PRODUCTION AND ANALYSIS OF HUMAN MONOCLONAL IgG ANTI-DNA ANTIBODIES

by

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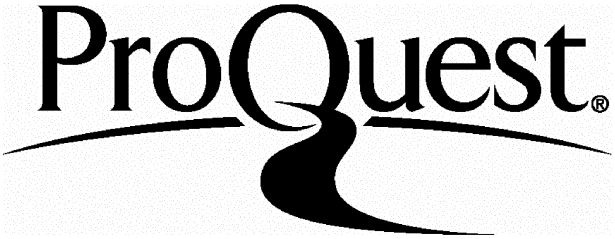

ABSTRACT

The hypothesis underlying the work described in this dissertation is that anti-deoxyribonucleic acid (DNA) antibodies of the immunoglobulin G class are pathogenic in the disease systemic lupus erythematosus (SLE), particularly in patients with renal involvement. The diversity of circulating antibodies in SLE prevents their precise study without producing monoclonal antibodies. Thus the objective was to generate and analyse human monoclonal IgG anti-DNA antibodies in terms of their fine specificity, idiotypes and biological properties.

Initial studies using a number of techniques demonstrated that a heteromyeloma cell line, designated CB-F7, when fused with the peripheral blood lymphocytes of lupus patients yielded the highest number of monoclonal IgG antibodies. Using this technique, five monoclonal IgG anti-DNA antibodies from one patient with active lupus were generated. A comparison with five monoclonal IgM anti-DNA antibodies, derived from the same patient when her disease was inactive, demonstrated a change to the IgG isotype associated with active disease and increased binding affinity to dsDNA. Two IgG anti-DNA antibodies, designated B3 and D5, were selected for further study.

To analyse the structure and distribution of the idiotypes related to these antibodies in the serum and tissue of patients with SLE and other diseases anti-idiotypic reagents were produced against B3 and D5. The idiotype associated with B3 was at or near the binding site for DNA, and may be partly encoded by two adjacent arginines in the complementarity determining region (CDR) 1 of the light chain. The expression of this idiotype in serum was associated with active arthritis in lupus patients. A polyclonal and monoclonal anti-idiotype were produced which identified idiotypes on D5 (D5-RId and D5-MId respectively) and although generated from different species each identified

overlapping structures on D5 which were at or close to the binding site for DNA. D5-MId, but not D5-RId, was found deposited in renal lesions of some SLE patients.

The biological properties of anti-DNA antibodies were investigated in terms of their ability to bind to murine renal and other tissue antigens *in vivo*. This was achieved by transferring hybridomas secreting the antibodies into mice with severe combined immunodeficiency. B3, but not D5, bound to cell surfaces in a variety of organs including the kidney and induced proteinuria. One anti-DNA antibody derived from another SLE patient deposited exclusively in the glomeruli of the mice but none of the antibodies caused glomerulonephritis.

In conclusion, human monoclonal IgG anti-DNA antibodies were produced which are representative of a proportion of antibodies found in the serum of SLE patients with active disease. The expression of an anti-DNA antibody associated idiotype, which was located in CDR1 region of the light chain, in the serum of SLE patients was associated with active arthritis. Only a proportion of IgG anti-DNA antibodies bound to tissue structures *in vivo*.

To Mirit, and Samuel

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Declaration. The work described in this thesis was performed by myself unless otherwise stated.

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ABBREVIATIONS

ANA	Antinuclear antibody
anti-Id	Anti-idiotype
APAAP	Alkaline phosphatase anti-alkaline phosphatase
Bic	Bicarbonate
BILAG	British Isles Lupus Assessment Group
BSA	Bovine serum albumin
°C	Degrees centigrade
C3	Complement component 3
CDR	Complementary determining region
CNS	Central nervous system
CVS/Resp	Cardiovascular / Respiratory
D	Diversity
dsDNA	Double stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylene diamino tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
Fab	Fraction of antibody which binds antigen
Fc	Fraction crystallisable (constant)
FCS	Foetal calf serum
FR	Framework
g	Gravity
GM	Growth medium
HAT	Hypoxanthine-aminopterin-thymidine
HLA	Human major histocompatibility complex
Id	Idiotype
IFA	Incomplete Freund's adjuvant

IgG	Immunoglobulin G
IgM	Immunoglobulin M
kD	Kilodaltons
λ	Lambda
κ	Kappa
mAb	monoclonal antibody
mg	Milligram
ml	Millilitre
M/S	Musculoskeletal
NSAID	Non-steroidal anti-inflammatory drug
(NZB/W)F1	New Zealand black x white F1 hybrid
OD	Optical density
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline/Tween
PBL	Peripheral blood lymphocytes
PEG	polyethylene glycol
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
RPMI	Rothwell Park Memorial Institute essential media
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SF	serum free (medium)
SLE	systemic lupus erythematosus
ssDNA	single stranded deoxyribonucleic acid
TBS	Tris buffered saline
TEMED	N,N,N',N'- Tetramethylethylene diamine

Tris	Tris-hydroxy-amino-methane
µg	Microgram
µl	microlitre
UV	Ultraviolet
V	Variable
Vasc	Vasculitis

CHAPTER 1. INTRODUCTION

General Comments

The introduction of this dissertation describes the possible role of anti-DNA antibodies in the disease systemic lupus erythematosus (SLE). In particular, four main areas are considered.

1. The difference between those anti-DNA antibodies found in health and disease with respect to isotype, binding properties and the idiotypes they possess.

2. The evidence that anti-DNA antibodies are important both in terms of revealing clues about the aetiology of the disease and in inducing the pathology seen in SLE.

3. The relevance of the study of monoclonal, as opposed to, polyclonal antibodies in relation to pathological anti-DNA antibodies found in SLE.

4. The clinical features of SLE and the animal models of this disease.

1.1 NATURAL VS PATHOLOGICAL AUTOIMMUNITY

For much of the 20th century the central dogma of immunology has been that the immune system does not normally react to self. When this tolerance to

self is disturbed the immune response can be directed against self resulting in autoimmunity. However, not all autoimmunity leads to disease, indeed some autoimmune phenomena are critical for the correct functioning of the immune system itself, such as the recognition of Major Histocompatibility Complex by thymocyte derived (T) cells, and the presence of antibodies and T cells that recognise the variable regions of other antibodies. This last phenomenon is known as idiotypic anti-idiotypic interaction and forms the idiotype network (section 1.12). Moreover, so called "natural autoantibodies" are found in normal individuals directed against a wide variety of host constituents, generally with low affinity (Daar and Fabre, 1981). The role of natural autoantibodies is controversial. They may act as a first line of defence against invading organisms or perhaps protect autoantigens from attack by the components of a more harmful secondary immune response. Recently, more evidence is accumulating that B cells secreting antibodies with a low affinity to self have a survival advantage, since stimulation of B cells through their antigen receptors (i.e. immunoglobulin) is essential for B cell development (reviewed in Schwartz and Stollar, 1994). Autoantibodies are also the hallmarks of many autoimmune diseases, where the autoimmune reactions are detrimental to host function. A key question is what differentiates pathological autoantibodies from natural autoantibodies.

