36</td>
</tr>
<tr>
<td>SVBU 5-18-5</td>
<td>0</td>
</tr>
<tr>
<td>SVBU 5-18-2</td>
<td>0</td>
</tr>
</tbody>
</table>

The highest producing clones for each supervector, SVB33 and SVBU, are shown in bold type. Each value in the table was derived, by reference to a standard curve of human IgG of known concentration, using the mean of the OD values of the supernatant, incubated in duplicate wells of a human IgG ELISA plate after the background diluent and sham coated well OD values had been subtracted.
Figure 4.12 DNase I digestion of CHO cell supernatants, containing recombinant human IgG, is necessary before measuring their binding to dsDNA in ELISA

Prior to their incubation in an anti-dsDNA ELISA (2.1.4.2), supernatants from cultures of CHO cells producing recombinant human IgG were treated with either 0, 7.5 or 15.0 U/ml of DNase I (as described in section 2.6.6) to remove both DNA from the supernatant and DNA which might be bound by the antibodies. Two clones of CHO cells expressing each of three supervectors were studied (the clones are summarised in Table 5.1). The human IgG concentration of each supernatant was measured using the human IgG ELISA (2.6.8).

Figure A: SVBL 5-40-24 ('wild type' B3V\textsubscript{H} and B3V\textsubscript{\lambda})
Figure B: SVBL 3-26 ('wild type' B3V\textsubscript{H} and B3V\textsubscript{\lambda})
Figure C: SVB33 3-15 (B3V\textsubscript{H} and B3V\textsubscript{\lambda} CDR1, 33.H11V\textsubscript{\lambda} CDR2 and CDR3)
Figure D: SVB33 3-15-23 (B3V\textsubscript{H} and B3V\textsubscript{\lambda} CDR1, 33.H11V\textsubscript{\lambda} CDR2 and CDR3)
Figure E: SVBU 5-18 (B3V\textsubscript{H} and B3V\textsubscript{\lambda} CDR1, UK4V\textsubscript{\lambda} CDR2 and CDR3)
Figure F: SVBU 5-18-6 (B3V\textsubscript{H} and B3V\textsubscript{\lambda} CDR1, UK4V\textsubscript{\lambda} CDR2 and CDR3)

The graphs illustrate that both SVBL and SVB33 bind to dsDNA but that SVBU did not bind to dsDNA. The DNase I treatment, clearly showed that the binding of SVBL and SVB33 to dsDNA, coated on the ELISA plate, is inhibited by DNA present in the supernatants and which is bound by the antibodies. 7.5 U/ml of DNase I is sufficient to remove the inhibitory DNA, as no increase of binding is seen when the DNase I concentration is increased to 15 U/ml.
Sequence features of V_L affect human IgG binding to DNA

A

B

C

D

E

F

- DNase I 7.5 U/ml
- DNase I 15.0 U/ml
- No DNase I
Figure 4.13 Binding to dsDNA of recombinant human IgG, from the supernatant of CHO cells transfected with supervectors

The supernatants from CHO cells transfected with the supervectors SVBL, SVB33 and SVBU were treated with 7.5 U/ml of DNase I (as described in 2.6.6) before being incubated in an anti-dsDNA ELISA (2.1.4.2). Two clones of CHO cells expressing each supervector were studied (the clones are summarised in Table 5.1).

Both SVB33 and SVBL showed binding to dsDNA and SVBU showed no binding to dsDNA. However the concentration of human IgG in the supernatant from SVBL transfected CHO cells was too low for the binding to dsDNA to be directly compared with that from SVB33 CHO cells. The human IgG concentration of each supernatant was measured using the human IgG ELISA (2.6.8).
4.10 Large-scale production and affinity purification of human IgG

In order to obtain large quantities of purified human IgG from the CHO cells, they were grown in their selective amplification medium in a hollow-fibre tissue culture system. The human IgG was then affinity purified from the supernatant using a protein A column. This large-scale culture and affinity purification was carried out by Chemicon Europe Ltd, Hampshire, UK. Thus far, 3 mg of recombinant human IgG has been supplied from each of the CHO cell lines SVBL ('wild-type' B3V\textsubscript{H}/B3V\textsubscript{\lambda}), SVB33 (B3V\textsubscript{H}/B3V\textsubscript{\lambda} CDR1 and 33.H11V\textsubscript{\lambda} CDR2 and CDR3) and SVBU (B3V\textsubscript{H}/B3V\textsubscript{\lambda} CDR1 and UK-4V\textsubscript{\lambda} CDR2 and CDR3) as well as 1-2 mg of SVBLX (B3V\textsubscript{H}/B3V\textsubscript{\lambda} with arginine to serine reversion at position 27a of the lambda chain).

4.11 Binding of affinity purified recombinant human IgG molecules to dsDNA

The affinity purified human IgG antibodies were tested for their binding to dsDNA, using the ELISA described in section 2.1.4.2, the results are shown in Figure 4.14. The human IgG concentration was determined by Chemicon and was verified in our laboratory using the whole human IgG ELISA 2.6.8. In order to confirm the findings of the first experiment (Figure 4.14 A, C & E), this experiment was repeated on a separate occasion (Figure 4.14 B, D & F). Overall, the OD values in the repeat experiment were higher than those obtained in the first experiment, and the standard deviation of the standard sera at 5 IU/ml was too high (±0.45 between the two experiments), so both experiments are plotted.

Figure 4.14 (A&B), shows the dsDNA binding of the affinity purified antibodies when simply diluted in SEC buffer but not treated with DNase I, under these conditions only SVB33 binds to dsDNA. Figure 4.14 (C&D), shows the effect of DNase I treatment of the affinity purified antibodies, diluted in SEC buffer, prior to testing in the dsDNA ELISA. The DNase I treatment appears to have abolished the binding of the affinity purified SVB33 to dsDNA. In order to investigate whether a 'cofactor' was necessary for DNA binding, which might be present in cell culture supernatant but which may have been removed by affinity purification, the affinity purified antibodies were also tested for binding to dsDNA after dilution in supernatant from a non-transfected COS-7 cell culture. The antibody/COS-7
Figure 4.14  Binding of affinity purified human IgG molecules to dsDNA

The affinity purified human IgG antibodies were tested for their binding to dsDNA, using the ELISA described in section 2.1.4.2 (human IgG concentration was determined by Chemicon and was verified in our laboratory using the whole human IgG ELISA 2.6.8). In order to confirm the findings of the first experiment (Figures A, C & E), this experiment was repeated on a separate occasion (Figures B, D & F). Diluted serum from a patient with SLE was run on every plate, this serum was calibrated using the WHO reference serum Wo/80 as described in 2.1.2.3. The standard deviation of the standard sera OD value between plates A, C & E was ±0.28 at a concentration of 5 IU/ml. The standard deviation of the standard sera OD value between plates B, D & F was ±0.07 at a concentration of 5 IU/ml. Overall, the OD values in the repeat experiment were higher than those obtained in the first experiment, and the standard deviation of the standard sera at 5 IU/ml was too high (±0.45 between the two experiments), so both experiments are plotted. These assays were carried out together with Nancy Lambrianides, who is another PhD student in our laboratory.

A&B  These graphs show the dsDNA binding of the affinity purified antibodies when diluted in SEC buffer, these antibodies were not treated with DNase I. Only SVB33 binds to dsDNA under these conditions.

C&D  These graphs show the dsDNA binding of the affinity purified antibodies when diluted in SEC buffer, and treated with DNase I prior to testing in the ELISA. The DNase I treatment appears to have abolished the binding of the affinity purified SVB33 to dsDNA.

E&F  These graphs show the dsDNA binding of the affinity purified antibodies diluted in supernatant derived from COS-7 cells, after dilution in the supernatant, the antibody solutions are treated with DNase I prior to testing in the ELISA. The addition of the COS-7 supernatant appears to have reinstated the binding of SVB33 to dsDNA and also allows the binding of SVBL and SVBLX to dsDNA. SVBU does not bind to dsDNA.
Sequence features of $\gamma_{L}$ affect human IgG binding to DNA.

A

B

C

D

E

F

![Graphs showing binding of IgG to DNA for different concentrations.](image)
supernatant solutions were pre-treated with DNase I, prior to testing in the dsDNA ELISA. Figure 4.14 (E&F), shows the reinstatement of dsDNA binding of the affinity purified SVB33, when the affinity purified antibodies are diluted in supernatant derived from COS-7 cells and pre-treated with DNase I, prior to testing in the ELISA. The addition of the COS-7 supernatant also allows the binding of SVBL and SVBLX to dsDNA (Figure 4.14 [E&F]), these affinity purified antibodies would not bind to dsDNA when only diluted in SEC buffer, either with or without pre-treatment with DNase I (Figures 4.14 [C&D] and [A&B] respectively). The affinity purified SVBU did not bind to dsDNA under any circumstances.

4.12 Anti-nuclear antigen (ANA) binding of recombinant human IgG produced by CHO cells.

The binding of affinity purified recombinant human IgG, produced by the transfected CHO cells, to *in vitro* ANA was assessed using slides containing a preparation of Hep2 cells (as described in section 2.8.13). Figure 4.15 shows the ANA binding of the affinity purified recombinant human IgG diluted in PBS. The affinity purified recombinant human IgG, SVB33, showed positive diffuse pattern ANA staining (C) and at the same concentration (12.5µg/ml) of human IgG, SVBL showed weaker positive diffuse pattern ANA staining (B). The affinity purified recombinant human IgG antibodies SVBU (D) and SVBLX (E), were negative for ANA staining even at twice the concentration (25µg/ml) of human IgG. Hybridoma cell supernatant containing human IgG antibody B3 was used as a positive control and showed strong positive diffuse pattern ANA staining (A). The negative control, commercial non-specific human IgG1λ, was also negative for ANA staining (F). The same degree of ANA staining was seen if instead of PBS, the affinity purified antibodies were diluted in supernatant from COS-7 cells (containing no human IgG) and pre-treated with DNase I before adding to the Hep2 cell preparations (data not shown). The same antibodies gave positive or negative staining using the in-house produced rat liver and kidney sections (data not shown).
**Figure 4.15  In vitro anti-nuclear antigen (ANA) binding of recombinant human IgG**

The binding of the recombinant human IgG, produced by the transfected CHO cells to *in vitro* ANA, was assessed using slides containing a preparation of Hep2 cells. As a positive control, hybridoma cell supernatant was used from the original B3 antibody producing cell line. Isotype matched human IgG1λ was used as a negative control. These figures show the Hep2 cells incubated with the affinity purified recombinant antibodies diluted in PBS:

A:  B3 hybridoma, neat cell supernatant (positive control)
B:  SVBL ('wild type' B3V̂ and B3V̂λ) 12.5μg/ml
C:  SVB33 (B3V̂ and B3V̂λ CDR1, 33.ΗI1V̂λ CDR2 and CDR3) 12.5μg/ml
D:  SVBU (B3V̂ and B3V̂λ CDR1, UK4V̂λ CDR2 and CDR3) 25 μg/ml
E:  SVBLX (B3V̂ and B3V̂λ with arginine to serine reversion at 27a) 25 μg/ml
F:  IgG1λ (negative control) 12.5μg/ml

The hybridoma cell supernatant containing human IgG antibody B3, showed strongly positive diffuse pattern ANA staining (A). The affinity purified recombinant human IgG, SVB33, showed positive diffuse pattern ANA staining (C) and at the same concentration of human IgG, SVBL showed weaker positive diffuse pattern ANA staining (B). The affinity purified recombinant human IgG antibodies SVBU (D) and SVBLX (E), were negative for ANA staining even at twice the concentration of human IgG. The negative control was also negative for ANA staining (F).
Sequence features of $V_{\lambda}$ affect human IgG binding to DNA
4.13 Discussion of results from chapter four

In summary, six new hybrid light chain constructs were successfully produced (two by Joanna Haley and four by myself) by switching the CDRs between B3V, 33.H11V, and UK4V in the light chain expression vector pLN10. These constructs were transiently co-expressed with B3V by COS-7 cells. In addition two new supervectors were constructed (SVB33 and SVBU), combining B3V with two of the new light chain hybrids. These supervectors were stably expressed in CHO cells. The development of these new expression vectors (along with the use of the pre-existing parent light chain vectors [B3V, 33.H11V, and UK4V] and two other supervectors [SVBL and SVBLX]) allowed further investigation of the importance for DNA binding (and potential pathogenicity, Chapter five), of particular sequence motifs in the light chain CDRs of the anti-DNA antibodies B3 and 33.H11.

The initial observation from the experiments (section 4.3) transiently expressing the nine different light chains in combination with B3V, was that the highest human IgG production (shown in Table 4.1) was observed in those combinations which did not bind to dsDNA, that is those IgG molecules containing the CDR2/CDR3 of UK4 (BUV and 33UV, as well as parent molecule UK4V). There is no obvious explanation for this phenomenon, which was seen during repeated electroporation experiments. However, this phenomenon of different yields from different VH/VL combinations has been seen previously both in this expression system and others (Rahman et al. 1998; Giles et al. 2003; Kettleborough et al. 1991).

The reduced human IgG in combinations which bound DNA, was not simply due to the IgG being sequestered in immune complexes with DNA, since increasing concentrations of DNase I did not further increase the detection of human IgG. Although it is possible, that if the IgG were involved in complexes with nucleosomes, through recognition of nucleosome epitopes, these might not be broken down by DNase I treatment. It could also be hypothesised, that human IgG levels were lower in COS-7 cell cultures producing IgG combinations that successfully bind DNA, due to these antibodies having some effect on the cells that produced them. However, these antibodies were not toxic to the COS-7 cells, since no substantial differences were found between the viable cell counts three days post-
electroporation (data not shown), irrespective of which IgG molecules the COS-7 cells were producing. It is possible, that the antibodies could be penetrating the cells and modifying their IgG production by some unknown means, since some anti-DNA binding antibodies appear to possess the ability to penetrate live cells (Ehrenstein et al. 1995; Vlahakos et al. 1992a; Zack et al. 1996), as discussed in section 1.5.5.

As described in sections 4.4 and 4.5, the recombinant human IgG molecules consisting of B3V\(\text{H}\) in combination with nine different light chains were tested for binding to dsDNA. The computer models of B3V\(\text{H}\)/B3V\(\lambda\) ('wild-type' B3, Figure 1.4) and B3V\(\text{H}\)/33.H11V\(\lambda\) (Figure 1.6) predicted the importance for dsDNA binding, of arginine residues at position 27a (R27a) of B3V\(\lambda\)CDR1 and position 92 (R92) of 33.H11V\(\lambda\)CDR3, these residues were derived by somatic mutation (Kalsi et al. 1996; Rahman et al. 2001; Rahman et al. 2002a). The importance of R27a in B3V\(\lambda\) (this arginine is not present in 33.H11V\(\lambda\), 2a2 germ-line gene derived serine instead), was confirmed previously by site-directed mutagenesis of this arginine to serine (S27a), as encoded by the germ-line gene 2a2 (Figure 1.5). This single amino acid substitution, reduced the mutated B3 antibody’s binding to dsDNA by 50%, as compared to ‘wild-type’ B3 (Rahman et al. 2001). B3V\(\text{H}\)/33.H11V\(\lambda\) bound better to dsDNA than the mutated (arginine to serine at position 27a) B3, but bound less well than ‘wild-type’ B3 (Rahman et al. 2001), hence the arginine at position 92 of CDR3 of 33.H11V\(\lambda\) might enhance the binding of this combination, as predicted in Figure 1.6.