When an autoimmune response is harmful to the host autoimmune disease ensues. There is considerable diversity in the aetiology, pathogenesis and expression of these diseases. Clinically they can be divided into organ specific disorders such as Graves' disease, myasthenia gravis and Type 1 diabetes mellitus; and non-organ specific disease which includes the autoimmune rheumatic diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis and Sjogren's syndrome. This clinical classification to some extent reflects the pathogenic mechanisms that lead to the different

diseases; in non-organ specific diseases a wide array of autoantibodies exist (e.g. SLE) whereas in organ specific diseases the specificity is more restricted (e.g. myasthenia gravis).

The importance of autoantibodies in the dissection of the processes leading to autoimmune disease is threefold. In some autoimmune diseases the antibodies are clearly pathogenic such as in myasthenia gravis and autoimmune thyrotoxicosis. Autoantibodies can provide clues to the aetiology of the disease. For instance, molecular mimicry is thought to provide a link between cardiac tissue and *Streptococcus* polysaccharides in rheumatic fever (Ehrenstein and Isenberg, 1991). Thirdly, autoantibodies directed against the variable regions of other antibodies, known as anti-idiotypes, are thought to play an immunomodulatory role controlling immune responses.

One of the most interesting challenges in the study of autoimmune disease is the connection between tolerance and health with autoimmunity and disease. In this respect anti-DNA antibodies are of particular interest. Circulating anti-DNA antibodies are strongly associated with SLE, particularly anti-dsDNA antibodies (Isenberg et al, 1994). Natural anti-dsDNA antibodies i.e. those occurring in healthy individuals are extremely rare. There is some evidence that these antibodies are important in the pathogenesis of SLE, particularly the renal manifestations (section 1.10). The question of the origin of these antibodies is central to the issues of tolerance since if tolerance prevents autoimmunity one would expect that the presence of antibodies against DNA, the substance of genetic code, would be a very rare event. However, anti-DNA (particularly ssDNA) antibodies are present in low titres in many normal individuals (Rubin and Carr, 1979). These antibodies tend to be of the IgM isotype and of low affinity. In contrast, the antibodies associated with active SLE, particularly renal disease, are of the IgG isotype and of high affinity for dsDNA. Thus the differentiation of tolerance and health from autoimmunity and

disease may be, in antibody (and therefore B cell) terms, the isotype, precise specificity and affinity of the antibody.

The study of anti-DNA antibodies can lead to answers about two equally important questions. The first is regarding the aetiology and pathogenesis of SLE, a common autoimmune disease. The second deals with the fundamental nature of antibody, and therefore B cell, tolerance and the development of autoimmunity.

1.2 SYSTEMIC LUPUS ERYTHEMATOSUS: epidemiology, clinical features and aetiology: one disease or many?

Systemic lupus erythematosus (SLE) is a multisystem disease which predominantly affects women, particularly during the child bearing years. A recent report from the UK shows an annual incidence rate of 4 per 100,000/year and a prevalence rate of 24.6 per 100,000 (Hopkinson et al, 1993) . This study showed an increased prevalence amongst those with Afro-Caribbean origin. Epidemiological data from the United States show a prevalence rate of between 14.6-50.8 cases per 100,000 persons, a sex ratio of women: men to be 6-10:1, and a prevalence rate of up to 1 in 250 amongst the black population (Hochberg, 1993).

The diversity of the disease is reflected both clinically and immunologically. SLE is characterised by relapses and remissions which may involve any organ of the body (Table 1.1, classification criteria). The episodic nature of the disease contrasts somewhat with other autoimmune rheumatic diseases such as rheumatoid arthritis which usually involves more constant and persistent disease activity. The disease is so protean in its manifestations that it could be considered to encompass several different conditions. Indeed,

TABLE 1.1 Revised criteria of the American Rheumatism Association for the classification of systemic lupus erythematosus (SLE).

1.	Malar rash
2.	Discoid rash
3.	Photosensitivity
4.	Oral ulcers
5.	Arthritis
6.	Serositis: (a) pleuritis, or (b) pericarditis
7.	Renal disorder: (a) proteinuria>0.5g/24h or 3+ persistently, or (b) cellular casts.
8.	Neurological disorder: (a) seizures or (b) psychosis (having excluded other causes e.g. drugs).
9.	Haematological disorder: (a) haemolytic disorder or (b) leucopenia or < $4x10^9$/l on two or more occasions (c) lymphopenia or < $105x10^9$/l on two or more occasions (d) thrombocytopenia <$100x10^9$/l
10.	Immunologic disorders: (a) positive LE cell or (b) raised anti-native DNA antibody binding or (c) anti-Sm antibody (d) false serologic test for syphilis, present for at least six months
11.	Antinuclear antibody in raised titre

A person shall be said to have SLE if four or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.
After (Tan et al, 1982).

physicians who treat SLE patients could be divided into 'lumpers' and 'splitters', the former considering SLE as a number of different diseases and the latter think of SLE as one disease with a variety of manifestations. There is very little data on what proportion of patients have only a limited number of clinical features. Patients are known to experience flares involving one system, for example the locomotor system, i.e. arthritis, but following a remission other organs or systems may become involved and the patient develops a skin rash or severe nephritis. Alternatively, all three clinical features could occur simultaneously. However, there are also patients whose disease is confined to only one organ albeit with haematological and immunological features of SLE thus fulfilling the criteria for the disease (see below).

Inevitably, lupus patients present with a wide variety of conditions to different physicians. Thus, when analysing information about patients with lupus the subspecialty of the consulting physician to whom these patients are referred must be considered. Not surprisingly reports emanating from renal units tend to imply that all the lupus patients have renal involvement. In contrast, a study carried out at the Bloomsbury Rheumatology Unit, from which the patients used in this thesis were derived, reported that only 30% had renal disease (Worral et al, 1990). However, whereas minor joint disease, i.e. arthralgia, is easy to determine and occurs in most series in 95% of the cases, minor renal disease is more difficult to detect and kidney involvement might have been underestimated. More recent data suggest that if lupus patients are followed up for long enough a much higher proportion (~ 70%) develop renal disease (Menon et al, unpublished data). An analysis of renal biopsies indicated that patients who have no symptoms, signs or abnormal clinical investigations suggestive of renal disease often have abnormal kidney histopathology (Gladman et al, 1989). In addition, whereas most clinical assessments of renal disease measure organ damage, rather than disease activity, the opposite is

true of joint disease where arthritis and tendinitis reflects disease activity. Only recently attempts have been made to measure organ damage as a separate entity from disease activity (Gladman et al, 1992a).