It was therefore hypothesised that combining these two critical arginines (R27a and R92) in one light chain, as in B33V\(\lambda\) (CDR1 of B3V\(\lambda\) and CDR2/CDR3 of 33.H11V\(\lambda\)), might further enhance this IgG molecule’s ability to bind dsDNA. This idea was also predicted by computer modelling, which suggested that both of these arginines (R27a and R92) might be in contact with dsDNA (Figure 4.9 A&B). The anti-dsDNA ELISA results confirmed this hypothesis, with B3V\(\text{H}\)/B33V\(\lambda\) binding more strongly than the original B3V\(\text{H}\)/B3V\(\lambda\) or indeed B3V\(\text{H}\)/33.H11V\(\lambda\) (Figure 4.7). The importance of these two arginines (R27a in CDR1 B3V\(\lambda\) and R92 in CDR3 33.H11V\(\lambda\)) in binding to dsDNA, was further confirmed by the finding that dsDNA
binding was reduced approximately two-fold in combinations lacking both of these particular CDR motifs (B3V\textsubscript{h}/33BV\textsubscript{\lambda} and B3V\textsubscript{h}/UBV\textsubscript{\lambda}).

UK4V\textsubscript{\lambda} has an asparagine at position 27a and a serine instead of the 2a2 germ-line glycine residue at position 29, however computer modelling (Figure 1.7) predicts that it is mainly the presence of an arginine molecule at position 94 (R94) of the CDR3 that is responsible for the observed lack of binding of B3V\textsubscript{h}/UK4V\textsubscript{\lambda} to dsDNA (Rahman et al. 2001). This hypothesis is supported by the data shown in Figure 4.7, since the combinations that contained the CDR2/CDR3 from UK4 (B3V\textsubscript{h}/BUV\textsubscript{\lambda} and B3V\textsubscript{h}/33UV\textsubscript{\lambda}) were the only combinations that could not bind to dsDNA, besides the parent molecule B3V\textsubscript{h}/UK4V\textsubscript{\lambda}. Furthermore the presence of the CDR1 region of UK4 (containing the somatic mutations of an asparagine at position 27a and serine at position 29), still allowed binding to dsDNA, as shown in the combinations B3V\textsubscript{h}/UBV\textsubscript{\lambda} and B3V\textsubscript{h}/U33V\textsubscript{\lambda} (Figure 4.7). The high binding of B3V\textsubscript{h}/U33V\textsubscript{\lambda} to dsDNA is especially striking. In this case, CDR swapping has converted a non-binding ‘wild-type’ light chain (UK4) into a DNA binding light chain (hybrid U33). This result supports the hypothesis that the V\textsubscript{\lambda} CDR3 regions of UK4 and 33.H11 exert opposite effects on DNA binding, despite the fact that the arginine residues are present at only slightly different positions in the two molecules (R92 in 33.H11 and R94 in UK4).

Taken together these data emphasise the role played by the light chain sequence of B3, and in particular the importance of arginine residues derived by somatic mutation in enhancing the binding of anti-dsDNA IgG antibodies. However, these data also demonstrate clearly that it is not just the number of arginine residues present, but also that their position in the sequence is crucial, since the arginine at position 94 of UK4 completely prevents binding to dsDNA, probably due to stearic hindrance, whereas the arginine at position 92 of 33.H11 enhances binding to dsDNA. Studies by other workers have shown that the precise location of arginine residues in the CDRs is important. Li et al, found that although the presence of arginine residues in the V\textsubscript{h}CDR3 of stably expressed monoclonal antibody 412.67 contributed to DNA-binding, an arginine residue in the kappa light chain CDR3 did not (Li et al. 2000). These effects were explained using a computer model which showed that the
V<sub>H</sub>CDR3 arginines were exposed on the surface of 412.67, and therefore available to bind DNA, whilst the inward orientation of the V<sub>K</sub>CDR3 arginine from the surface of the antigen-binding site, prevented it from binding to DNA.

The contribution of the B3 and 33.H11 CDR motifs (R27a and R92) to DNA binding was further demonstrated, by experiments utilising the nine light chains (3 parent light chains and 6 light chain CDR hybrids) in combination with the heavy chain of 33.H11 (these experiments are not shown in this thesis, as although I prepared four of the light chain hybrids, these particular electroporations and assays were carried out by Joanna Haley). These experiments showed that 33.H11V<sub>H</sub> only bound DNA when paired with the B33V<sub>K</sub> hybrid chain, emphasising the importance of the sequence motifs present in this light chain (Haley et al. 2004). Interestingly, pairing of the 33.H11V<sub>H</sub> with either B3V<sub>K</sub> or 33.H11V<sub>K</sub> produced IgG molecules, which did not bind dsDNA. ‘Wild-type’ 33.H11 has previously been shown to bind dsDNA by ELISA (Winkler et al. 1991), although it does not bind Crithidia, and these experiments support the theory that it is at best a low affinity anti-dsDNA antibody, as discussed in section 1.4.7.1. These experiments therefore also emphasised the dominant role of the B3 heavy chain in binding to dsDNA.

Using a bacterial expression system to express cloned antibody cDNA sequences in the form of Fab, our group has previously shown that dsDNA binding is reduced when the heavy chain of B3 is replaced by that of 33.H11 (Kumar et al. 2000). Also when COS-7 cells were used, to express IgG molecules consisting of B3V<sub>K</sub> paired with the V<sub>H</sub> of a different antibody WRI176 (human IgM anti-ssDNA antibody), this combination could not bind ssDNA or dsDNA (Rahman et al. 1998). This finding accords with the original B3/dsDNA computer model (Figure 1.4) which (as discussed in section 1.4.5) predicts that the heavy chain of B3, and particularly the arginine at position 53 of V<sub>H</sub>CDR2, is important in creating the antigen-binding site (Kalsi et al. 1996). In B3V<sub>H</sub>, the arginine residue, R53, is the product of somatic mutation from a germline serine residue. In 33.H11V<sub>H</sub> this residue is also mutated but to a neutral glycine. However, in 33.H11 the lack of an arginine residue at position 53 may be balanced by an extra somatic mutation from a negatively charged glutamic acid to a positively charged lysine at another centrally located antigen.
contact site, position 56 in \( V_{H} \) CDR2. The \( V_{H} \) regions of B3 and 33.H11 are derived from different germline genes, \( V_{H}3-23 \) and \( V_{H}3-07 \), respectively. Therefore it is possible that \( V_{H}3-23 \), prior to any somatic mutation, may be predisposed to higher DNA binding than \( V_{H}3-07 \). Alternatively, the somatic mutations in B3\( V_{H} \) may enhance binding to dsDNA more than those in 33.H11\( V_{H} \). Both \( V_{H} \) sequences are extensively mutated, compared to their germline genes, with clustering of replacement mutations in the CDRs suggestive of antigen drive. The importance of the heavy chain sequence for the binding of both murine and human anti-DNA antibodies to DNA, has been shown by several researchers as discussed in section 1.4.7 (Mockridge et al. 1996; Pewzner-Jung et al. 1996; Radic et al. 1991; Radic et al. 1993).

Therefore in the case of B3, the data reported in this thesis and by Haley et al (2004), show that both the heavy and light chains are important in binding to dsDNA. The somatic mutations present in the heavy and light chain sequence of B3 (R27a in \( V_{\lambda} \) CDR1 and R53 in \( V_{H} \) CDR2) which have been shown to be important in binding to DNA are therefore likely to have arisen as a result of binding to DNA (or antigen containing DNA such as nucleosomes or chromatin) in a process of antigen-driven affinity maturation.

As described in section 4.6, supervectors (SVB33 and SVBU) were produced which combined B3\( V_{H} \) with the most interesting of the light chain hybrids, B33\( V_{\lambda} \) containing R27a and R92 which were shown to enhance binding to dsDNA and BU\( V_{\lambda} \) containing the R94 which blocked dsDNA binding. The transient expression of these supervectors in COS-7 cells seemed to increase (2-4 fold) the production of human IgG, as compared to transfection with the separate heavy and light chain vectors. As seen previously, the levels of IgG produced by cells transfected with SVBU (producing non-DNA binding IgG) were higher than those transfected with SVB33 (producing DNA-binding IgG). However, stable transfection of CHO cells with the supervectors seemed to remove this 'DNA-binding related' difference in human IgG production, although great variation was seen between different clones of CHO cells (Table 4.3). The variation between the clones was especially marked after increased human IgG production due to methotrexate amplification (Table4.4).
The dsDNA binding of the human IgG molecules produced by the transient (COS-7 cells) and stable expression (CHO cells) of the supervectors is shown in Figures 4.11 and 4.13, respectively. The results concur with the findings obtained using the transient expression, of separate heavy and light chain vectors on COS-7 cells, showing again the strong dsDNA binding of B3VH/VH33 and the lack of dsDNA binding by B3VH/VH33BU. The lack of dsDNA binding of the recombinant IgG antibodies (SVBL and SVB33) unless the supernatants were pre-treated with DNase I (Figure 4.12), illustrated that the binding sites were occupied by a factor present in the culture supernatants, such as DNA or nucleosomes.

The results shown in Figure 4.13, were obtained by assaying the dsDNA binding of the recombinant antibodies whilst still in situ in the cell culture supernatants in which they were produced. However, intriguing results were observed, when testing the human IgG binding of the same recombinant antibodies to dsDNA, following their affinity purification on protein A (Figure 4.14). The difference in the anti-dsDNA binding of the affinity purified antibodies diluted either in SEC buffer or COS-7 cell supernatant (both DNase I treated), shown in Figures 4.14 [C&D] and [E&F] respectively, implies that the purified antibodies (SVBL, SVB33 and SVBLX, not SVBU) cannot bind to dsDNA without the presence of a ‘cofactor’ derived from the COS-7 cell supernatant. This ‘cofactor’ is not removed (or not completely removed) by DNase I treatment of the COS-7 supernatant.

This result, suggests that B3 (SVBL) and the mutants (SVB33 and SVBLX) are not pure dsDNA binders, it is possible that they actually recognise an epitope comprising DNA with histone, such as occurs in nucleosomes. The original studies (using B3 hybridoma cell supernatant) of B3 (Ehrenstein et al. 1993), did not test the binding of purified antibody to dsDNA, and although DNase I treatment of the B3 hybridoma supernatant was necessary for dsDNA binding, this may not have removed all the material from the antibody binding site (Kramers et al. 1994), just enough of the free factor (for example nucleosomes) from the supernatant to prevent competitive inhibition of binding. It is also interesting to note, that in the previous study (Ehrenstein et al. 1993), binding of B3 to a heterologous mixture of histones (histone type IIS, Sigma) was decreased by DNase I treatment and increased by the addition of DNA to the supernatant. In support of B3 recognising an epitope comprising both
DNA and histone regions, as found in nucleosomes, an earlier study (Ehrenstein et al. 1995) showed the strong binding of B3 hybridoma supernatant to nucleosomes (nucleohistone, Sigma).

The difference in dsDNA binding between the affinity purified antibodies, diluted in SEC buffer but either with or without pre-treatment with DNase I (shown in Figure 4.14 [C&D] and [A&B] respectively), was also interesting. It is likely, that during affinity purification of the recombinant human IgG containing CHO supernatants, the majority of the supernatant components are removed. However, Figure 4.14 [A&B] suggests that some of the 'cofactor', probably nucleosomes, remained bound to SVB33 allowing binding to dsDNA on the solid-phase of the ELISA. The fact that SVBL and SVBLX do not bind to dsDNA under these conditions, suggests that they may have a lower affinity for the 'cofactor', such that purification removed the 'cofactor' from the binding site of these antibodies. This theory is potentially supported, by the abrogation of the binding of SVB33 to dsDNA by DNase I treatment (as shown in Figure 4.14 [C&D]). This hypothesis is based on the assumption that if only a small quantity of nucleosome was bound to the purified SVB33, DNase I digestion of the DNA component might dissociate the complex and prevent binding to DNA via the histone component. If this analysis of the data is correct, then it is possible that the combination of the arginine residues R27a and R92 as found in B3V_{H}/B33V_{L} (SVB33) have actually increased the binding to nucleosomes, rather than to pure dsDNA.

An alternative, but less attractive, explanation for the difference between the results shown in Figure 4.14 [A&B] and [C&D] is that DNase I is acting as a competitive inhibitor in the assay shown in Figure 4.14 [C&D]. There is some evidence that anti-DNA antibodies can bind to DNase I (Puccetti et al. 1995). However, if DNase I is a competitive inhibitor in the assay shown in Figure 4.14 [C&D], why did this not also occur in the assay shown in Figure 4.14 [E&F]? Although, in the assay shown in Figure [E&F] there is likely to be large amounts of DNA present in the supernatant, which might reduce the likelihood of DNase I binding to the antibodies.

The binding of B3V_{H} in combination with the nine light chains (3 parent light chains and 6 light chain CDR hybrids) to histones (coated on ELISA plates), was also tested.
by Joanna Haley (Haley et al. 2004). The relative ability of the various B3V\textsubscript{H} and different light chain combinations to bind histones differed from their ability to bind dsDNA. The highest binding to histone, was observed for the original B3V\textsubscript{H}/B3V\textsubscript{\lambda} combination. This study also suggested that for binding to histones, the presence of the B3V\textsubscript{\lambda} CDR1 was not as important as the presence of B3V\textsubscript{\lambda} CDR2/CDR3, since replacement of this region with 33.H11V\textsubscript{\lambda} CDR2/CDR3 (as in B3V\textsubscript{H}/B33V\textsubscript{\lambda}, the IgG produced by cells transfected with supervector SVB33) dramatically reduced the binding to histones, but reversion of R27a to a serine (as in IgG from cells transfected with SVBLX) only slightly reduced the binding to histones.

Thus although the combination of the arginine residues R27a and R92, as found in B3V\textsubscript{H}/B33V\textsubscript{\lambda} (SVB33), have actually increased the binding to dsDNA via histone (as in nucleosomes), the addition of R92 appears to reduce the binding to histone alone. It is possible that the positively charged R92 residue in 33.H11 V\textsubscript{\lambda} CDR3 may inhibit binding to positively charged histones while enhancing binding to negatively charged DNA. As with DNA, the UK-4 R94 CDR motif blocked binding to histones. However, the IgG combinations which contained B3V\textsubscript{H} with the light chains UK-4V\textsubscript{\lambda}, 33UV\textsubscript{\lambda} and BUV\textsubscript{\lambda} were found to bind to another self-antigen, Ro60 (Haley et al. 2004). This finding showed that the inability of these light chains to bind to DNA or histones, was not due to the experiments having caused such a major change in structure, that the heavy/light chain combinations containing UK-4V\textsubscript{\lambda} CDR3 could not function as antibodies at all.

The importance of the arginine at position 27a of B3 light chain was underlined by the lack of in vitro ANA binding of human IgG purified from the supernatant of CHO cells transfected with SVBLX (Figure 4.15). There was also no ANA binding seen when IgG purified from the CHO cells transfected with SVBU, confirming the ability of the arginine at position 94 to block binding. ANA binding was observed in Hep2 cells incubated with purified human IgG from the CHO cells producing ‘wild-type’ B3 (SVBL transfected) and B3VH/V\textsubscript{\lambda}B33 (SVB33 transfected). The binding of affinity purified SVBL and SVB33 (diluted in PBS) to nuclear antigens, did not require the presence of a ‘cofactor’ from cell supernatant. This result was because the substrate was not pure DNA, but the whole range of nuclear antigens.
Based on these findings, it was interesting to try and compare the pathogenicity of these recombinant human IgG molecules *in vivo*, as described in Chapter Five.
RESULTS CHAPTER FIVE

Investigation of the pathogenicity of recombinant anti-DNA IgG in SCID mice
CHAPTER FIVE

INVESTIGATION OF THE PATHOGENICITY OF RECOMBINANT ANTI-DNA IgG IN SCID MICE

5.1 Introduction and aims of this chapter

The aim of this chapter was to investigate, whether the differences in dsDNA binding between the different light chain variants of B3 (as seen in chapter 4), would result in different degrees of pathogenicity in vivo.