Most studies do not analyse individual patient assessments, whether patients had organ involvement simultaneously or separately or the severity of the different clinical features. In other words, instead of solely analysing individual organ involvement in a group of patients as a whole, it is also necessary to analyse individual organ involvement separately in individual patients. Thus two instruments are necessary to satisfy both the "lumpers" and the "splitters". To satisfy the former classification criteria are required and these have been available for two decades. It has been more difficult to develop a mechanism to satisfy the splitters but a relatively new activity index which assesses disease activity in each organ system separately has been developed. Classification criteria and activity indices are discussed later in this thesis.

Over the last forty years most of the mortality associated with SLE has been attributable to renal and CNS disease and to intercurrent infection. The cumulative survival rates of patients with SLE have progressively increased with better treatment. In 1954 the 5 year survival was 51% (Merrel and Shulman, 1955) whereas a recent study in 1989 showed a survival rate at 5 years of 95% and at 10 years of 87% (Swaak et al, 1989). As survival improves increasing attention has been paid towards potentially preventable causes of mortality such as steroid associated coronary heart disease. Indeed in 1976 it was first suggested that the mortality in SLE followed a bimodal distribution and that late death was due to atherosclerotic cardiovascular disease and acute myocardial infection (Urowitz et al, 1976).

The treatment of SLE usually involves combinations of non-steroidal anti-inflammatory drugs (NSAID) anti-malarials (hydroxychloroquine),

corticosteroids and cytotoxic drugs. NSAID are used particularly for joint disease, fever and muscle pain; hydroxychloroquine is effective for joint and skin disease. In general the first two are given for mild disease and the last two are reserved for more serious disease, particularly renal disease. The treatment of CNS disease is far less satisfactory. Worrall et al (1989) found that fifty five percent of patients received prednisolone doses in excess of 10 mg/day.

As with many autoimmune diseases the aetiology of SLE is considered to be multifactorial, involving genetic susceptibility, endocrine and environmental components. Amongst monozygotic twins the concordance rate has been reported to be as high as 70% whilst dizygotic twins have a concordance rate of 15% (Block et al, 1975). More recently a study based in the community has shown a lower concordance figure, though some of the patients were identified by self diagnosis and the duration of follow up to monitor for the development of lupus was shorter than in the first study (Deapen et al, 1992). The haplotype A1, B8, DR3 is particularly associated with SLE in Caucasians. Some autoantibodies are linked to HLA markers such as HLA-DR3 (DRw17) and DQw2 with anti-Ro/anti-La antibodies. Recently anti-DNA antibodies have been linked to three HLA-DQβ alleles (*0201, *0602, *0302) occurring in 96% of patients with high levels of anti-dsDNA antibodies (Khanduja et al, 1991). Retroviruses have received attention recently as possible trigger factors for SLE (Venables and Brooks, 1992). Autoantibodies may arise by molecular mimicry as has been proposed for anti-U1 sn RNP antibodies and influenza B virus (Guldner et al, 1990). A number of drugs, such as hydralazine, have been linked to the disease but drug induced lupus is different in several aspects such as equal male/female preponderance and generally absent renal disease. Interestingly antibodies against dsDNA are not seen, but anti-ssDNA and anti-histone antibodies are found (Tan, 1989). Ultraviolet light is known to

exacerbate the cutaneous manifestations of SLE.

As with all aspects of this disease the immunopathology of SLE is diverse (Table 1.2). At the cellular level, SLE is characterised by abnormalities in T and B lymphocytes, natural killer cells, and accessory cells (antigen presenting cells). Cytokine production has been studied extensively both in SLE patients and in animal models of SLE. Changes have been noted in almost every cytokine, though there is much conflicting data. The role of B cells and in particular anti-DNA antibodies will be discussed in detail later in this thesis.

1.3 SLE CLASSIFICATION CRITERIA AND DISEASE ACTIVITY INDICES.

In order to study the disease, classification criteria have been established by the American Rheumatism Association (Table 1.1). A patient may be considered to have SLE if four or more of the 11 criteria are present, serially or simultaneously, during any interval of observation (Tan et al, 1982). The sensitivity and specificity of these criteria are approximately 95%. Disease activity indices have been developed by a number of independent groups primarily to assist clinicians to treat patients (Hay et al, 1993b). Whereas disease criteria place patients with a variety of manifestations into one group, there have been attempts to separate patients into different categories based on organ involvement when assessing disease activity. Apart from allowing clinicians to make more rationale choices regarding management of patients, judging disease activity according to organ involvement may reflect the underlying pathological processes more accurately. In other words different disease manifestations may have different aetiologies. For example, certain autoantibodies found in SLE have been found to be associated with different

Table 1.2 Cellular abnormalities and cytokine dysregulation (adapted from Isenberg and Horsfall, 1993)

Cell type/cytokine	Dysregulation
Monocyte/macrophage	↓ TNF α production-genetic defect[a]
Lymphocytes	↑ Numbers of activated B cells
B cells: (see section 1.7)	→ hypergammaglobulinaemia
	→ IgG autoantibodies reactive with self antigens (cell membrane, cytoplasmic proteins, nuclear antigens, extracellular proteins) and non-self (polyclonal).
	↑ IL-2 receptor, ↓ CR 1 expression, ↑ surface expression of hsp 90[b] but not hsp 70 compared to normal cells.
T cells:	↓ $CD4^+CD45R^+$ (subset T helper, suppressor/inducer)
	↑↑ $CD4^-8^-$ T cell receptor $\alpha\beta^+$ Th (escape thymic deletion since double negative?)
	Activated T cells are Class II^+ (DP, DR)
	Defective suppression
	Impaired cytotoxicity
	Activated peripheral T cells (only → 15% anti-DNA help),
	cloned Tγδ cells reactive with hsp 60 and → help to anti-DNA^+ B cells (not class II restricted)(blocked by antibody to hsp 65 but not hsp 70)
Cytokines	
IL-1[c]	↓ or ↑ . ↓ responsiveness of T cells
IL-2	↓ or ↑ . Reduction in vitro observed may reflect transient exhaustion of in vivo-activated T cells. IL-2 → autoimmunity in neonatally thymectomised mice, ameliorates disease in lpr mice.
IL-4	May a play a role in B cell hyperactivity.
IL-6	Elevated in SLE and correlates with disease activity. Note: acute phase reactants such as CRP, which are induced by IL-6 are not elevated in SLE. ?abnormality in IL-6 receptor.
TNFα	MHC -linked production:
	↓ in HLA-DR2, DQw1 associated with nephritis
	↑ in HLA-DR3, DR4, no nephritis.
	Accelerates or ameliorates nephritis in animal models depending on dosage.
IFN-γ[d]	↑ in SLE. Augments disease in animal models. Many biological effects similar to manifestations of SLE e.g. fever, malaise, alopecia, polyclonal B cell activation, lymphopenia.

a TNFα = tumour necrosis factor; b hsp = heat shock protein; c IL = interleukin; d IFN = interferon.

manifestations, for instance anti-Ro antibodies are associated with photosensitivity and some dermatological manifestations.