SCID mice have previously been used to study the pathogenicity of both murine and human anti-DNA antibodies as in the experiments described in chapter 3. As discussed in the introduction (section 1.4.8.3), the human anti-dsDNA antibody RH-14 was the most nephritogenic human IgG molecule, when produced by hybridoma cells implanted into SCID mice (Ravirajan et al. 1998). However, due to technical difficulties, RH-14 has not yet been cloned. However, another IgG1\(\lambda\) anti-dsDNA antibody, B3, was sequenced and cloned prior to the start of my PhD. The binding properties of B3 have already been extensively discussed earlier in this thesis. When human hybridoma cells secreting B3 were implanted into SCID mice, the antibody was shown to penetrate cells and bind to their nuclei, both in the kidney and in other organs (Ehrenstein et al. 1995). Although the mice implanted with B3 developed proteinuria, histological examination of their kidneys, by light and electron microscopy, did not show evidence of glomerulonephritis. Hence, the mechanism by which B3 induced proteinuria production is not clear.

As described in chapter 4, our group now has a number of CHO cell lines producing recombinant B3 and variants of B3, which exhibit differing capacity for binding to DNA. It was therefore of interest to compare their pathogenicity in vivo and since the SCID mouse had previously been successful for this purpose using hybridoma cells, it was decided to try implanting the CHO cells into these mice. This chapter
describes four experiments implanting SCID mice with CHO cell lines producing recombinant human IgG, the implanted cell lines are summarised in Table 5.1.

As discussed later in this chapter, during the course of the experiments implanting the CHO cells into the SCID mice it became apparent that there were a number of problems inherent in this system. An alternative approach was to inject the SCID mice directly with purified recombinant human IgG antibodies. The advantage was that intravenous (i.v.) injection could avoid pathology that might result from implanting the CHO cells. The disadvantage was that the IgG molecules were known to have a half-life of only a few hours, being rapidly cleared by the innate immune system, and also that a large quantity of purified IgG was required. Thus, i.v. injection of the human IgG would only be able to yield information regarding deposition of different recombinant IgG molecules in the kidney, but not their longer-term pathology. Initially, due to the lack of sufficient quantities of recombinant IgG, implanting the cells into SCID mice was the only option. Once several milligrams of purified recombinant IgG were obtained by large-scale culture of several CHO cells lines (as described in section 4.8.3) it was possible to attempt the i.v. injection experiment.

5.2 Implanting recombinant B3 (SVBL) and mutated B3 (SVBLX) into SCID mice

Our group previously showed that a single arginine to serine reversion at position 27a of the light chain of B3, was sufficient to cause a significant reduction in dsDNA binding by ELISA (Rahman et al. 2001). The initial two experiments implanting CHO cells producing recombinant human IgG into SCID mice, were conducted to compare the potential in vivo pathogenicity of ‘wild-type’ B3 (produced by CHO cells stably transfected with SVBL) with that of the point mutated B3 (arginine to serine reversion at 27a, referred to as R27aS, produced by CHO cells transfected with SVBLX). Prior to implanting the CHO cell lines, it was noted that their human IgG production was 130 and 250 ng / 1x10^6 cells / day for SVBL 5-40-24 and SVBLX 29-11-11 respectively (data from Dr. Joanna Haley’s thesis).
<table>
<thead>
<tr>
<th>Name (Supervisor transfected)</th>
<th>Cell Clone No.</th>
<th>Recombinant human IgG produced by cell line</th>
<th>Number of rounds of methotrexate amplification</th>
<th>Methotrexate concentration cell line is maintained in (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVBL</td>
<td>5-40-24*</td>
<td>B3VH and B3Vλ ‘wild type’</td>
<td>2</td>
<td>1 x 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>3-26*</td>
<td></td>
<td>1</td>
<td>1 x 10⁹</td>
</tr>
<tr>
<td></td>
<td>29-11-11*</td>
<td></td>
<td>2</td>
<td>1 x 10⁻⁷</td>
</tr>
<tr>
<td>SVBLX</td>
<td>10-47*</td>
<td>B3VH and B3Vλ (R27aS)§</td>
<td>1</td>
<td>1 x 10⁻⁹</td>
</tr>
<tr>
<td></td>
<td>6-26*</td>
<td></td>
<td>1</td>
<td>1 x 10⁻⁹</td>
</tr>
<tr>
<td>SVB33</td>
<td>3-15</td>
<td>B3VH and light chain hybrid B33 (B3Vλ CDR1, 33.H11Vλ CDR2 and CDR3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3-15-23</td>
<td></td>
<td>1</td>
<td>1 x 10⁻⁸</td>
</tr>
<tr>
<td>SVBU</td>
<td>5-18</td>
<td>B3VH and light chain hybrid BU (B3Vλ CDR1, UK4Vλ CDR2 and CDR3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-18-6</td>
<td></td>
<td>1</td>
<td>1 x 10⁻⁸</td>
</tr>
<tr>
<td>pG1D210*</td>
<td></td>
<td>Non-secreting referred to as ‘empty-vector’</td>
<td>2</td>
<td>1 x 10⁻⁷</td>
</tr>
</tbody>
</table>

* These supervectors and CHO cell lines were produced by Joanna Haley

§ R27aS: Vλ chain contains a point mutation of arginine to serine at position 27a of CDR1, which represents a reversion of a somatic mutation of B3 to the 2a2 germline sequence
5.2.1 Preliminary implantation of SVBL and SVBLX

In this first experiment, 10 days after pristane priming, the SCID mice were implanted with CHO cells containing either SVBL 5-40-24 (n=5 mice) or SVBLX 29-11-11 (n=5), a control group received non-transfected CHOdhfr− cells (n=4) and a further group received only the pristane priming injection (n=4).

The results of this experiment are summarised in Table 5.2. The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test, and the proteinuria values for all the groups of mice were compared using a one-way ANOVA, followed by Bonferroni’s multiple comparison test. The ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, P<0.0001. The Bonferroni post-test indicated that the significant differences in proteinuria were between mice implanted with SVBL and SVBLX (P<0.001); SVBL and the non-transfected CHOdhfr− cells (P<0.001); and SVBL and pristane only controls (P<0.001). Proteinuria was increased in mice implanted with SVBL despite the fact that the highest levels of human IgG, up to 260ng/ml, were detected in the sera of the mice implanted with SVBLX.

Unfortunately the non-transfected CHOdhfr− cells did not survive in the SCID mice, no evidence of the cells was found on autopsy. This observation was possibly due to the fact that these non-transfected cells did not contain a functional dihydrofolate reductase (dhfr) gene, since they did not contain the expression vector containing this gene. These cells therefore were unable to grow in the absence of deoxyribonucleosides and ribonucleosides, and it is plausible that the availability of these nutrients in the peritoneum of the mice was insufficient for survival of these cells. It might also be postulated that the presence of the expression plasmid DNA inside the SVBL and SVBLX CHO cells, could potentially be toxic and cause pathogenic effects in the mice. To address these issues, the repeat experiment (section 5.2.2) included mice implanted with CHO cells that contained the ‘empty-vector’ pG1D210, thus incorporating the functional dhfr gene.
Table 5.2 Summary of results from initial experiment implanting CHO cell lines SVBL and SVBLX into SCID mice

These results are from the initial experiment described in section 5.2.1. Proteinuria was assessed using Albustix which give a semi-quantitative measure based on colour change, proteinuria level is scored as negative or trace, (+) 0.3g/L, (++) 1.0g/L, (+++) 3.0g/L. Based on these given values, in order to calculate the estimated* g/L, it was assumed that trace = 0.1g/L, trace/+ = 0.2g/L, +/++ = 0.65g/L and ++/+++ = 2.0g/L. The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test and compared using a one-way ANOVA, followed by Bonferroni’s multiple comparison test. The ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, P<0.0001. The Bonferroni post-test indicated that the significant differences in proteinuria were between mice implanted with SVBL and SVBLX [P<0.001]; SVBL and the non-transfected CHOdhfr<sup>-</sup> cells [P<0.001]; and SVBL and pristane only [P<0.001]. Key to symbols: $, these mice were still healthy when terminated; #, these cells did not survive/grow in the mice.
5.2.2 Repeat implantation of SVBL and SVBLX

In the repeat experiment, 5 mice each were implanted with CHO cells containing either SVBL 5-40-24, SVBLX 29-11-11 or ‘empty-vector’ pG1D210, an additional 3 mice received only pristane priming. In the second experiment, 3 additional mice were also implanted per group to be terminated early at days 2, 7, and 14 after implantation. This was done to investigate human IgG levels and any pathological changes that might be transient and not seen at termination.

The results of the repeat experiment are summarised in Table 5.3. The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test and compared using a one-way ANOVA, followed by Bonferroni’s multiple comparison test. The ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, P=0.0003. The Bonferroni post-test indicated that the significant differences in proteinuria were between mice implanted with SVBL and SVBLX (P<0.05); SVBL and the CHO cells containing the ‘empty vector’ pG1D210 (P<0.001); and SVBL and pristane only controls (P<0.01). However, the mice that received CHO cells containing the ‘empty-vector’ control pG1D210, did have some proteinuria in the range of trace to ++. Figure 5.1, shows the proteinuria production, in each group of mice, over the duration of the experiment. This graph (Figure 5.1) also shows why, due to the differing survival of the mice in different groups, it was necessary to compare the terminal proteinuria values in the mice in the summary Table 5.3.

5.2.3 Human IgG production in the SCID mice implanted with CHO cells

In the repeat experiment as in the first, the maximum levels of human IgG were found in the group implanted with the SVBLX containing CHO cells. This reflected the in vitro human IgG expression of these cell lines, since the SVBLX containing CHO cells consistently had a slightly higher level of human IgG expression than the SVBL containing cell line. The control ‘empty-vector’ produced no human IgG in culture and no human IgG was detected in any of the mice implanted with CHO cells containing the ‘empty-vector’.

Secretion of human IgG does not appear to be transient in this system, since additional mice killed earlier at either 2, 7 or 14 days after implantation, had even
Table 5.3 Summary of results from repeat experiment implanting CHO cell lines SVBL and SVBLX into SCID mice

These results are from the repeat experiment described in section 5.2.2. Proteinuria was assessed using Albustix which give a semi-quantitative measure based on colour change, proteinuria level is scored as negative or trace, (+) 0.3g/L, (++) 1.0g/L, (+++) 3.0g/L. Based on these given values, in order to calculate the estimated* g/L, it was assumed that trace = 0.1g/L, trace/+ = 0.2g/L, +/- = 0.65g/L and +++ = 2.0g/L. The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test and compared using a one-way ANOVA, followed by Bonferroni’s multiple comparison test. The ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, P=0.0003. The Bonferroni post-test indicated that the significant differences in proteinuria were between mice implanted with SVBL and SVBLX [P<0.05]; SVBL and the CHO cells containing the ‘empty vector’ pG1D210 [P<0.001]; and SVBL and pristane only [P<0.01]. Key: $, these mice were still healthy when terminated.

<table>
<thead>
<tr>
<th>Implanted Cells</th>
<th>Mean Human IgG in sera (ng/ml) (number of mice/group)</th>
<th>Terminal Proteinuria (range of group)</th>
<th>Mean Terminal Proteinuria (estimated* g/L)</th>
<th>Mean Day of Death</th>
<th>Mean weight change Day 0-21 (g/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVBL 5-40-24</td>
<td>105 (4/5), NR (1/5)</td>
<td>++ - +++</td>
<td>2.4</td>
<td>26</td>
<td>0.64</td>
</tr>
<tr>
<td>SVBLX 29-11-11</td>
<td>420 (5/5)</td>
<td>+ - +++/+</td>
<td>0.99</td>
<td>39</td>
<td>1.24</td>
</tr>
<tr>
<td>'Empty-vector' pG1D210</td>
<td>0</td>
<td>trace - +/-</td>
<td>0.38</td>
<td>48</td>
<td>0.64</td>
</tr>
<tr>
<td>Pristane only</td>
<td>0</td>
<td>trace/+</td>
<td>0.2</td>
<td>56$</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Figure 5.1 Proteinuria production over time in mice implanted with CHO cells producing recombinant human IgG

These results are from the repeat experiment described in section 5.2.2. Proteinuria was assessed using Albustix which give a semi-quantitative measure based on colour change, proteinuria level is scored as negative or trace, (+) 0.3g/L, (++) 1.0g/L, (+++)
3.0g/L. Based on these given values, in order to calculate the estimated* g/L, it was assumed that trace = 0.1g/L, trace/+ = 0.2g/L, +/+ = 0.65g/L and +/+++ = 2.0g/L. Each point on the graph is the mean estimated proteinuria, of the surviving mice in each group at each time point. The error bars show the standard deviation (SD). The mice were pristane primed on day -10, and the cells were implanted on day 0.
lower serum human IgG levels than those mice whose sera was measured on termination (data are not shown as OD values were too close to the lower limit of the assay to accurately calculate the human IgG concentrations).

5.2.4 Reduced survival of mice implanted with CHO cells containing SVBL

In both experiments, as shown in Tables 5.2 and 5.3, the mice that had been implanted with CHO cells containing SVBL became ill and died earlier than mice in the groups that received either CHO cells containing SVBLX or any of the control groups. However mice implanted with the CHO cells containing the 'empty-vector' pG1D210, also died earlier than the pristane only control mice (Table 5.3). The percentage survival of mice over time, in the second repeat experiment, is shown in Figure 5.2. On termination a few large tumour masses and many small lumps were found in the peritoneum of all of the mice implanted with CHO cells (except non-transfected CHOdhfr cells in experiment 1), but no differences were observed between groups. Some of the mice had enlarged spleens or more peritoneal/ascitic fluid than others but this occurred within all groups of mice regardless of the cells implanted. As a rule, the culture of CHO cells in the SCID mice did not result in the formation of significant amounts of ascitic fluid in the peritoneum, as is usually found on culture of hybridoma cells in SCID mice. An enlarged spleen is a common feature of SCID mice (Vladutiu 1993).

5.2.5 Histopathology of SCID mice implanted with CHO cells

Despite the significant levels of proteinuria observed in the mice implanted with CHO cells containing SVBL, H&E staining (section 2.3.5.1) of kidney sections showed no evidence of 'lupus-like' morphology in any of the groups of mice. Staining with rabbit anti-human IgG-HRP developed with DAB (section 2.3.5.2), also failed to detect any human antibody deposition in kidneys from any of the mice.

However there was evidence of non-SLE related pathology, in terms of neutrophil infiltration of kidney glomeruli and the liver, as well as liver necrosis (the neutrophils were confirmed by staining for the presence of chloroacetate esterase, section 2.9.4, as well as by their characteristic morphology after H&E staining). The neutrophil infiltration occurred in all groups of mice, irrespective of whether they had been implanted with CHO cells containing SVBL, SVBLX or pG1D210 the
Figure 5.2 Percentage survival over time of mice implanted with CHO cells producing recombinant human IgG

This figure shows the percentage of mice, in each group, that were still alive at each time point. There was reduced survival of mice in the groups that were implanted with CHO cells producing SVBL and SVBLX, as compared to those mice in groups that were implanted with CHO cells containing the empty vector or were only primed with pristane. The mice were killed when they became ill, there was no evidence of ascites growth in these mice. (These results are from the repeat experiment described in section 5.2.2.)
'empty-vector' control. The infiltration of neutrophils was most marked in the CHO cells containing the 'empty-vector', but this might be because these mice were sacrificed later since they were less ill. Mice killed earlier at days 2, 7 or 14 showed consistently less pathology, with immature neutrophil infiltration, in the same groups of mice.

Electron microscopy of kidney sections (section 2.3.5) revealed very limited morphological changes including mesangial cell interposition, splitting of the basement membrane and some microvillus transformation. However, these changes were present in all groups (SVBL, SVBLX and pG1D210 'empty-vector'). In all groups the foot processes were normal, there was no thickening of the basement membrane and no evidence of immune deposits. Myelin figures were also observed in the kidneys examined, these were possibly due to the pristane-priming.

5.2.6 Further observations of the SCID mice implanted with CHO cells

The mean change in weight of the mice in each experimental group, over the 21 days following CHO cell implantation, are shown in Table 5.3. The mice which received only pristane, gained the most weight at 1.8 g/mouse, this is a normal healthy weight gain. Mice implanted with CHO cells containing SVBL put on less weight than those implanted with cells containing SVBLX. These results could be interpreted in two ways, either the SVBLX containing CHO cells are growing faster in vivo than the SVBL containing cells, or that the mice which received the SVBL CHO cells, underwent greater weight loss due to cachexia than the SVBLX group. The latter is perhaps more likely in view of the greatest weight gain being seen in the control mice given pristane only. It seems that the effects of cachexia in the mice, mask any changes in weight due to growth of the CHO cells. There was however, no difference between the weight change observed in the mice implanted with SVBL and those receiving the CHO cells containing the empty vector.

The levels of murine immunoglobulins were also checked in the repeat experiment. The SCID mice were slightly 'leaky' on termination of the experiment (mice then aged 3-4 months) with low levels of murine IgM found in the terminal bleeds of all of the mice, irrespective of their experimental group. Murine IgG was only found in
one mouse, which had been implanted with CHO cells containing the ‘empty-vector’ (data not shown).

5.2.7 Initial assessment of the first two experiments testing the pathogenicity of recombinant human IgG produced by CHO cells implanted into SCID mice

After assessing the results of the first two experiments implanting SCID mice with CHO cells, producing B3 (wild-type, from SVBL transfected cells) and mutated B3 (arginine to serine reversion, from SVBLX transfected cells), the predominant finding was the reproducible increased proteinuria and decreased survival of the mice implanted with CHO cells containing SVBL. It was notable that the mice implanted with CHO cells containing SVBL, also had less human IgG in their sera than those receiving SVBLX containing cells. Despite the apparent background pathology caused by the CHO cells and or incorporated plasmid DNA, both groups of mice exposed to low levels of recombinant human IgG, developed higher levels of proteinuria and died earlier than mice which received CHO cells containing the ‘empty-vector’ control. It was important to consider whether these data implied that the secreted antibody, caused the enhanced proteinuria and death in these mice and whether the arginine to serine reversion in the mutated B3, was responsible for the differences between mice implanted with CHO cells containing SVBL and SVBLX.

Although the results of the two experiments were reproducible, the same cell lines were implanted in both experiments. It was conceivable that the difference between mice implanted with CHO cells containing SVBL or SVBLX might be due to a coincidental difference between the CHO cell clones, irrespective of which supervector DNA they contain. Thus the next experiment examined whether several different clones of CHO cells, producing the same recombinant IgG, might also give different results in the SCID mice.

5.3 Testing clonal variability of CHO cell lines producing recombinant human IgG on implantation into SCID mice

A third experiment was carried out to test any difference in vivo between different clones of CHO cells producing the same recombinant human IgG molecule. The original clones used in the first two experiments were re-tested, alongside ‘new’ clones derived from different cell foci during the original transfection of the CHO
cells, with the supervectors SVBL and SVBLX. In total two clones of SVBL (5-40-24 and 3-26) and three clones containing SVBLX (29-11-11, 10-47 and 6-26) were used (clones were at different stages of methotrexate amplification, as summarised Table 5.1).

It was observed in the initial two experiments that the CHO cells did not grow in ascites fluid as hybridoma cells would. This lack of growth, might be because the CHO cells are adherent when grown in vitro. In adherent growth, cells can stop dividing when they contact with other cells (at confluence) hence this could be another factor in the low level of human IgG which was produced in the mice. In an effort to increase the human IgG produced in vivo, additional mice were implanted with ten times (1 x 10⁷ cells) the number of cells.

The numbers of mice receiving each cell line and the results of this experiment are shown in Table 5.4, part (A) shows the results from mice implanted with 1 x 10⁶ cells and part (B) refers to mice receiving 1 x 10⁷ cells. Part (C) of Table 5.4, shows the human IgG production of the cells in vitro, prior to implanting into the SCID mice.

5.3.1 Human IgG production in vitro and in vivo

Unfortunately, as shown in Table 5.4 (C), the production of human IgG in vitro by clone SVBL 5-40-24 was very low (< 4.0 ng/1x10⁶ cells/day), compared with this clone's previous production prior to experiments 1 and 2 (130 ng/1x10⁶ cells/day). The other SVBL clone SVBL 3-26, also had a low in vitro human IgG production (2.6 ng/1x10⁶ cells/day). However, despite the low in vitro production, the human IgG levels in the mouse sera at the end of this experiment were actually higher than those observed in experiments 1 and 2, possibly due to the inclusion of methotrexate in the media injected with the cells in this experiment (maintaining the selective pressure as described in sections 2.8.3 and 2.9.2).

As in experiments 1 and 2, SVBLX 29-11-11 produced higher levels of human IgG both in vitro and in vivo, than either SVBL clone. A second clone of SVBLX, 10-47, produced an even higher level of human IgG both in vitro and in vivo, on the other hand the third clone SVBLX 6-26 did not produce well in culture and human IgG was undetectable in the murine sera of mice implanted with this clone. Excepting
Table 5.4 Summary of data from experiment implanting ‘multiple-clones’ of CHO cell lines into SCID mice

Proteinuria was assessed using Albustix which give a semi-quantitative measure based on colour change, proteinuria level is scored as negative or trace, (+) 0.3g/L, (++) 1.0g/L, (+++) 3.0g/L. Based on these given values, in order to calculate the estimated* g/L we assumed that trace = 0.1g/L, trace/+ = 0.2g/L, +/+ = 0.65g/L and ++/+++ = 2.0g/L. N/A = not applicable. * These mice remained healthy.
SVBLX 6-26, overall the levels of recombinant human IgG in the murine sera were substantially higher in mice implanted with SVBLX clones than those implanted with SVBL clones.

5.3.2 **Do proteinuria levels in the SCID mice reflect variation between CHO cell clones?**

The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test and compared using a one-way ANOVA, followed by Bonferroni’s multiple comparison test. The ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, \( P = 0.004 \). However, the Bonferroni post-test indicated that the significant differences in proteinuria were only between those mice implanted with SVBL 5-40-24 and those mice which received pristane only (\( P < 0.05 \)) or were unimmunised (\( P < 0.05 \)). In experiments 1 and 2, mice implanted with CHO cells containing SVBL 5-40-24 had significantly higher levels of proteinuria than mice implanted with SVBLX 29-11-11 (\( P < 0.001 \) and \( P < 0.05 \) respectively). Once again in this third experiment (mice implanted with \( 1 \times 10^6 \) cells, Table 5.4 [A]) the mean proteinuria was higher in those mice receiving SVBL 5-40-24 than those receiving SVBLX 29-11-11, but this was not significantly different (\( P > 0.05 \)). It was also notable, that mice that had received CHO cells containing SVBLX 6-26 had high proteinuria, despite human IgG being undetectable in their sera. There was also no significant difference (\( P > 0.05 \)) between the proteinuria observed in those mice that received pG1D210 ‘empty vector’ CHO cells and any of the other groups of mice receiving the CHO cell clones containing the supervectors. Thus, it seems that the CHO cells themselves were contributing significantly to the production of proteinuria in the mice. The proteinuria results, from the individual mice implanted with \( 1 \times 10^6 \) cells as in Table 5.4 (A), are shown in Figure 5.3.

5.3.3 **Other observations from the ‘multiple-clone’ experiment**

As observed in experiments 1 and 2, histological examination of H&E sections of the kidneys from these mice, revealed no differences between mice implanted with different CHO cell lines. All groups of mice had some hypercellularity of the glomeruli and evidence of congestion or haemorrhage. Once again the presence of neutrophils in the glomeruli was noted.
**Figure 5.3 Proteinuria levels in mice implanted with CHO cells producing recombinant human IgG**

Proteinuria was assessed using Albustix which give a semi-quantitative measure based on colour change, proteinuria level is scored as negative or trace, (+) 0.3g/L, (++) 1.0g/L, (+++) 3.0g/L. Based on these given values, in order to calculate the estimated* proteinuria in terms of grams/Litre, we assumed that trace = 0.1g/L, trace/+ = 0.2g/L, +/+ = 0.65g/L and ++/+++ = 2.0g/L.

A: This graph shows the terminal proteinuria of each mouse in the experiment described in section 5.3, these data are from the mice which received $1 \times 10^6$ cells. The horizontal bars show the mean proteinuria of each group of mice. The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test and compared using a one-way ANOVA, followed by Bonferroni’s multiple comparison test. The ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, $P=0.004$. However, the Bonferroni post-test indicated that the only significant differences in proteinuria were between those mice implanted with SVBL 5-40-24 and those mice which received pristane only ($P<0.05$) or were unimmunised ($P<0.05$).

B: This graph shows the production of proteinuria over time, following implantation of the CHO cells on day 0. These data are also from the experiment described in section 5.3.
Investigation of the pathogenicity of recombinant anti-DNA IgG in SCID mice

A

Proteinuria (g/L)

SVBL 5-40-24  SVBL 3-26  SVBLX 29-11-11  SVBLX 10-47  SVBLX 6-26  pG1D210  Pristane only  Unimmunised

B

Proteinuria (g/L)

SVBL 5-40-24  SVBL 3-26  SVBLX 29-11-11  SVBLX 10-47  SVBLX 6-26  'Empty vector' pG1D210  pristane only  unimmunised
There was no marked difference between the survival of mice in different experimental groups in this 'multi-clone' experiment, unlike the difference observed in experiments 1 and 2. The unimmunised mice or those given only pristane, were still healthy after all other groups of mice given CHO cells (including pG1D210 control) were dead, by day 21 irrespective of which clone or human IgG they produced. This implied that the use of the CHO cells in these mice was not viable.

The implantation of ten times the number of cells ($1 \times 10^7$ cells) as shown in Table 5.4 (B) did not give any increased human IgG in the sera, and simply lead to the earlier death, by day 12, of these mice. The levels of proteinuria were also higher than those seen in mice given $1 \times 10^6$ cells, further implicating the CHO cells themselves as the major cause of the pathology observed in these mice.

5.4 Implantation of SCID mice with CHO cells producing recombinant human IgG after stable transfection with supervectors SVB33 and SVBU

Before the inherent difficulties of implanting CHO cells in the SCID mice became apparent, a further in vivo experiment was underway. This experiment was to assess the two new supervectors, SVB33 and SVBU, which were produced and transfected into CHO cells as described in chapter 4. The ‘new’ vectors were tested alongside two clones of cells producing SVBL. The SVB33 and SVBU recombinant human IgG producing clones, are summarised in Table 5.1. Instead of two clones of different origin, in this experiment the same original clone was used before and after amplification with $1 \times 10^{-8}$ M methotrexate (as shown in Table 5.1). Once again each mouse was implanted with $1 \times 10^6$ cells in 0.5 ml of media and the methotrexate concentration was maintained.

The concentrations of human IgG that the CHO clones were producing prior to implantation were 83 ng/$1 \times 10^6$ cells/day (SVB33 3-15), 6700 ng/$1 \times 10^6$ cells/day (SVB33 3-15-23), 71 ng/$1 \times 10^6$ cells/day (SVBU 5-18) and 148 ng/$1 \times 10^6$ cells/day (SVBU 5-18-6) as shown in Tables 4.3 and 4.4. The results of implanting the cells in the SCID mice are summarised in Table 5.5. Unfortunately, as in the previous experiment (section 5.3) the results confirmed that the CHO cells were contributing to pathology by themselves, irrespective of the vector DNA that they contained. Because of this background pathology, no conclusions could be drawn regarding the
## Table 5.5 Summary of data from SCID mice implanted with CHO cells containing the 'new' supervectors SVB33 and SVBU

Proteinuria was assessed using Albustix which give a semi-quantitative measure based on colour change, proteinuria level is scored as negative or trace, (+) 0.3g/L, (++) 1.0g/L, (+++) 3.0g/L. Based on these given values, in order to calculate the estimated* g/L we assumed that trace = 0.1g/L, trace/+ = 0.2g/L, +/- = 0.65g/L and +++/+++ = 2.0g/L. The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test. One-way ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, P=0.019. However, the Bonferroni post-test indicated that the only significant difference in proteinuria was between those mice implanted with SVBL 5-40-24 and those that received pristane only (P<0.05). Key: $, these mice were still healthy when terminated.

<table>
<thead>
<tr>
<th>CHO cell clone name</th>
<th>Number of mice (n)</th>
<th>Human IgG in sera (mean of group, ng/ml)</th>
<th>Terminal proteinuria (range of group)</th>
<th>Mean proteinuria (estimated* g/L)</th>
<th>Day of death (mean of group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVBL 5-40-24</td>
<td>5</td>
<td>165</td>
<td>++ - +++/+++</td>
<td>1.2</td>
<td>12.4</td>
</tr>
<tr>
<td>SVBL 3-26</td>
<td>5</td>
<td>208</td>
<td>+ - ++</td>
<td>0.65</td>
<td>11.5</td>
</tr>
<tr>
<td>SVB33 3-15</td>
<td>5</td>
<td>127</td>
<td>trace/+ - ++</td>
<td>0.84</td>
<td>11</td>
</tr>
<tr>
<td>SVB33 3-15-23</td>
<td>5</td>
<td>184</td>
<td>+ - ++</td>
<td>0.44</td>
<td>11.6</td>
</tr>
<tr>
<td>SVBU 5-18</td>
<td>5</td>
<td>1575</td>
<td>trace - ++</td>
<td>0.68</td>
<td>11</td>
</tr>
<tr>
<td>SVBU 5-18-6</td>
<td>5</td>
<td>800</td>
<td>+ - ++</td>
<td>0.51</td>
<td>12.8</td>
</tr>
<tr>
<td>pG1D210</td>
<td>5</td>
<td>0</td>
<td>trace/+ - +++</td>
<td>0.54</td>
<td>17</td>
</tr>
<tr>
<td>Pristane only</td>
<td>5</td>
<td>0</td>
<td>trace/+ - +/+</td>
<td>0.33</td>
<td>$</td>
</tr>
</tbody>
</table>

*Mean of group values were calculated using the formula: (trace = 0.1g/L, trace/+ = 0.2g/L, +/- = 0.65g/L and +++/+++ = 2.0g/L)
potential pathological effects of the different human IgG produced by the different lines.

The proteinuria data were found to be normally distributed using the Kolmogorov-Smirnov test and compared using a one-way ANOVA, followed by Bonferroni’s multiple comparison test. The ANOVA showed that there was a significant difference between the mean proteinuria values of all the groups, \( P=0.019 \). However, the Bonferroni post-test indicated that the only significant difference in proteinuria was between those mice implanted with SVBL 5-40-24 and those mice that received pristane only (\( P<0.05 \)). Those mice that had received the ‘empty-vector’ pG1D210 control, survived marginally longer (day 17) and the mice that were only pristane primed were still healthy at day 17. Once again the histological examination of the kidneys showed pathology the same as that described in section 5.3.3. The observed pathology was presumably due to the CHO cells, since this was not pathology typical of lupus and was present in all of the groups that had been implanted. With the possible exception of those mice implanted with pG1D210 the mice implanted with CHO cells in this experiment (Table 5.5), died earlier than those in the experiment described in section 5.3 (Table 5.4). There is no obvious explanation for this, both experiments were carried out in the CB-17 SCID mice and used the same media containing methotrexate to maintain selective pressure for human IgG production.