The British Isles Lupus Assessment Group (BILAG) designed disease activity criteria based on the principle of the physician's intention to treat (Hay et al, 1993a)(see Appendix). This index assesses the activity of the disease in eight major organs or systems avoiding the drawbacks of a global scoring system. The BILAG index also makes an allowance for change in activity over time . The organ/systems are general features, locomotor system, nervous system, renal involvement, dermatological involvement, pleuro- pericardial involvement, vasculitis, and haematological involvement. Immunological tests do not contribute to the score. From this assessment scores are generated ranging from A, most active, to E, no activity in that particular organ system. Although not designed for this purpose the BILAG index can be adapted to provide a global score using the following scoring system, A=9 points, B=3, C=1, D=0, E=0. The BILAG index has been validated against other global scores (Gladman et al, 1992b). A and B scores are considered to represent active disease in individual systems and a total score of greater than 6 indicates active disease as a whole. The apparent complexity of BILAG reflects the disease but in practice the questionnaire that needs to be completed at each clinic visit is easy to use and can be undertaken in 2-3 minutes. In addition, the development of a computer programme has facilitated quick and easy recording and analysis of data.

1.4 ANIMAL MODELS OF SLE

Much of the data regarding the aetiology of SLE has come from the study of murine models of SLE. Much exciting information has been gleaned from the study of these inbred strains of mice, particularly about the processes involved

in autoimmunity. However, the clinical features of these models do differ from each other and more importantly from SLE itself (Table 1.3). All of the models tend to have relentless progression of disease (as opposed to relapses and remissions), all have nephritis, and some of the non-renal manifestations of human SLE do not occur (Andrews et al, 1978). If SLE is a collection of many diseases it would be difficult to model all of them with one animal system and it may be that some murine lupus strains correlate better with some patients than with others. In the same way in which some animal models of SLE also have features of other disorders as will be outlined below, some patients with SLE overlap clinically with other autoimmune rheumatic diseases. Another complicating factor is that patients with SLE are treated by a variety of immunosuppressive and immunomodulatory drugs whereas mice are not exposed to these agents. One notable example where treatment complicates the picture is in the incidence of coronary heart disease. Whereas the incidence of polyarteritis involving the coronary vessels is 50% in MRL/lpr mice (Andrews et al, 1978) it is a very rare event in the human disease. However, the use of corticosteroids has increased the incidence of heart disease secondary to atherosclerosis both directly and indirectly due to the patients living longer and being subject to the occurrence of diseases that affect the older population in general. One study compared the autopsy findings of lupus patients treated with steroids compared to those who died before steroid treatment was introduced (Bulkey and Roberts, 1975). The steroid treated group had a much greater degree of significant ischaemic heart disease.

Some of the models have distinct features that are not seen in patients with SLE. The MRL/lpr mouse, for instance, develops massive lymphadenopathy which has been attributed to a defect in the Fas antigen which is involved in the process of apoptosis (Watanabe-Kukunaga et al, 1992). Neither the massive proliferation of lymphocytes nor a defect in the Fas antigen

Table 1.3 Animal models of SLE

Strain	Haplotype	Mean life span (months)	Major clinical features	Autoantibody specificities	Other immunological abnormalties
NZB	H-2d	15 to 18	Haemolytic anaemia, lung infiltrates, GN	DNA, RF crtoglobulins erythrocyte	Thymic atrophy, high IgM/IgG, TNF deficiency
(NZB/NZW) F (females)	H-2d/x	7 to 9	Severe GN, lung infiltrates	DNA, RF	Thymic atrophy, high IgM/IgG
MRL-lpr/ lpr (females)	H-2k	3 to 5	GN vasculitis, arthritis, MI	DNA, Sm RF(++) cryoglobulins	Thymic atrophy, high IgM/IgG, lymphoid hyperplasia
BXSB (males)	H-2b	4 to 6	GN, MI haemolytic aneamia.	DNA, erythrocyte	Thymic atrophy, high IgM/IgG, lymphoid hyperplasia
Moth-eaten	H-2b	1	lung infiltrates hair loss, mild GN	DNA, RF erythrocyte	Thymic atrophy, high IgM/IgG, general immunosuppression leading to infections
Palmerston - North	H-2q	10 to 12	polyarteritis nodosa, GN	DNA	high IgM/IgG
Swan	H-2k	18	Mild GN	DNA	Early thymic atrophy

are found in patients with SLE (Mysler et al, 1994). Where the animal models do reflect SLE more precisely is in some of the immunological processes particularly the appearance of anti-dsDNA antibodies. A class switch from IgM to IgG anti-DNA antibodies heralds the onset of disease in (NZB/NZW)F1 mice. The aetiology of these diseases is far better understood than human SLE and it is important to determine whether similar processes are occurring in the murine and human forms of the disease. Most of the data regarding the immunopathology of murine 'SLE' discussed in this thesis has not been reproduced in human studies. Studying human SLE is obviously more difficult for ethical reasons. For instance, although human spleen tissue has been shown to be a better source of producing monoclonal antibodies (Watts et al, 1990b) it is rarely available whereas the spleen is invariably used for generating murine monoclonal antibodies.

1.5 OTHER AUTOIMMUNE RHEUMATIC DISEASES

Serum from patients suffering from other autoimmune rheumatic diseases are often used as disease controls in studies of SLE. Some of the features of these diseases will be discussed briefly particularly with reference to SLE. Rheumatoid arthritis (RA) is an inflammatory disease affecting primarily the synovial joints associated with a symmetrical polyarthritis and bony erosions. It is more common in women (F:M=3:1) and commonly affects an older age group. Although rheumatoid arthritis can occasionally affect other organs the manifestations differ from SLE. The kidneys for example can be affected by amyloid deposition in rheumatoid arthritis but nephritis occurs very rarely. Although rheumatoid factors and anti-nuclear antibodies are present in both diseases, the former predominate in RA but the latter in SLE. There is some evidence that a specific immune response against collagen type II is

present in some patients with RA which may suggest an aetiological role.

Sjogren's syndrome is a chronic inflammatory disorder of exocrine glands and is manifested by dry eyes and a dry mouth. It may occur as a primary event or secondary to other autoimmune disease notably SLE and rheumatoid arthritis. Primary Sjogren's syndrome has a number of features in common with SLE, notably the same female to male ratio, 9:1, the same associated HLA haplotype: HLA-A1, B8, DR3, hypergammaglobulinaemia and a predisposition to fatigue and arthralgia. There is also an increased risk of a B cell lymphoma. A narrower range of autoantibodies is observed in Sjogren's syndrome, commonly with a specificity against extractable nuclear antigens Ro (SS-A) and La (SS-B).

Scleroderma is a group of diseases characterised by progressive hardening of the skin, again with a similar female predominance to SLE. As with RA and SLE there is multisystem involvement but the features contrast with both. Greatly increased collagen production can occur in organs such as the kidney and lung which causes most of the mortality in this rare disease. Antibodies to an antigen Scl-70, identified as topoisomerase 1, are disease specific but only occur in 25% of patients.

Idiopathic myositis is a chronic progressive condition characterised by inflammation of the muscles (F:M= 3:1). Up to ninety percent of patients have anti-nuclear antibodies, up to 85% have antibodies to a 56kDa nuclear antigen and 30% have antibodies to the Jo-1 antigen.

Two points are worth emphasising about these diseases in relation to SLE. Evidence for a direct role for antibodies in the pathogenesis of SLE is stronger than in the other diseases and the fluctuating nature of disease is most marked in SLE. It should be pointed out, however, that there is considerable overlap in these conditions and some patients have features of more than one disease. In addition, members of the same family can have different

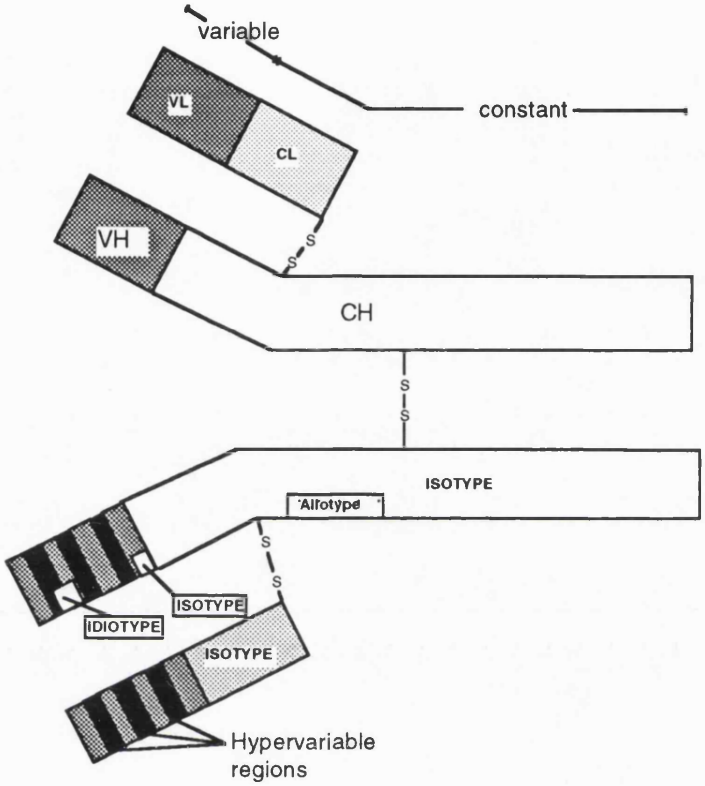
autoimmune rheumatic diseases indicating a genetic link between these diseases.

1.6 IMMUNOGLOBULIN STRUCTURE AND GENES

Antibodies are made up of heavy and light chains joined together by disulphide bonds. IgG consists of two heavy and two light chains (Figure 1.1). The variable regions of the heavy and light chain comprise the antigen binding site while the constant region of the heavy chain mediates effector functions such as the fixation of complement. The isotype of the antibody is defined by the heavy chain constant region. The V region protein sequences each contain three complementary determining regions (CDR), which have been shown in the crystal structure to form the antigen binding site and four framework regions (FW) which maintain the overall structure of the binding site. There may be some overlap in that the FW regions may occasionally be involved in direct binding with the antigen. The first two CDRs of the heavy chain and all three CDRs of the light chain are formed by the V region gene whilst the the third CDR of the heavy chain is formed by a D and a J segment gene joining to the V region at the 3' prime end. Chromosome 14 encodes the heavy chain, 2 encodes the kappa chain and 22 encodes the lambda chain.

The ability of the antibody to recognise a vast array of different antigens is due to many tandemly arrayed V region genes, four J region and 12 or more D regions which can join together, theoretically, in any combination. In addition, non germline-encoded N sequences can be introduced enzymatically at the junctions during this process. The precise number of germline V genes is unknown, particularly for the light chains. Even for the VH gene segments the estimates vary from as few as 100 to as many as 1000. Some of these estimates do not take into account pseudogenes which are irrelevant to the

Figure 1.1 The structure of the antibody molecule showing the variable and hypervariable regions. The position of an idiotype may be anywhere in the variable region.



expressed repertoire. Recently, Tomlinson and colleagues found 83 VH segments with open reading frames, some of which may represent duplicates (Tomlinson et al, 1992). The V region genes had been grouped together in terms of families on the basis of sequence homology. Six families have been assigned to the VH gene region. From two recent estimates, there are approximately 25 VH gene segments each assigned to VH1 and VH3, 1-3 in VH2, VH5 and VH6, and 10-14 VH4 segments (Tomlinson et al, 1992; Walter et al, 1990).

Most germ line encoded antibodies are of low affinity and are thought to be useful in neutralising viruses and toxins and acting as a first line of defence. Natural autoantibodies are thought to be predominantly in this category. To produce higher affinity antibodies the process of somatic mutation occurs whereby nucleotides are randomly introduced into the germ line genes. Somatic mutation can be divided into random (or intrinsic) and those that result in an increase in affinity for antigen, and thus presumably selected by antigen. Somatic mutations most frequently occur in the CDR regions of both the heavy and the light chain. By determining the nucleotide sequence of antibodies and comparing them to the germ-line the number of mutations can be calculated. If the ratio of the number of replacement to silent mutations exceeds 2.9 antigen selection is thought to have occurred (Shlomchik et al, 1987). However, this guide may not always apply since once the maximum affinity towards antigen has been achieved, replacement substitutions are no longer selected for, and silent mutations begin to accumulate. In addition some replacement substitutions could be neutral in terms of affinity.