The sera from the mice in this experiment were also tested for binding to dsDNA and \( \alpha \)-actinin as described in section 2.9.3. Despite human IgG having been shown to be present in all mice implanted with SVBL, SVB33 and SVBU (shown in Table 5.5), and despite repeating the assays after treatment of the sera with DNase I, there was no binding of any of these sera to either dsDNA or \( \alpha \)-actinin.

5.5 Intravenous (i.v.) injection of recombinant human IgG into SCID mice

A total of 10 SCID mice were injected with 100 \( \mu g \) of affinity purified recombinant human IgG as described in section 2.9.5. Two mice received each of the following antibodies: ‘wild-type’ B3 (purified from CHO cells transfected with SVBL); B3\( V_H \) combined with light chain hybrid B33 [B3\( V_\lambda \) CDR1, 33.H11\( V_\lambda \) CDR2 and CDR3]
(purified from CHO cells transfected with SVB33); B3V\textsubscript{H} combined with light chain hybrid BU [B3V\textsubscript{\lambda}, CDR1, UK4V\textsubscript{\lambda}, CDR2 and CDR3] (purified from CHO cells transfected with SVBU); B3V\textsubscript{H} and B3V\textsubscript{\lambda} with arginine to serine point mutation at position 27a in CDR1 of the light chain variable region (purified from CHO cells transfected with SVBLX) and two control mice received a commercial isotype matched IgG\textsubscript{1\lambda} (Sigma).

The mice were sacrificed 6 hours after injection of the antibodies and the sera and organs collected for analysis. Frozen sections of kidneys from the mice were stained using a direct immunofluorescence technique (described in section 2.9.6) to detect any human IgG that might have bound or deposited in the kidney. As a positive control for the immunofluorescence, kidney sections were also stained from mice which had been implanted with RH-14 (as described in chapter 2) and had developed hyaline thrombi which stained positively for human IgG. The positive control kidney showed very strong positive staining of the hyaline thrombi in the glomeruli, however all of the mice injected i.v. with the different purified recombinant human IgG were negative.

The human IgG antibody B3 had previously been affinity purified from hybridoma cell supernatant and injected i.v. into SCID mice, the antibody was shown to penetrate cells and bind to their nuclei, both in the kidney and in other organs (Ehrenstein \textit{et al.} 1995). However in this previous experiment, ten times as much B3 (1mg) was injected by Ehrenstein \textit{et al.} I was limited to only 100µg per mouse in 100µl, due to the fact that the recombinant antibodies (purified from the CHO cell supernatants) were supplied at concentrations between 1-2mg/ml (by Chemicon) and 100µl was the maximum volume which could be injected, under our Home Office licence. However, it was feasible to try injecting a lower concentration of IgG, since another group had shown successful detection of IgG deposition in the kidney after injection of only 75µg/mouse of the murine anti-DNA antibody R4A (Gaynor \textit{et al.} 1997). Ideally, as a positive control when injecting the recombinant IgG purified from the CHO cell supernatants, I would have also injected both 100µg and 1mg [1mg was the dose that was injected in the previously published experiment
(Ehrenstein et al. 1995) of affinity purified B3 derived from human hybridoma cells. However, this original antibody was no longer available in our laboratory.

5.6 Discussion of results from chapter five

The overall conclusion, from the experiments implanting SCID mice with CHO cells stably producing recombinant human IgG, was that any potential differences caused by the different light chain mutants of B3 were obscured in this system by an immune response against the CHO cells. There was no reason to predict this adverse response against the CHO cells in the SCID mice, since a previous paper had implanted CHO cells (producing a potential anti-cancer agent, Mullerian inhibiting substance) into SCID mice (for up to 4 weeks) and had reported no adverse effects due to the presence of the CHO cells (Stephen et al. 2001).

In the initial experiments (described in section 5.2) it had appeared that there was greater pathology, in the form of proteinuria and reduced survival, in mice exposed to the ‘wild-type’ B3 (SVBL). However, the proteinuria could not be related to any human IgG deposition or histological changes within the kidney, and indeed later experiments exposed clonal variation in the levels of proteinuria, which was not related to the serum concentration of human IgG in the implanted mice. As well as an immune response of the SCID mice against the CHO cells, the rapidly growing CHO cells may have released inflammatory mediators, which could also have caused the neutrophil infiltration and proteinuria. It must be acknowledged that the estimated proteinuria values used throughout these experiments are based on assumed as well as actual concentrations as given in the ‘Albustix’ data sheet. In addition these recorded proteinuria levels are assessed by the subjective judgement of a colour change, rather than a quantitative assay technique.

It is possible that the variation in proteinuria and survival between different clones could be due to differing rates of in vivo growth of the various CHO cell clones. Measuring the weight change after implantation was carried out in the experiment described in section 5.2, however due to the occurrence of cachexia, it was not feasible to use weight change as an indicator of rate of cell growth in the mice. In addition, although the in vitro growth of the cells cannot predict their in vivo behaviour, the clones which were most vigorous in vitro (data not shown) are not
clearly the lines which caused the most proteinuria or earliest death *in vivo*. Indeed CHO cells containing pG1D210 often grew rapidly in culture, but these mice generally had less proteinuria and survived longer.

An additional variable between the groups of mice in the experiment described in section 5.3 (and 5.4) that did not exist in the earlier experiments (section 5.2) was the presence of methotrexate (MTX). The level of MTX varied between $1 \times 10^{-7}$ M and $1 \times 10^{-9}$ M in different cell clones as shown in Table 5.1. MTX was not included in the cell suspensions, inoculated into the SCID mice, in the experiments described in section 5.2. However, due to the low concentration of human IgG observed in the murine sera in these experiments it was thought possible that removal of MTX had compromised the production of human IgG, hence it was decided to maintain the MTX concentration in subsequent experiments. MTX is excreted in the kidneys but is not nephrotoxic at therapeutic doses in humans. The highest level of MTX present in the cell cultures was $1 \times 10^{-7}$ M, this is at the lower end of the MTX concentration range which is potentially toxic (to liver and lungs) in humans. The actual concentration of MTX in mice would be even lower, since only 0.5 ml of cell suspension was inoculated in mice with a blood volume of approximately 2 ml. In addition the level of MTX would rapidly fall within a few hours of inoculation in the mice and proteinuria was not observed until several days after cell implantation. The mice inoculated with CHO cells containing the empty vector (pG1D210), which were suspended in the highest concentration of MTX, had slightly less proteinuria than other groups of mice, some of which had lower levels of MTX. In summary the effect of MTX in these experiments was probably limited in comparison to the probable pathogenic effects of the infiltrating neutrophils or the CHO cells themselves. However, it would be judicious to avoid the use of MTX in any future studies, especially in view of its immunosuppressive ability.

The influx of neutrophils in all groups of mice implanted with CHO cells is interesting. Experiments in a rat model of antibody-mediated glomerulonephritis have shown that proteinuria was dependent on the neutrophil influx (Cochrane *et al.* 1965). In the histology for the experiment described in section 5.2.2, the neutrophils were stained to allow counts to be made (data not shown), of the numbers of neutrophils present in the kidney sections of mice implanted with the different CHO
cell lines. The most neutrophils were observed in the mice implanted with pG1D210 (empty-vector), although these mice did survive the longest. However, this data did suggest that the neutrophil number was not solely responsible for proteinuria, since these mice generally had the lowest levels of proteinuria in this experiment. There is evidence that endotoxin (bacterial lipopolysaccharide, LPS) can cause proteinuria in rats (Karkar and Rees 1997), and a recent paper has shown synergy between nephrotoxic immunoglobulin (polyclonal rabbit anti-mouse glomerular antigens) and endotoxin in causing a neutrophil influx in antibody-mediated glomerulonephritis in mice (Robson et al. 2003). It is possible, that the CHO cell cultures were contaminated with endotoxin, this was not measured in my experiments. This possibility is something to consider in future cell implantation experiments. Endotoxin is removed by protein A purification (Karkar and Rees 1997) and would not be a problem in experiments involving i.v. injection of purified antibodies.

The CHO cells also did not appear to produce very much human IgG in the mice, the levels detected in the murine sera were considerably lower (approximately 1000 fold) than those obtained in SCID mice implanted with human hybridoma cells (in chapter 3). However, this finding was consistent with the relative in vitro production of both CHO and hybridoma cell lines in culture. The low level of human IgG expression in this system, thus adds to the unsuitability of using these cells in mice. Although for the purposes of obtaining quantities of recombinant human IgG in vitro, the low level of human IgG production by these cells, can be overcome by large-scale culture and affinity purification.

The fact that there was no detectable binding of human IgG, from the sera of mice implanted with SVBL and SVB33 (in 5.4), to either dsDNA or α-actinin by ELISA, even after DNase I treatment, was interesting. Since in the assay used to detect human IgG in the sera (2.1.2), both the capture and detecting antibodies are γ-chain specific, any antigens present in the binding site will not affect the assay result. However, this is not the case in either the anti-dsDNA or anti-α-actinin ELISAs, since capture of antigen specific human IgG molecules relies on binding to the antigen immobilised on the plate. The sera were therefore treated with DNase I prior to assaying, which was previously effective in increasing the detection of specific
recombinant human IgG in COS-7 and CHO cell supernatants. It seems however, that when trying to detect these recombinant IgG in the murine sera, DNase I treatment was not sufficient. One explanation could be that the recombinant antibodies present in the murine sera are involved in binding to antigens not available in the cell supernatants, and that these form complexes which are not broken down by DNase I treatment, such as large immune complexes of human IgG and nucleosomes. Alternatively, something in the sera is inhibiting the action of DNase I. Actin is known to be an inhibitor of DNase I, and at inflammatory sites, platelet actin has been shown to inhibit endogenous DNase I in humans and mice (Lachmann 2003). A third possibility, is that the recombinant human IgG molecules require the presence of a cofactor, in order to bind to dsDNA and α-actinin (similar to some anti-phospholipid antibodies requiring the cofactor β2-glycoprotein I, in order to bind cardiolipin). Perhaps such a cofactor is present in the cell supernatants but not freely available in the sera. It is possible that this ‘cofactor’ could be nucleosomes, which might be freely available in cell supernatant, especially due to the large number of dying cells, but which would have been rapidly cleared from the murine sera in vivo.

Since study of the in vivo effects of human IgG molecules, secreted by CHO cells implanted into SCID mice, was found not to be feasible due to the inherent background pathology contributable to the CHO cells, direct injection of the purified antibodies into the mice was investigated. There was a disappointing lack of any detectable human IgG, in the kidneys of the mice injected i.v. with purified recombinant IgG variants of B3. However, there could have been a number of reasons for this result, particularly too low an amount of IgG. The possibility exists of repeating this experiment with greater concentrations of purified human IgG, and giving repeated injections over a number of days. However, the large amounts of purified antibody required would make this a very costly endeavour. This method would obviously be impractical for longer-term study of pathology in the kidney.
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IS α-ACTININ A TARGET OF PATHOGENIC ANTI-DNA ANTIBODIES IN LUPUS NEPHRITIS?

6.1 Introduction and aims of this chapter

It is not known why some anti-dsDNA antibodies deposit preferentially in the kidney. The nature of the target antigen(s) and the binding interactions involved, remain a matter of conjecture. It has been suggested that some anti-dsDNA antibodies do not bind directly to DNA deposited in the kidney but are cross-reactive and bind directly to kidney antigens (Madaio et al. 1987; Raz et al. 1993). As discussed in section 1.5.3.2, one recently proposed candidate antigen, is the 100KD actin-binding and cross-linking protein, alpha-actinin (α-actinin). Two groups have reported that pathogenic, but not non-pathogenic, murine anti-DNA monoclonals bind directly to α-actinin (Mostoslavsky et al. 2001; Deocharan et al. 2002). Deocharan et al also reported unpublished preliminary studies, showing that pathogenic murine anti-DNA antibody R4A binds to α-actinin in human mesangial cells, and that sera from active lupus patients have high titres of anti-α-actinin antibodies [no data were shown, but these studies were reported in (Deocharan et al. 2002)]. Following these reports that pathogenic murine anti-DNA antibodies bind to α-actinin, it was obviously of interest to assess the ability of human pathogenic anti-dsDNA antibodies to bind this antigen. Human monoclonal anti-DNA antibodies and antibodies affinity purified from the sera of patients with SLE were both investigated. The recombinant anti-DNA antibodies, SVBL, SVB33, and SVBU produced by CHO cells, were also tested for binding to α-actinin.

6.2 ELISA to measure human immunoglobulin binding to α-actinin

An ELISA was developed to measure the binding of human IgG and IgM to α-actinin, as described in chapter 2.10.2. Figure 6.1 shows a representative titration of
Figure 6.1: Titration of human IgM anti-DNA mAb 3-5 in a representative anti-α-actinin ELISA

Main graph shows titration of mAb 3-5 from 8µg/ml - 0.031µg/ml during optimisation of assay. Three curves are shown, the binding of 3-5 to α-actinin, the binding of 3-5 on the uncoated (Sham ‘coated’ with 50µl/well of BIC buffer) side of the plate and the blue line shows the binding of 3-5 to α-actinin after subtraction of the background binding to the uncoated side. The inset graph shows greater detail of lower portion of curve, 3-5 was subsequently run on every anti-α-actinin ELISA plate from 1µg/ml. The points are the mean of optical density (OD) values of duplicate wells, error bars show the standard deviation from the mean.
the binding of the positive control human IgM anti-DNA antibody 3-5 to α-actinin coated on the plate. Since the majority of the following experiments involve the measurement of human IgG binding to α-actinin, it would have been ideal to have a human IgG anti-α-actinin antibody as a positive control but no suitable antibody was available. The positive control 3-5 was not used to quantify any of the results, it was just used as a check that the assay was working and to standardise between plates.

6.3 Binding of human anti-dsDNA monoclonals to α-actinin.

6.3.1 Binding of human IgG monoclonal antibodies

The hybridoma derived human anti-DNA IgG monoclonal antibodies, RH-14, B3 and DIL-6, are described in section 2.2, Table 2.1. The human IgG binding of the hybridoma cell culture supernatants to α-actinin was measured using the ELISA described in section 2.10.2 and the human IgG concentration of the supernatants was measured using the human IgG ELISA described in section 2.1.2. The pathogenic anti-dsDNA antibodies RH-14 and B3 bound strongly to α-actinin in an ELISA, whilst non-pathogenic DIL-6 bound very weakly (Figure 6.2). An isotype matched, non-specific control IgG1λ (Sigma I-5029), diluted to 10µg/ml, showed no binding to α-actinin.

6.3.2 Inhibition of binding to α-actinin

The ability of dsDNA to inhibit specific IgG binding to α-actinin was assessed using a modification of the anti-α-actinin ELISA (described in section 2.10.3). The binding of RH-14 to α-actinin (on the solid-phase), could be partially inhibited by pre-incubation (in the fluid-phase) of RH-14 with increasing concentrations of purified calf thymus dsDNA, the maximum percentage inhibition was 67% (Figure 6.3).

6.3.3 Binding of human IgM monoclonal antibodies

The binding of the hybridoma derived human IgM anti-DNA monoclonal antibodies (properties shown in Table 2.2) to α-actinin was measured using the ELISA described in section 2.10.2 and the concentration of IgM in the supernatants was measured using the assay described in section 2.1.3. All seven IgM human anti-DNA antibodies bound to α-actinin. However, RT84 had a α-actinin binding
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**Figure 6.2: Binding of human anti-dsDNA mAbs to α-actinin**

The pathogenic anti-dsDNA mAbs RH-14 and B3 bound α-actinin but the non-pathogenic anti-dsDNA mAb bound only very weakly. There was no significant difference between the binding of RH-14 and B3 to α-actinin (P=1.0), but both bound α-actinin significantly more than DIL-6 (P=0.0079 in both cases, using Mann-Whitney test). Anti-α-actinin binding was measured by solid-phase ELISA, the OD values for each point are the mean of duplicates and the error bars show the standard error of the mean (SEM). In cases when the supernatants were tested on different plates the OD values of the positive control antibody A52 were within 10%. The binding to sham coated wells was deducted from the OD values of the α-actinin coated wells. A non-specific human IgG1 λ isotype matched (to RH-14 and B3) control, showed no binding to α-actinin.
Figure 6.3 Inhibition of binding of RH-14 to α-actinin

Inhibition of the binding of RH-14 to α-actinin on the solid-phase by pre-incubation in the liquid-phase with increasing concentrations of purified calf thymus dsDNA. RH-14 is diluted to 25% of neat hybridoma cell supernatant (s/n). The inset graph shows percentage inhibition, the maximum inhibition was 67%.
Figure 6.4  Binding of human IgM anti-DNA monoclonal antibodies to α-actinin

Binding of IgM anti-DNA monoclonal antibodies all derived from the same patient with lupus nephritis. Binding to α-actinin is expressed as ‘anti-α-actinin activity’, meaning the ratio of binding to α-actinin in terms of optical density (OD) value divided by the IgM concentration of the supernatant (in terms of OD from the linear part of the IgM standard curve). RT84 clearly shows the greatest binding to α-actinin. A non-specific IgM control antibody showed no binding to α-actinin (data not shown).
activity (ratio of α-actinin binding to IgM concentration) which was at least six times higher than that exhibited by the other six antibodies (Figure 6.4). A nonspecific control IgM (Sigma I-8260) showed no binding to α-actinin, when measured at a concentration of 10μg/ml.

6.3.4 Binding of recombinant human IgG produced by CHO cells to α-actinin.

The supernatants from CHO cell cultures, transfected with the supervectors SVBL, SVB33 and SVBU as described in Chapter four, producing the recombinant human anti-DNA antibodies, B3VII/B3Vλ, B3VII/B33Vλ and B3VII/BUVλ respectively, were tested for binding to α-actinin by ELISA (2.10.2). Figure 6.5 demonstrates the necessity for DNase I treatment (2.6.6) of the supernatants prior to incubation in the ELISA. Without DNase I treatment there was no binding by any of the antibodies to α-actinin, but after treatment with 7.5 U/ml of DNase, SVB33 bound to α-actinin. Increasing the DNase I concentration to 15 U/ml, did not further increase the binding of SVB33 to α-actinin. Figure 6.6, compares the α-actinin binding of the recombinant IgG molecules in the DNase I pretreated CHO cell supernatants. SVB33 showed strong binding to α-actinin. SVBL did not bind to α-actinin, but was only present at a much lower human IgG concentration that SVB33, so could not be directly compared. SVBU did not bind to α-actinin, SVBU was tested at the same range of human IgG concentrations as SVB33 3-15. The specificity of the binding of SVB33 to 100KD α-actinin was confirmed by western blotting as discussed in section 6.4.4.
Figure 6.5 The effect of DNase I digestion of CHO cell supernatants containing recombinant human IgG on their binding to α-actinin in ELISA

Prior to their incubation in an anti-α-actinin ELISA, supernatants from cultures of CHO cells producing recombinant human IgG were treated with either 0, 7.5 or 15.0 U/ml of DNase I (as described in section 2.6.6) to remove both DNA from the supernatant and DNA which might be bound by the antibodies. Two clones of CHO cells expressing each of three supervectors were studied (the clones are summarised in Table 5.1). The human IgG concentration of each supernatant was measured using the human IgG ELISA (2.6.8).

Figure A: SVBL 5-40-24 (‘wild type’ B3Vh and B3Vλ)
Figure B: SVBL 3-26 (‘wild type’ B3Vh and B3Vλ)
Figure C: SVB33 3-15 (B3Vh and B3Vλ CDR1, 33.H11Vλ CDR2 and CDR3)
Figure D: SVB33 3-15-23 (B3Vh and B3Vλ CDR1, 33.H11Vλ CDR2 and CDR3)
Figure E: SVBU 5-18 (B3Vh and B3Vλ CDR1, UK4Vλ CDR2 and CDR3)
Figure F: SVBU 5-18-6 (B3Vh and B3Vλ CDR1, UK4Vλ CDR2 and CDR3)

The graphs illustrate that SVB33 binds to α-actinin but that SVBU did not bind to α-actinin. SVBL did not bind to α-actinin either, however the human IgG concentration of these supernatants was very low. The effect of DNase I treatment clearly showed that binding of SVB33 to α-actinin is inhibited by DNA present in the supernatants. 7.5U/ml of DNase I is sufficient to remove the DNA from the supernatant as no increase of binding is seen when the DNase I concentration is increased to 15U/ml.
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NO DNase I
DNase I 1.5 U/ml
DNase I 7.5 U/ml
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Figure 6.6  Binding to α-actinin of recombinant human IgG, from the supernatant of CHO cells transfected with supervectors

The supernatants from CHO cells transfected with the supervectors SVBL, SVB33 and SVBU were treated with 7.5 U/ml of DNase I (as described in 2.6.6) before being incubated in an anti-α-actinin ELISA.

Two clones of CHO cells expressing each of three supervectors were studied (the clones are summarised in Table 5.1). The human IgG concentration of each supernatant was measured using the human IgG ELISA (2.6.8).

SVB33 showed binding to α-actinin but SVBL and SVBU showed no binding to dsDNA. However the concentration of human IgG in the supernatant from SVBL transfected CHO cells was very low and could not be directly compared with that from the SVB33 or SVBU CHO cells.
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6.4 Binding of IgG from patients with SLE to α-actinin.

6.4.1 Preliminary comparison of sera from SLE patients and healthy controls

The initial experiment was simply to compare the binding to α-actinin, in an IgG anti-α-actinin ELISA (described in section 2.10.2), of sera from patients with SLE with that of sera taken from healthy controls. The initial sera were all from patients with SLE but included both patients with and without renal disease and their levels of anti-DNA antibodies ranged from 18-1703 IU/ml. Figure 6.7 shows that there is a significant difference (P<0.05) between the IgG binding to α-actinin of sera from patients with SLE as compared with sera from healthy controls. This experiment also established that 1:200 was the optimal dilution of sera when measuring their IgG binding to α-actinin.

6.4.2 Analysis of whole serum from patients with and without lupus nephritis.

Patients were selected who had high levels of anti-DNA antibodies and were divided into ‘renal’ (n=12) or ‘non-renal’ (n=11) as described in section 2.10.1. The clinical data of the selected patients are summarised in Tables 2.3 and 2.4. There was no significant difference between the two patient groups in terms of anti-DNA antibody levels, duration of disease since diagnosis (date when patient fulfilled at least 4 ACR revised criteria for diagnosis of SLE (Tan et al. 1982)) or age as shown in Figure 6.8 (A-C respectively). The ‘renal’ group had a slightly higher proportion of Afro-Caribbean and patients of mixed-ethnicity (Figure 6.8 [H]). The patients could be divided into three treatment regimens, (I) those whom received no immunosuppressive drugs, (II) those on prednisolone up to 10 mg/day and (III) those receiving another immunosuppressive, either with or without prednisolone, or those that received over 10 mg/day of prednisolone alone. The patients with renal involvement were receiving more aggressive immunosuppressive drug treatment than the non-renal patients, as shown in Figure 6.8 [E&F]. However, there was no significant difference between the ‘renal’ and ‘non-renal’ patients when comparing the mean dose of prednisolone received by each group (Figure 6.8 [D]). As expected when studying patients with SLE, the majority of patients were female (Figure 6.8 [G]).
Figure 6.7 Comparison of the binding of IgG from whole sera of patients with SLE and sera from healthy controls

Sera were titrated from 1:100 to 1:800 on both α-actinin and sham coated wells of an ELISA plate and binding was detected with goat anti-human IgG-alkaline phosphatase conjugated antibody. The binding in the sham coated wells was deducted from the binding in the α-actinin coated wells. There is a significant difference (P<0.05) between the binding to α-actinin of IgG from the sera of patients with SLE (n=15) and the sera of healthy controls (n=8). Using the Mann Whitney test P=0.029. The error bars show standard error of the mean (SEM).
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Figure 6.8 Comparison of clinical properties of ‘Renal’ and ‘Non-renal’ patients from whom sera were derived for experiments described in section 6.4.2

These graphs compare the clinical data shown in Tables 2.3 and 2.4. (A) No significant difference in anti-DNA antibody level (P<0.05); (B) No significant difference in duration of disease since diagnosis (P<0.05); (C) No significant difference in age (P<0.05); (D) No significant difference in daily dose of prednisolone (P<0.05). The data shown in A-D were compared using the Mann-Whitney test. (E) Number of patients in each group receiving treatment regimens I-III; (F) Number of patients in each group receiving other drugs, instead or as well as prednisolone; (G) Number of patients of each sex; (H) Number of patients from each ethnic group.
Using the ELISA described in section 2.10.2, the initial analysis of the binding to α-actinin of whole sera from 'renal' versus 'non-renal' patients showed no significant difference in their binding to α-actinin (Figure 6.9). However, both groups of patients showed significantly higher binding to α-actinin than the healthy control sera samples (P<0.05).

6.4.3 Specific binding of purified anti-DNA antibodies to α-actinin.

Since it was not possible to study the binding of anti-dsDNA IgG to α-actinin specifically, when using whole serum, those elements which bound dsDNA were affinity purified from the sera of patients, using dsDNA-cellulose as described in section 2.10.4. Subsequently the affinity purified samples from each patient, was measured for human IgG binding to α-actinin using the ELISA described in section 2.10.2. The clinical data of the patients selected are summarised in Tables 2.5 and 2.6, and 'renal' and 'non-renal' patient groups are compared in Figure 6.10. As with the sera studied in section 6.4.2 there were no significant differences between the 'renal' and 'non-renal' groups in terms of anti-DNA IgG levels, disease duration, age or mean daily dose prednisolone. Differences between the 'renal' and 'non-renal' groups in terms of their drug treatment regimen, sex and ethnicity were as to be expected (as seen in the previous patient groups described in 6.4.2).

Following affinity purification, it can be seen (in Figure 6.11) that there is a significantly greater binding to α-actinin of anti-dsDNA IgG antibodies affinity purified from the sera of 'renal' patients with SLE, as compared with those IgG antibodies purified from 'non-renal' patients (P=0.034). The IgG binding of anti-dsDNA antibodies, purified from both 'renal' and 'non-renal' patients, to both α-actinin and dsDNA was compared as shown in Figure 6.12. It was found that a substantially greater proportion (6/10) of IgG anti-dsDNA binding antibodies derived from 'renal' patients bound to α-actinin, than from those purified from the 'non-renal' patient sera (2/8). The Chi-square test for independence, was used to test the dependence of α-actinin binding of patients anti-dsDNA IgG on the presence of renal disease, the null hypothesis was that α-actinin binding by anti-dsDNA IgG is NOT dependent on the presence of renal disease (or vice versa). The Chi-square value was 2.11 (significance P<0.05 required a Chi-square value >3.84) therefore the
There was no significant difference between the anti-α-actinin binding of sera from SLE patients who had renal disease compared with those patients who had never had renal involvement (P=0.31, Mann Whitney test). Sera from both groups of SLE patients bound to α-actinin significantly more than sera from healthy controls (P<0.05). Human IgG binding to α-actinin was measured in a solid-phase ELISA as described in section 2.1.2 The sera were all measured at 1:200 dilution and binding to sham coated wells was deducted from that of coated wells.
Chapter Six  
Is α-actinin a target of pathogenic anti-DNA antibodies in lupus nephritis?

Figure 6.10 Comparison of clinical properties of ‘Renal’ and ‘Non-re nal’ patients from whom anti-DNA Abs were derived for experiments described in section 6.4.3

These graphs compare the clinical data shown in Tables 2.5 and 2.6. (A) No significant difference in anti-DNA antibody level (P<0.05); (B) No significant difference in duration of disease since diagnosis (P<0.05); (C) No significant difference in age (P<0.05); (D) No significant difference in daily dose of prednisolone (P<0.05). The data shown in A-D were compared using the Mann-Whitney test. (E) Number of patients in each group receiving treatment regimens I-III; (F) Number of patients in each group receiving other drugs, instead or as well as prednisolone; (G) Number of patients of each sex; (H) Number of patients from each ethnic group.
Figure 6.11  Binding to α-actinin of IgG anti-dsDNA antibodies purified from the sera of patients with SLE

There is a significantly (P=0.034) greater binding to α-actinin of anti-dsDNA IgG antibodies affinity purified from the sera of ‘renal’ patients with SLE, as compared with those IgG antibodies purified from ‘non-renal’ patients. The P value is from a Mann-Whitney test comparing the OD values between the ‘renal’ and ‘non-renal’ groups. The mean of the OD values of the ‘renal’ and ‘non-renal’ groups is indicated by the red and blue lines respectively. The line denoted ‘x’ indicates the OD value 0.158, which represents the mean binding, plus two standard deviations, to α-actinin of healthy control sera (n=12, taken from figure 6.9). Points above this line are considered as positively binding to α-actinin. The binding to sham coated wells was deducted from the binding to α-actinin coated wells.
Figure 6.12 Binding to \( \alpha \)-actinin and dsDNA of IgG anti-dsDNA antibodies purified from the sera of patients with SLE

This figure shows the relationship between the human IgG binding to \( \alpha \)-actinin and binding to dsDNA of anti-dsDNA antibodies purified from patients with SLE. Increased binding to \( \alpha \)-actinin of anti-dsDNA IgG affinity purified from the serum of 'renal' SLE patients was observed compared to those antibodies derived from 'non-renal' patients with lupus. The line denoted ‘x’ indicates the OD value 0.158, which represents the mean binding of healthy control sera (taken from figure 6.9) to \( \alpha \)-actinin plus two standard deviations. Points above this line are considered as positively binding to \( \alpha \)-actinin. Anti-\( \alpha \)-actinin and anti-dsDNA binding of human IgG were measured by solid-phase ELISAs as described in sections 2.10.2 and 2.1.2 respectively. In each assay, the binding to sham coated wells was deducted from the binding to \( \alpha \)-actinin or dsDNA coated wells.
null hypothesis could not be rejected. So although a greater proportion of the anti-dsDNA specific IgG samples from patients with renal disease bound to α-actinin, than those samples from ‘non-renal’ SLE patients, it could not be concluded that binding to α-actinin was dependent on the patient having renal disease or vice versa. To test this hypothesis further it would be necessary to increase the number of patients studied. It could be hypothesised, that there is a subset of high affinity anti-dsDNA antibodies which are pathogenic and cause renal disease, and that this might be facilitated by their binding to α-actinin present in kidney cells. However, there was no significant correlation between IgG binding to dsDNA and binding to α-actinin, either in patients who had renal disease or who did not have renal disease. However, there was a slight trend towards correlation between binding to α-actinin and to dsDNA in the renal group. Again, much larger numbers of patients need to be added to the study, to either confirm or refute this hypothesis.

6.4.4 Western blotting

Since the α-actinin derived from the chicken gizzard is reported by the suppliers (Sigma) to be 80% pure, the specificity of the anti-dsDNA IgG binding to the 100KD α-actinin molecule was confirmed by western blotting, as described in section 2.10.5. Figure 6.13 shows representative binding of affinity purified anti-dsDNA IgG from two renal and two non-renal patient sera to α-actinin. This binding to α-actinin was greatly reduced by prior absorption, of the affinity purified antibodies, with dsDNA.