During the initial phases of an immune response germ line encoded IgM antibodies predominate. As the immune system is exposed to the antigen it reacts by producing higher affinity antibodies. Those B cells producing antibodies that react strongly with antigen will be helped by T cells to proliferate

and differentiate. The average affinity of the circulating antibody increases as this process proceeds. The process of somatic mutation and affinity maturation is accompanied by antibody isotype switching leading to the generation of IgG antibodies. These IgG antibodies are the hallmark of the secondary immune and ongoing immune responses characteristic of autoimmune disease.

1.7 B CELLS AND AUTOANTIBODIES IN SLE

A large array of autoantibodies are found in the serum of SLE patients. Studies indicate that in some murine models of SLE there is a widespread breakdown in the control of autoantibody production. This suggests that the presence of these autoantibodies is not simply through an antigen driven mechanism, rather there seems to be a loss of control over the immune system. Mice carrying the lpr mutation have defects in the Fas gene (Watanabe-Kukunaga et al, 1992). The product of the Fas gene which is absent in the lpr strain of mice is important in apoptosis (programmed cell death). The authors concluded that in this mouse model autoreactive T and B cells are not deleted from the repertoire and widespread autoimmunity occurs. Other work has shown that the MRL/lpr mouse has a primary fault in the B cell indicating that it is not purely a break in T cell tolerance (Sobel et al, 1991). Further support for a primary defect in B cells causing autoimmunity comes from Strasser and colleagues who have used a transgenic model in which a single dysregulated gene, bcl-2, capable of enhancing B cell lifespan, provokes hypergammaglobulinaemia and widespread, lupus like, systemic autoimmunity (Strasser et al, 1991). The model was dependent on the genetic background of the mouse since other authors have not produced autoimmune disease when bcl-2 is transfected into normal mice (McDonnell et al, 1989). Strasser and colleagues suggest that the rapid turnover of B cells in the normal mouse

prevents the development of autoimmunity. One study has shown that bcl-2 is overexpressed in human lymphocytes in SLE (Grainger, 1992), though another report found no differences between bcl-2 expression in lymphocytes in normals and SLE patients (Rose et al, 1993). It may be important to study the B and T cell compartment separately; Kumagai and colleagues have found bcl-2 overexpression in the B cell but not in the T cell compartment (Kumagai et al, 1993).

Perhaps the most persuasive evidence to date that B cells alone (i.e. without the involvement of T cells) can cause a lupus like syndrome is the report by Reininger et al (1992) demonstrating that pre-B cell clones derived from lupus prone NZB/NZW F1 mice (but not normal mice) can differentiate in severe combined immunodeficiency (SCID) mice and secrete IgG anti-DNA antibodies, although mostly anti-ssDNA. In some of these mice a lupus like syndrome was also observed. This implies that (NZB/NZW) F1 B cells do not require T cell help to undergo a class switch to IgG.

If the above observations relating to murine lupus are valid in the human form of the disease it would imply that there is a general loss of tolerance in SLE. However, there are two aspects which alter this apparent dysregulation of the immune system. Firstly, that there is no general increase in all antibodies. Whereas the level of antibodies to exogenous antigens is increased several fold, the increase in autoantibodies is several thousand fold (ter Borg et al, 1991). Further, although there are approximately 2000 antigens in the cell, only about 30 have associated antibodies present in SLE patients (Gharavi et al, 1988). The polyclonal activation that occurs in SLE does not result in an increase in antibodies that bind antigens which the host has not previously encountered, or autoantigens not associated with SLE such as thyroglobulin, as determined by in vitro antibody production (Dar et al, 1992). This suggests that there the widespread production of autoantibodies that is observed in MRL/lpr

mice does not occur. Secondly, there is evidence of an antigen driven response against DNA and probably some other autoantigens. Experiments in (NZB/NZW) F1 mice (a model for lupus) reveal a specific rise in IgG anti-dsDNA antibodies and show that the polyclonal B cell activation precedes by several weeks, and reliably predicts, an antibody response skewed towards DNA and the development of nephritis (Klinman, 1990). This data suggests that B cell hyperactivity is an early phenomenon and that an antigen driven response occurs later in the disease.

Apart from antibodies to DNA a number of other autoantibodies have been detected in the serum of lupus patients often with clinical associations (Table 1.4). For instance, anti-cardiolipin antibodies have been linked with recurrent abortion, chorea and thromboembolic events. Some investigators have suggested that the presence of anti-cardiolipin activity in patients with anti-DNA antibodies is due to the cross reactivity of these antibodies. However, this conclusion is based on studies of IgM monoclonal antibodies, whereas serological studies have demonstrated that they are two separate populations (Smeenk et al, 1987). Another association is between the presence of anti-Ro antibodies with skin manifestations and with congenital heart block. Some of these antibodies also show evidence for somatic mutation in murine SLE (Kita et al, 1993; Bloom et al, 1993).

1.8 ANTI-DNA ANTIBODIES

Anti-DNA antibodies were first reported in the sera of SLE patients in 1957 (Robbins et al, 1957). The presence of certain subsets of anti-DNA antibodies are thought to be specific for SLE. Those that bind dsDNA and are of the IgG isotype are associated with active disease, particularly renal disease. In contrast, IgM anti-ssDNA antibodies are considered to be part of the natural

Table 1.4 Autoantibodies found in SLE and their clinical associations

Antibody	Antigen/epitope	Prevelance(%)	Clinical and other associations
Intracellular			
DNA	dsDNA (ssDNA) (ZDNA)	40 to 90	IgG, cationic, present in renal eluates
Histone	H1,2A, 2B, 3, 4	30 to 80	Drug-induced lupus+anti-ssDNA
Sm	A	Overall~ 35	SLE specific
	B/B'	Afro-Caribean 50to70	Afro-Caribean
	D	Caucasian 10 to 20	80% Sm+, also HLA-DR2
	N		
U1 RNP	68-kDa RNP	20 to 35	Mild disease, renal involvement HLA DR4
Ro SS-A	60, 52-kDa protein bound to cytoplasmic RNA		Rash, congenital heart block HLA-DR2, DQw1 HLA-DQw3-protective?
La SS-B	47-kDa protein bound to a variety of RNAs	10 to 15	DR3 2°SS
Heat shock proteins (hsp)	hsp 70 hsp 90	40 50	IgM>IgG Surface expression on monocytes & CD4+ T and B lymphocytes
Cell membrane			
Cardiolipin	Phospholipids DNA	20 to 40	Recurrent abortion, thrombosis
Neuronal antigen	Expressed on neuronal cell line grown in vitro	70 to 90 (CNS) ~10 (non CNS)	Some cross-react with lymphocyte cell surface
Lymphocyte	T cells>Bcells	~ 74 (IgM)	Cytotoxic (80%)
	HLA components	~47 (IgG)	Some cross-react with cell surface antigens of CNS
Red cell	Non-Rh related	<10	Haemolytic anaemia
platelet		<10	Idiopathic thrombocytopenic purpura
Extracellular			
Rheumatoid factor	Fc region of IgG	~ 25	Usually IgM, cross-react with histones Ro/SS-A