Figure 6.14 shows the binding to 100KD α-actinin of the recombinant human IgG from the supernatants (after DNase I treatment) of CHO cells producing SVBL and SVB33. The antibody produced by CHO cells transfected with SVBII did not bind to 100KD α-actinin.
Figure 6.13: Western blot showing the binding of anti-DNA IgG from patients with SLE to 100KD α-actinin

Lane 1: The binding of the positive control, rabbit anti-α-actinin detected with goat anti-rabbit-HRP. Lanes 2 & 3: Show the binding to α-actinin of anti-dsDNA IgG (0.5μg/ml) purified from a patient with renal lupus, in lane 3 the binding has been partially absorbed by pre-incubation with 50μg/ml dsDNA. Lanes 4 & 5: Show the binding to α-actinin of anti-dsDNA IgG (0.5μg/ml) purified from a second patient with renal lupus, in lane 5 the binding has been partially absorbed by pre-incubation with 50μg/ml dsDNA. Lanes 6&7: Show the relative lack of binding to α-actinin of anti-dsDNA IgG (0.5μg/ml) purified from two individual non-renal lupus patients. These two patients did not bind α-actinin in the ELISA. Lane 8: Shows no binding of the negative control, human IgG1λ at 1μg/ml.
Figure 6.14 Western blot showing the binding of recombinant human IgG from CHO cell supernatants to 100KD α-actinin

Cell supernatants from CHO cells transfected with supervectors SVBL, SVB33 and SVBU. The supernatants were treated with 7.5U/ml of DNase 1 (as section 2.6.6) before incubating on western blot of α-actinin.

Lane 1: SVBL (‘wild type’ B3VH and B3VL)
Lane 2: SVB33 (B3VH and B3VL CDR1, 33.H11VL CDR2 and CDR3)
Lane 3: SVBU (B3VH and B3VL CDR1, UK4VL CDR2 and CDR3)

The recombinant human IgG antibodies SVBL and SVB33 bind to 100KD α-actinin purified by SDS-PAGE but SVBU showed no binding.
6.5 Discussion of results from chapter six

Human anti-DNA antibodies have been shown to be present in the kidneys of patients with lupus nephritis (Sabbaga et al. 1990) and a few human anti-DNA monoclonal antibodies have been shown to deposit and cause proteinuria in SCID mice (Ravirajan et al. Isenberg 1998; Ehrenstein et al. 1995). However, as discussed in section 1.5, the precise mechanism by which some anti-dsDNA antibodies preferentially bind to the kidney is not known and neither are the actual antigenic targets of these pathogenic antibodies. There are a number of hypotheses proposing how anti-dsDNA antibodies deposit in the kidney, including the possible cross-reactive binding of anti-dsDNA antibodies to glomerular antigens.

Recently, the 100KD actin-binding protein α-actinin has been cited as playing a role in several human (Kaplan et al. 2000) and experimental nephrotic syndromes (Michaud et al. 2003; Smoyer et al. 1997; Shirato et al. 1996; Kos et al. 2003). Alpha-actinin has also attracted interest as a target of pathogenic murine anti-dsDNA antibodies (Mostoslavsky et al. 2001; Deocharan et al. 2002). Proteinuria is a common feature of patients with lupus nephritis, but the precise defects that allow protein to cross the filtration barrier in the kidney are not clear. It has been suggested that disruption of components of the podocyte cytoskeleton, of which α-actinin is an integral part, might result in a lack of slit diaphragm integrity resulting in leakage of protein into the urinary space (Khoshnoodi and Tryggvason 2001; Somlo and Mundel 2000). It has been hypothesised that pathogenic anti-DNA antibodies may exhibit cross-reactive binding to α-actinin, thus affecting podocyte function, resulting in the foot process effacement and proteinuria observed in patients with lupus nephritis.

The human anti-dsDNA monoclonal antibodies RH-14 and B3, have previously been shown to be pathogenic, that is they deposit in the kidney and cause proteinuria in SCID mice (Ravirajan et al. 1998; Ehrenstein et al. 1995). On the other hand DIL-6 is not pathogenic. It does not bind to the kidney or cause proteinuria in SCID mice (Ravirajan et al. 1998). The results in Figure 6.2 showed that RH-14 and B3, the two pathogenic human anti-dsDNA antibodies, were able to bind to α-actinin by ELISA, and that non-pathogenic antibody DIL-6 showed virtually no binding to α-
actinin. Obviously, this study only involved a very small number of human anti-DNA monoclonal antibodies, but it does concur with the findings in studies involving murine monoclonal antibodies. Deocharan et al, found that the pathogenic murine anti-DNA antibody R4A, but not a non-pathogenic mutant, bound by ELISA to the commercial α-actinin derived from chicken gizzard (Sigma) that was used in the studies described in this thesis (Deocharan et al. 2002). Mostoslavsky et al, showed that pathogenic murine anti-dsDNA antibodies (J25 and ID9) bound to the same commercial α-actinin in a western blot, but that two non-pathogenic anti-dsDNA antibodies, of equal dsDNA binding ability (J64 and J38) did not bind to α-actinin (Mostoslavsky et al. 2001). It was also shown in this study that serum from a nephritic BWF1 mouse with high titres of anti-dsDNA antibodies bound to the commercial α-actinin, whereas serum from a BALB/c mouse did not bind (Mostoslavsky et al. 2001). The commercial preparation of α-actinin, should consist of the smooth muscle α-actinin isoforms, α-actinin-4 and α-actinin-1, since chicken gizzard is composed of smooth muscle (Endo and Masaki 1982). Studies have shown that only α-actinin-4 (not α-actinin-1) is present in human kidney lysates and this is mainly concentrated in the podocyte foot processes (Kaplan et al. 2000), although α-actinin is also present in some blood vessels and mesangial cells (Drenckhahn and Franke 1988).

Of the human IgM anti-DNA clones (Ravirajan et al. 1992), RT84 the highest α-actinin binder had also previously been shown to bind DNA with the highest affinity (as shown in Table 2.2). It was also interesting that the three IgM clones which bind only ssDNA (RT16, RT79 and RT115) had the lowest binding activity to α-actinin, as compared to the other four RT clones which bind both dsDNA and ssDNA. These hybridoma derived IgM antibodies have not been tested for pathogenicity in SCID mice. These data are supported by the reported binding of three human IgM anti-DNA antibodies to both commercial α-actinin by ELISA, and to a 100KD band in a western blot of human glomerular cell lysates [meeting abstract, (Marambio et al. 2002)].

The recombinant human anti-DNA antibodies, B3V\textsubscript{H}/B3V\textsubscript{λ}, B3V\textsubscript{H}/B33V\textsubscript{λ} and B3V\textsubscript{H}/BUV\textsubscript{λ} (produced by CHO cell cultures, transfected with the supervectors
SVBL, SVB33 and SVBU) were also tested for binding to α-actinin. Only the supernatant containing B3Vh/B33Vλ (SVB33) bound to α-actinin by ELISA (Figure 6.6). However, the recombinant ‘wild-type’ B3Vh/B3Vλ (SVBL) was only present at very low concentrations of human IgG and did show binding to purified α-actinin in the western blot, as did B3Vh/B33Vλ (Figure 6.14). Thus, the two recombinant human IgG antibodies which bound to dsDNA, also bound to α-actinin, but B3Vh/BUVλ (SVBU) which does not bind to DNA (or ANA) did not bind to α-actinin. Figure 6.5, showed that DNase I treatment of the cell supernatants was necessary for B3Vh/B3Vλ and B3Vh/B33Vλ to bind to α-actinin. This finding indicates that the antibody binding site was occupied by DNA (or DNA containing complexes, such as nucleosomes, derived from the culture supernatant) which prevented binding to α-actinin. This result also suggests that the binding of these antibodies to α-actinin is truly cross-reactive, and is not mediated via a DNA or nucleosome-bridge. These results do not, however, prove this hypothesis since DNase I treatment may not remove all complexed antigens, such as nucleosomes, from the antibodies (Kramers et al. 1994). This result agrees with previous reports, that prior DNase I treatment of the glomerular or mesangial cell extracts to remove any bound DNA, did not affect the binding of purified (DNA free) murine anti-DNA antibodies to them (Mostoslavsky et al. 2001; Deocharan et al. 2002). These data also suggested that the binding to α-actinin was truly cross-reactive and not mediated by a DNA or nucleosome-bridge.

Figure 6.3 showed the partial inhibition, up to 67%, of the binding of RH-14 to α-actinin by pre-incubation with increasing concentrations of purified calf-thymus dsDNA. Deocharan et al showed that the binding of both R4A and MRL-lpr sera to α-actinin, could be inhibited by up to 80% by pre-incubation with dsDNA. The partial inhibition of RH-14’s binding to α-actinin by dsDNA in the fluid-phase, supports previous suggestions that anti-DNA antibodies exhibit cross-reactive binding to α-actinin, perhaps by recognition of a shared epitope or structural mimicry (Mostoslavsky et al. 2001; Deocharan et al. 2002). Mostoslavsky et al, point out the fact that α-actinin is a very acidic protein (pI is approximately 5) which like DNA is negatively charged, and even suggest that α-actinin’s anti-parallel
dimeric helices (Ylanne et al. 2001) could be a structural mimic of the sugar-phosphate backbone of the dsDNA helix (Mostoslavsky et al. 2001). However, these authors also point out that their pathogenic and non-pathogenic subsets of anti-DNA antibodies show no apparent difference in terms of their pI values or the number of basic arginine residues that are present in the V<sub>H</sub>CDR3, but only the pathogenic subset of antibodies actually bind to α-actinin. However, there are only five pathogenic and two non-pathogenic antibodies compared, also the basic residues present in the light chain CDRs are not compared, and as we have shown with B3 (in Chapter four), the light chain residues can be critical for dsDNA binding.

The studies described in section 6.4, investigated the α-actinin binding of human IgG in the sera and specifically the α-actinin binding of polyclonal anti-dsDNA IgG antibodies purified from the sera of patients with SLE. Two groups of SLE patients were compared, those who had active renal disease and those who did not have renal disease, both groups of patients had high levels (>200 U/ml) of IgG anti-DNA antibodies. Figure 6.7, showed that patients with SLE had higher binding to α-actinin than healthy controls, irrespective of the level of anti-dsDNA antibodies in their sera. When the IgG binding to α-actinin, was measured in patients with SLE that were selected for high levels of anti-DNA antibodies, there was no significant difference in the α-actinin binding of those patients with renal disease as compared with those patients without renal disease (Figure 6.9). Although as in Figure 6.7, the patients with SLE had significantly higher binding to α-actinin than healthy controls.

However, when the polyclonal IgG antibodies binding to dsDNA were purified from the two groups of patients with SLE, the human anti-dsDNA specific IgG from those patients with renal disease, did exhibit significantly higher binding to α-actinin than those from patients without renal disease (Figure 6.11). However, although a greater proportion of the patients with renal disease, showed cross-reactive binding of their anti-dsDNA antibodies with α-actinin, it was not possible to prove conclusively that this was related to the presence of renal disease in these patients, possibly due to the limited numbers of patients tested. There was also no correlation, between the ability of the IgG antibodies to bind dsDNA and their ability to bind α-actinin, in either the patients with renal disease or those without renal disease. There was
however a trend, towards correlation of binding to dsDNA and α-actinin, in the patients with renal involvement. Thus I was not able to prove the hypothesis, conclusively, that a subset of the highest binding anti-DNA antibodies might cause renal disease, due to their ability to deposit in the kidney by cross-reactive binding to α-actinin. It does however remain a reasonable possibility.

There is some supporting evidence in preliminary experiments showing that immunoglobulin eluted from the kidneys of lupus patients binds to both DNA and α-actinin (personal communication, C. Putterman). However future studies using an improved method described by Xie et al (2003), reputed to yield larger quantities of eluted immunoglobulin from kidney tissue, will be necessary to determine whether patients with lupus nephritis have selective enrichment in the binding to α-actinin of anti-DNA binding IgG from the kidney eluates, versus the same patient’s serum.

The group of patients without renal disease obviously had no evidence of renal disease at the time of bleeding, although they all had high levels of anti-dsDNA antibodies. It is possible that in some patients these antibodies could be pre-cursors to those that would be pathogenic in the future, and therefore these antibodies may have already shown enhanced binding to α-actinin. By analogy, a large study (63 with lupus nephritis, 15 with active non-renal lupus and 33 with inactive SLE), investigating the anti-glomerular basement membrane binding of sera from patients with SLE, found that most of the patients with active nephritis had glomerular binding activity, but so did some of the active non-renal patients, although at lower levels (Lefkowith et al. 1996).

It is also important to consider, that there was no significant difference between the 'renal' and 'non-renal' patient groups in the prescribed level of the immunosuppressive steroid prednisolone. Glucocorticoid receptors have been shown to be upregulated in the podocytes of patients treated with another glucocorticoid, dexamethasone, that has been shown to upregulate the expression of the podocyte molecules, nephrin and tubulin-alpha (Mathieson 2003). It is unknown how prednisolone or indeed some of the other drug treatments being taken by the
patients in the studies described in this chapter, may affect the expression of α-actinin.

The function of α-actinin as an integral part of the podocyte cytoskeleton, and thus located within the cell cytoplasm, poses the question of how α-actinin would be accessible for binding by anti-DNA antibodies? Two of the antibodies which have been shown to bind to α-actinin, human monoclonal anti-DNA antibody B3 and murine monoclonal anti-DNA antibody R4A, may be able to penetrate live cells and could thus bind to cytoplasmic α-actinin (Ehrenstein et al. 1995; Deocharan et al. 2002). However, as discussed in section 1.5.3.2, there is evidence showing that α-actinin is also expressed on the cell surface, in the plasma membrane. Deocharan et al. showed that R4A binds to the surface of both live mesangial cells derived from MRL/lpr mice and to a kidney podocyte cell line (Deocharan et al. 2002). Both of these groups (Mostoslavsky et al. 2001; Deocharan et al. 2002), also showed (by western blotting) that pathogenic murine anti-dsDNA monoclonal antibodies only bound to the membrane and membrane cytoskeletal fractions of glomerular cell lysates.

An interesting theory, which could explain why some patients with SLE have high-levels of anti-dsDNA antibodies but do not develop lupus nephritis, is that α-actinin is differentially expressed, and perhaps α-actinin is upregulated in susceptible individuals. For example, α-actinin could be readily visualised by immunofluorescent staining in the kidney glomeruli of diseased BWF1 mice, but not in young pre-nephritic BWF1 or healthy BALB/c mice (Mostoslavsky et al. 2001). Differential expression of α-actinin was also demonstrated in murine mesangial cell (MC) lysates, with R4A binding strongly to 100KD α-actinin in MC lysates from MRL/lpr mice, but only weakly binding to MC lysates from BALB/c mice (Deocharan et al. 2002). As discussed in section 1.5.3.2, several factors (such as drug treatment, cytokines, chemokines or growth factors) may affect the expression of α-actinin, which may ultimately relate to an individuals genetic or environmental susceptibility to lupus nephritis. Thus the hypothesis is that cross-reactive binding of anti-dsDNA antibodies to α-actinin may cause proteinuria by disruption of the cytoskeletal regulation of the slit-diaphragm, and also cause tissue damage by
antibody deposition, complement activation and initiation of inflammation. These processes could also increase the availability of α-actinin, by upregulation of α-actinin expression due to local mediator release. Alternatively (or in addition), the upregulation of α-actinin for binding by pathogenic anti-dsDNA antibodies, as seen in autoimmune mouse models, may be secondary to ongoing inflammation in the kidney due to anti-dsDNA antibodies binding to glomerular antigens by one of the alternative mechanisms (discussed in section 1.5). In summary, further studies investigating both the glomerular expression of α-actinin, and of autoantibodies binding to α-actinin are therefore warranted in patients with lupus nephritis.
CHAPTER SEVEN

Conclusions

and

Ideas for Future work
CHAPTER SEVEN

CONCLUSIONS AND IDEAS FOR FUTURE WORK

The work described in this thesis furthers our understanding of the critical effect that the position of arginine residues in the antigen binding site, may have on the binding properties of anti-DNA antibodies. Combining two somatic mutation derived arginine molecules R27a and R92, that are important in the dsDNA binding of their respective parent antibodies (B3 and 33.H11), into one novel IgG molecule (B3V\(h\)/B33V\(\lambda\)) resulted in increased binding to dsDNA. The importance of the precise position of such critical arginine molecules was demonstrated since the presence of an arginine at position 94 in the absence of R92, totally abolished binding of B3V\(h\)/BUV\(\lambda\) to dsDNA. Since R27a and R92 enhance the binding of antibodies to dsDNA, it seems likely that the acquisition of these somatic mutations during affinity maturation of the B cell clones producing B3 and 33.H11, was driven by specific binding of their BCRs to dsDNA.

However, the abolition of dsDNA binding by the affinity purification and DNase I treatment of antibodies, which had previously bound dsDNA when suspended in supernatant, and the subsequent reinstatement of dsDNA binding by reconstitution with supernatant, suggested that B3 might in fact recognise an epitope comprising both dsDNA and histone, such as occurs in nucleosomes or chromatin. This concurs with recent findings concerning prototypic murine ‘anti-dsDNA’ antibody 3H9, which when highly purified was actually found to recognise a complex of H2A/H2B/dsDNA but not dsDNA in the solid-phase or solution (Guth et al. 2003). Also these authors found that 3H9 did bind to dsDNA when used in the form of culture supernatant or after incomplete purification using protein G, this was exactly as I have shown for SVB33 in Figure 4.14. These data support the hypothesis that ‘anti-dsDNA’ antibodies like 3H9 and B3 may bind to DNA containing complexes such as nucleosomes or chromatin, which facilitate their apparent binding to dsDNA in the solid-phase.

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The same recombinant B3-derived antibodies that bound to dsDNA, also bind to histones (Haley et al. 2004), although the importance of certain CDR motifs was slightly different. These results emphasise the difficulties inherent in measuring and classifying such antibodies in patients with SLE, since it is likely that these antibodies exist as complexes in vivo. Such complexes may not be completely removed by either affinity purification or DNase I treatment in isolation (Kramers et al. 1994). It might be useful to try and determine the nature of the factor, or factors, in the supernatant that are required for dsDNA binding of the purified antibodies. An attempt could be made by the reconstitution of the affinity purified and DNase I treated antibodies, prior to assaying for dsDNA binding, with for example pure nucleosomes, or pure histone fractions. In the light of the lack of DNA binding by the highly purified antibodies, it would also be interesting to assay the binding of these antibodies to nucleosomes on the solid-phase. The additional binding of B3 and RH-14 (anti-DNA, anti-histone and anti-nucleosome antibody) to α-actinin suggests that these 'anti-dsDNA' antibodies actually recognise a conformational epitope that allows limited cross-reactive binding. Using the computer model of B3, it has been hypothesised that B3 may recognise Z-dsDNA in a binding site which is at right angles to the binding site which is hypothesised to bind native B-dsDNA (Kalsi et al. 1996). Thus it is possible that anti-dsDNA antibodies may possess more than one binding site.

Although the anti-DNA ELISA used in this thesis was sufficient to determine the effects on DNA binding of sequence changes in the light chain CDRs, better information regarding the affinity of the antibodies for dsDNA or ssDNA could be obtained using surface plasmon resonance (for example BIAcore). In surface plasmon resonance the antibody affinity for an antigen is measured through the calculation of kinetic rate constants detected for the binding and dissociation of an antibody when it is passed over a chip coated with antigen. Alternatively the dissociation constant (Kd), as a measure of antibody affinity, can be determined by incubation of the antibodies with varying concentrations of dsDNA prior to assaying in an ELISA with dsDNA on the solid-phase (Friguet et al. 1985). In this system the concentration of antigen must be in great excess of the concentration of antibody. Functional affinity of the antibodies can also be measured by addition of the
chaotropic agent diethylamine (DEA) to inhibit the binding of low affinity antibodies to the dsDNA coated on the solid-phase (Devey et al. 1988). The higher the affinity of the antibodies tested, the smaller the shift in binding caused by the presence of DEA in the assay. The results are expressed in terms of the inhibition index, which is the reciprocal $\log_{10}$ of the shift, thus a small shift in binding gives a high inhibition index for a high affinity antibody.

The supervectors I have described were constructed to allow transfection of CHO cells with a single vector, resulting in expression of both heavy and light chains of IgG. This endeavour was time consuming but more efficient than co-expression of two single vectors, one for each immunoglobulin chain. However, there is still a risk that there will be an imbalance in heavy/light chain expression due to the design of the supervectors. This imbalance is because the construction of the supervectors involves a light chain expression cassette (containing the HCMV promoter, the $\lambda$ constant region gene and the $\lambda$ variable region gene of the chosen antibody) being transferred into the heavy chain expression vector directly upstream from the heavy chain HCMV promoter. As a result, the light chain and heavy chain are being expressed in sequence. When two promoters in sequence are used to express two genes in this way it is very likely that the second gene will be expressed at a lower level than the first. Therefore it is possible that the heavy chain in our supervectors will be expressed at lower levels than the light chain.

This concern is important as although not actually detected in the ELISA (since the conjugated secondary antibody is specific for heavy chain), excess light chains could interfere in the ELISA assays. If the light chains alone could bind to DNA they might reduce the binding observed in the ELISA, by competing with whole antibody for binding to dsDNA on the solid phase. It is unlikely that the light chains bind DNA effectively without the presence of the heavy chain, since the binding site is formed by both immunoglobulin chains and an arginine residue from the heavy chain of B3 is known to be important in stabilising DNA binding (Kalsi et al. 1996). Excess light chains may also cause proteinuria in the in vivo experiment, since light chain deposition in the kidney can cause nephropathy, this can be associated with multiple myeloma (Randall et al. 1976). However, since proteinuria was also
observed in mice inoculated with CHO cells that contained the 'empty vector' and hence could not produce light chains, this is unlikely to be a factor in my experiments. In addition no immune deposits were observed in any of the inoculated mice.

The antibody production of the CHO cells transfected with the supervectors was higher than that of the COS-7 cells co-transfected with separate heavy and light chain vectors. The main disadvantage of the transient system obviously being that antibody was only obtained for a few days. However, the human IgG production of CHO cells transfected with SVBL 5-40-24 had decreased significantly (from 130 to less than 4.0 ng/1x10^6 cells/day), when they were grown up from frozen for the in vivo experiment testing multiple CHO cell clones of each supervector. This decreased production of human IgG occurred despite the level of methotrexate in the culture medium being carefully maintained. Several workers have shown long-term production of IgG by recombinant CHO cells. However, it is documented that copies of the inserted genes were lost and thus also production of IgG reduced over time, especially when methotrexate was omitted from the culture medium (Kunert et al. 2000; Strutzenberger et al. 1999). Clonal variability in IgG production over time has been reported, even in sub-clones derived from the same original clone (Kim et al. 1998). So although these lines are stable compared with the transient expression system, it needs to be determined how stable they are in our laboratory, over long-term passage and post-thawing from frozen storage. For future studies, an efficient method has been reported to screen for the sub-clones of CHO cells which are the highest producers of IgG, this method based on flow cytometry and cell sorting may be much more efficient than ELISA screening, since it can measure secreted IgG trapped on the surface of single cells (Borth et al. 2000). However, the CHO cell lines reported in this thesis have already been used successfully to produce several milligrams of affinity purified antibodies that can be used in subsequent experiments.

The CHO cells that were implanted in the SCID mice were still growing in adherent cultures, which may have limited their ability to grow in the peritoneum and may have caused more adverse pathology than cells grown in suspension. It is possible to convert the recombinant CHO cell line gradually to both serum free and to suspension culture (Miescher et al. 2000), both of which may avoid some of the
pathology that was observed even with the control CHO cell line transfected with 'empty vector'. Pathology that might be attributed to the CHO cells or their culture medium, could also be avoided by the direct i.v. injection of the anti-DNA antibodies into mice. However, this experiment would require large amounts of antibody, need repeated regular injection (since rapid clearance results in a short half-life of such antibodies) and would be costly. A single i.v. injection of a relatively low dose (100μg) of the affinity purified recombinant anti-DNA antibodies in this thesis, was not sufficient to cause renal deposition in SCID mice.

The use of CHO cells derived from hamster ovary in mice may present a problem, even in immunodeficient SCID mice. In the experiments where SCID mice were implanted with the mouse-human heteromyeloma fusion partner cell line CB-F7, I did not observe neutrophil infiltration or the marked cachexia that was seen in mice inoculated with CHO cells. There are no reports of CB-F7 being transfected with expression vectors, but such an expression system has been produced using another mouse-human heteromyeloma cell line F3B6 (Li et al. 2000). F3B6 was used to produce human IgG containing the $V_{H}$ and $V_{\kappa}$ chains of an IgA1κ anti-dsDNA monoclonal antibody originally derived from a lupus patient. In order to produce the B3 recombinant antibodies in either CB-F7 or F3B6 cells, the DNA sequences would have to be re-cloned into a suitable vector that would allow selection of successfully transfected cells. The pG1D1 supervector does not contain the neomycin resistance gene as this system relies on selection by the addition of the dhfr gene (present in pG1D1) to CHOdhfr

The development of a human anti-DNA IgG transgenic mouse might be a better way to study the long-term in vivo pathogenicity of a human anti-DNA antibody. A transgenic mouse would allow the study of the antibody without the interference of other factors introduced into the mouse such as the foreign host cells and culture medium components like cell debris, foetal calf serum or methotrexate. The disadvantage is that if it was possible to produce a human anti-DNA transgenic mouse it would only be feasible to study one antibody at a time, since it would be much more time consuming and costly to produce multiple transgenic mouse lines, than to produce several cell lines secreting a variety of antibodies. The advantage is
that the human anti-dsDNA transgenic could be studied by backcrossing to different murine backgrounds, such as autoimmune or normal strains. Studying anti-DNA antibodies on an autoimmune background may have advantages since other defects only present in an autoimmune animal may facilitate pathogenicity due to the anti-DNA antibody. For example, α-actinin was expressed in the kidney glomeruli of BWF1 lupus-prone mice but not in ‘normal’ BALB/c mice (Mostoslavsky et al. 2001). However, the expression of α-actinin observed in diseased mice could be secondary to the disease process, having been upregulated in response to inflammatory mediators released due to binding of anti-DNA antibodies.

The different pathology observed on implanting RH-14 in SCID mice of different ages, suggests that one should not assume that a particular anti-DNA antibody will always deposit in the same location within the kidney. It would be interesting to investigate this phenomenon further to determine whether it was age related factors, such as altered expression of target antigens or changes to capillary dynamics, or the presence of an additional factor such as endotoxin in the implanted cell culture, which caused the different immune deposition.

The use of murine models to study human diseases may be criticized especially since studying the role of human anti-DNA antibodies in normal, immunodeficient or autoimmune mice is clearly complex. Given their increased availability, it would seem logical to consider using simpler in vitro systems, such as human kidney cells. For example, immortalised human kidney cell lines that express the same phenotype as the cells from which they were derived, such as a human podocyte cell line (Saleem et al. 2002) and human glomerular endothelial cells (Satchell et al. 2004) could be utilized. It would be interesting to investigate whether the recombinant human anti-DNA antibodies bind to these human kidney cells and whether they bind to the cell surface or penetrate the cells to bind to cytoplasmic or nucleic antigens. The podocyte is especially interesting since several hereditary human nephrotic syndromes have been shown to be attributable to defects in podocyte specific genes (Pavenstadt et al. 2003). Affected genes include those for nephrin, podocin, WT-1, CD2-associated protein and α-actinin-4.
As well as the binding to the individual cells, antibody binding could be assessed in
the human glomerular basement membrane assay (Lefkowith et al. 1996), the rat
perfused kidney system (Raz et al. 1989) and to sections of human kidney if
available. The perfused rat kidney has previously been used to measure the effect of
both murine and human anti-DNA antibodies on induction of proteinuria, this was
achieved by measurement of increased albumin excretion after perfusion with anti-
DNA antibodies (Raz et al. 1989).

The results presented in this thesis suggest that α-actinin could well be a target of
human anti-DNA antibodies. However, more extensive studies in a larger number of
carefully selected patients need to be conducted to determine whether anti-DNA
antibodies binding to α-actinin is a mechanism which results in renal pathology in
patients with lupus nephritis. In addition to testing the binding of anti-DNA
antibodies to commercial α-actinin it will be important to look at the binding to
lysates of human kidney tissue and specific glomerular cells (podocytes and
endothelial cells) by western blotting. It will be especially interesting to see if anti-
dsDNA antibodies can bind to other podocyte molecules (e.g. nephrin, podocin, WT-
l and CD2-associated protein) which have been implicated in other human nephrotic
diseases as discussed earlier. It will also be interesting to see if other autoantibodies
that occur in patients with SLE, such as anti-Sm/RNP, anti-Ro/anti-La among others,
also bind to α-actinin or whether this is limited to cross-reactive anti-dsDNA
antibodies.

It is arguable how much one can extrapolate from the study of a few human
monoclonal anti-DNA antibodies. B3 is a highly relevant antibody to study in the
context of SLE as it has been derived from a patient with active SLE, although it is
interesting to note that this patient did not have renal disease at the time of the fusion.
B3 has an IgG1 isotype and it has high affinity for dsDNA. B3 is likely to be typical
of pathogenic anti-DNA antibodies found in other patients with SLE since its V_l and
V_H are encoded by V_l,2a2 and V_H,3-23 respectively. V_l,2a2 and V_H,3-23 are the two
most commonly rearranged V_l and V_H genes, in both healthy individuals and SLE
patients (Brezinschek et al. 1997; Ignatovich et al. 1999). Therefore the conclusions
drawn from this thesis are likely to be relevant to a significant proportion of human anti-DNA antibodies found in patients with SLE.

RH-14, which binds nucleosomes, histones as well as ssDNA and dsDNA was also derived from a patient with active SLE including glomerulonephritis, since this antibody has been shown to be the most pathogenic in SCID mice this would be the most interesting to study. Unfortunately this antibody has not yet been successfully sequenced, so no conclusions can be made regarding the relationship between its amino acid sequence and its pathogenic function.

If a large proportion of the human anti-dsDNA antibodies found in patients with lupus nephritis, possess common sequence motifs in their antibody binding sites or recognise common antigenic target(s) in the kidney, then the long-term therapeutic goal of this work would be to design effective antagonists to block the effects of these autoantibodies in patients with SLE.
# Appendix A  Amino Acids: One letter code and charge of side chain at neutral pH

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>ONE LETTER CODE</th>
<th>SIDE CHAIN CHARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>A</td>
<td>uncharged</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
<td>positive</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
<td>uncharged</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
<td>negative</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C</td>
<td>uncharged</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
<td>uncharged</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>E</td>
<td>negative</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
<td>uncharged</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
<td>uncharged</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>I</td>
<td>uncharged</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
<td>uncharged</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
<td>positive</td>
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<tr>
<td>Methionine</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Valine</td>
<td>V</td>
<td>uncharged</td>
</tr>
</tbody>
</table>
Appendix B  Publications arising from the work described in this thesis


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