immune repertoire. These IgM autoantibodies are thought to be polyreactive and are probably derived from germ line genes with little somatic mutation. Kazatchkine and colleagues purified anti-DNA antibodies from the sera of SLE patients and normal individuals. Both anti-DNA fractions expressed the same degree of cross-reactivity with a panel of autoantigens and the authors concluded that anti-DNA antibodies purified from SLE patients and normals were the same (Hurez et al, 1993). However, without isolating individual antibodies in the preparations i.e. producing monoclonal antibodies, clear differentiation in terms of antigen specificity cannot be made.

Current knowledge suggests that a process of affinity maturation, somatic mutation and associated isotype switching leads to the production of IgG anti-dsDNA antibodies. This concept is almost wholly based on observations in the mouse such as those made by Weigert and colleagues who have studied the products of a single B cell precursor and have observed these antibodies through the course of disease in a mouse model of SLE (Shlomchik et al, 1990). Tillman et al (1992) have prepared hybridomas from a single SLE prone mouse at different times. Before the onset of disease most of the hybridomas generated IgM antibodies. Later, during the course of the disease, IgG antibodies were produced that were clonally related to some of the IgM antibodies generated earlier and showed a particular amino acid change in CDR3 of the heavy chain: aspartate to asparagine. A significant higher percentage of IgG clones, compared to IgM clones, produced antibodies that bound to both ss and dsDNA or dsDNA alone compared to ssDNA alone. Thus the antibody specificity of the IgG anti-DNA antibodies clearly changes as a result of class switching and accumulated somatic mutations. Site directed mutagenesis of a disease related anti-DNA antibody indicated that the removal of one arginine which was the result of an N region addition abrogated the DNA binding activity of the antibody (Radic et al, 1993). It appears that the immune

response was driven towards dsDNA binding, perhaps by dsDNA containing complexes. From these studies one can draw several conclusions: anti-DNA antibodies from MRL/lpr and (NZB/NZW) F1 mice are oligoclonal, they have sequentially accumulated somatic mutations, and the pattern of somatic mutations shows evidence of antigen selection. However the IgM anti-DNA antibodies from these mice can also possess some somatic mutations and thus class switching to the IgG isotype does not occur at the first moment of somatic mutation. Earlier work has shown that the switch from IgM to IgG anti-DNA antibodies in the serum is associated with the onset of disease in (NZB/NZW) F1 mice (Steward and Hay, 1976).

Although the work by Tillman et al (1992) suggests that the IgG anti-DNA antibodies arise from the IgM anti-DNA antibodies no information was given regarding the structure of antibodies with other specificities arising from these mice. This is an important weakness of their work since Diamond and Scharff (1984) have shown that an anti-pneumococcal antibody can change specificity with the substitution of one amino acid and bind to dsDNA.

Studies of anti-DNA antibodies in humans (and mice) have been largely based on monoclonal antibody technology. The large range of autoantibodies present in the sera prevents the examination of a single specificity. Further, the cross-reactivity of anti-DNA antibodies may be due to a number of anti-DNA antibodies of different specificities. The production of a large quantity of homogeneous antibody has allowed precise delineation of antibody structure, specificity and DNA sequence. Since monoclonal antibody technology has profoundly influenced concepts regarding anti-DNA antibodies it is important to consider how well the monoclonal antibodies generated reflect the antibodies thought to be an essential component of the aetiopathogenesis? This is a difficult question to answer since monoclonal antibodies are essential to determine the direct links between SLE and anti-DNA antibodies. The only

means to assess the relevance of these monoclonal antibodies at present is to compare them with the anti-DNA binding immunoglobulins in the serum which, by association, are linked to active SLE.

The vast majority of human monoclonal anti-DNA antibodies produced are of the IgM isotype. Some of these IgM antibodies are polyreactive suggesting a link with the natural autoantibodies found in normal individuals, many bind ssDNA but not dsDNA. However, some of these IgM antibodies do partially reflect the specificities of the antibodies detected in the sera. In one study the splenocytes from two patients were used; patient RSP had anti-cardiolipin antibodies but no anti-dsDNA antibodies; whereas patient RT had anti-dsDNA antibodies but no anti-cardiolipin antibodies. Patient RSP yielded anti-cardiolipin antibodies but no anti-DNA antibodies, but patient RT had both specificities (Ravirajan et al, 1992). What is clear is that the technology used to produce these antibodies somehow selects for IgM secreting cells rather that IgG antibodies (see section 1.16). It has been suggested that this simply reflects the paucity of IgG anti-DNA antibodies in SLE patients (Casali and Notkins, 1989) but two studies do not support this explanation. Using an ELISPOT to enumerate the number of IgG and IgM secreting cells in the peripheral blood two groups have found a roughly equal proportion of IgG to IgM secreting cells in normal individuals and SLE patients. Klinman et al (1991) found that 20% of the IgG and 8% of the IgM secreting pool were committed to anti-DNA antibody production in patients with very active disease but roughly equal numbers were detected in the patients with less active disease. Dar and colleagues studied 16 patients and found for the group as a whole there were approximately twice the number of IgM anti-DNA secreting cells compared to the number of IgG anti-DNA secreting cells (Dar et al, 1990). IgM anti-DNA antibodies are found in the sera of SLE patients but it is not known whether they are relevant to the pathogenesis of the disease, or the

byproduct of oligoclonal activation.

There is indirect evidence to support the concept that IgM anti-DNA antibodies found in the sera of lupus patients are different from those present in normal individuals. IgM antibodies bearing a particular anti-DNA associated idiotype derived from myeloma patients did not bind to DNA, whereas those derived from lupus patients did bind to DNA (Manheimer-Lory et al, 1991a). However, this particular idiotype is encoded by germ-line structures and thus cannot be used to support the hypothesis that the idiotype positive IgM anti-DNA antibodies were hypermutated in lupus but not in the myeloma patients.

Apart from examining the monoclonal antibodies in terms of their isotype, specificity is also important. Again the circular argument regarding monoclonal antibodies as being the only source of exact information applies, but it also important to consider the techniques used to detect anti-DNA antibodies as this has been a source of controversy for several years. Debate continues as to which of the various detection methods correlates best with active SLE. Both the Farr assay and the Crithidia immunofluorescent method have been suggested. Isenberg et al (1987) found that the Crithidia test was the most specific when compared with four other methods including the Farr assay. ELISAs using dsDNA as the antigen can detect low, as well as high, affinity anti-DNA antibodies and can be used to measure the affinity of the antibody. Friguet et al (1983) has shown that by using a fluid phase inhibition ELISA to measure the affinity of monoclonal antibodies to *Escherishia coli* the results obtained are very similar to those obtained by more conventional methods such as immunoprecipitation, the equivalent of the Farr assay. This ELISA method is only strictly applicable to monoclonal antibodies but will yield an average affinity of the antibody population contained in polyclonal preparations (Nieto et al, 1984).

Brinkman et al (1991) have studied one of the most commonly used

ELISAs for detecting DNA antibodies. Since DNA does not adhere very well to the plastic of an ELISA plate an intermediate layer is commonly used, either protamine sulphate or poly-L-lysine (as in this thesis). Brinkman et al found that 18% of anti-DNA antibodies had an intrinsic affinity for the intermediate layer, nearly all of which were of the IgM isotype. Thus it is important to test putative anti-DNA antibodies against the intermediate layer in these type of assays. Whatever criticism is levelled at the ELISA it remains the only practical method for screening large numbers of hybridomas generated from SLE patients for DNA reactivity.

1.9 IS DNA THE INITIATING ANTIGEN IN THE ANTI-DNA RESPONSE?

One of the key questions regarding the production of anti-dsDNA is the identity of the inciting antigenic stimulus. A number of early attempts to induce an immune response with mammalian DNA have been unsuccessful in a variety of animal species (Stollar, 1989). However, sequence analysis of anti-DNA antibodies has shown features of antigenic selection by dsDNA (section 1.8). A number of hypotheses have been proposed to explain this. In contrast to the lack of immunogenicity of mammalian DNA, bacterial DNA can induce a strong antibody response in mice. Further, when normal mice were immunised with bacterial DNA, proteinuria and glomerulonephritis developed, though no antibodies were directed against mammalian DNA (Gilkeson et al, 1993b). Interestingly, the molecular analysis of these anti-DNA antibodies induced by bacterial DNA revealed differences from spontaneous anti-DNA antibodies in the content and location of VH CDR3 arginine residues (Gilkeson et al, 1993a). The fact that some anti-DNA antibodies cross-react with components of infectious agents such as E. Coli and that some anti-DNA antibodies are similar

in structure to that of anti-bacterial antibodies (Watts and Isenberg, 1990) support the hypothesis that foreign DNA may be the eliciting antigen. However, these antibodies may equally represent natural antibodies, providing the first line of defence against infectious agents, known to have a wide range of specificities including self antigens and not be representative of the antibodies found in active lupus. Some of the observed cross-reactive binding patterns of murine monoclonal anti-DNA antibodies have been shown to be due to the presence of DNA/anti-DNA immune complexes in the monoclonal antibody preparations (Brinkman et al, 1989). Monoclonal IgG anti-antibodies have been shown to have a much more limited range of specificities (Winkler et al, 1991).

In order to stimulate an immune response DNA may be altered by a variety of mechanisms such as exposure to UV light, a known factor in the exacerbation of the disease; or by exposure to reactive oxygen species. In a recent study Casciola-Rosen et al (1994) have shown that ultraviolet light induces apoptosis of keratinocytes and the formation of clusters of nuclear and cytoplasmic antigens at the cell surface. Monoclonal anti-DNA antibodies are known to bind DNA exposed to reactive oxygen species, sometimes preferentially (Ara and Ali, 1992; Blount et al, 1992). DNA linked to a protein may be the true antigen such as DNA-histone complexes which are found in increased concentrations in the circulation of patients with SLE (Rumore and Steinman, 1990). This type of DNA which exist as multiples of 200 base pairs is released by cell apoptosis rather than cell necrosis (Wyllie, 1980). Therefore in SLE, cell apoptosis may be induced by a variety of mechanisms, and the nucleosomes released from those cells provide the antigenic stimulus that results in somatically mutated high affinity IgG anti-DNA antibodies.

Most recently, mammalian DNA linked to a DNA-binding peptide (Fus1) derived from *Trypanosoma cruzi* has been shown to induce an anti-DNA response similar to that observed in (NZB x NZW)F1 autoimmune mice (Desai

et al, 1993). In this study, however, 9/42 of the control mice injected with Fus1 alone developed anti-DNA antibodies. Interestingly, although antibodies to ssDNA appeared in the sera of these animals relatively early in the immunisation schedule, antibodies to dsDNA often appeared more than 100 days after the first immunisation which reflects the temporal development of anti-DNA antibodies in NZB/NZW mice. Further, the molecular analysis of these immune antibodies reveal that they are similar to the autoimmune antibodies arising spontaneously in lupus prone mice (Krishnan, 1993). This observation taken together with the fact bacterial induced anti-DNA antibodies are different from autoimmune anti-DNA antibodies suggest that mammalian DNA may be more closely related to the initiating antigen than bacterial DNA.

1.10 ARE ANTI-DNA ANTIBODIES PATHOGENIC?

Most studies relating to the pathogenesis of anti-DNA antibodies have focused on the kidney. Amongst the reasons for this perhaps the most important has been that nearly all the mouse models of SLE develop severe nephritis. Investigators have used a variety of ways to support the notion of direct pathogenicity of anti-DNA antibodies. These include the circumstantial support from the fluctuation of antibody levels in tandem with lupus disease activity. More direct evidence comes from studies of the renal lesions in both murine lupus and the human disease. All classes of immunoglobulin have been found deposited in the kidney but, in general, the severity of nephritis correlates with quantity of glomerular IgG rather than with IgM (Donadio et al, 1978; Clough et al, 1980). Madaio and colleagues found that polyreactive antibodies, rather than monospecific, anti-DNA antibodies were eluted from two patients with diseased kidneys, one of whom had a concurrent renal abscess (Sabbaga et al,

1990). However, whereas the serum was affinity purified to isolate the anti-DNA antibody component, the renal eluate was not. Thus it is not surprising that the latter preparation was more cross-reactive. Of interest, 95% of these antibodies were IgG. Based on four patients, Winfield et al (1977) found that the anti-DNA immunoglobulin eluted from kidneys had a higher binding avidity to DNA than the anti-DNA fraction in the same patients' serum. In these studies the kidney was exposed to acid elution and the antibody may be denatured thereby altering its combining sites which may not reflect those initially present. Antibodies that do not bind to DNA are also present in the immunoglobulin eluted from murine lupus kidneys (Pankewycz et al, 1987). However, the presence of antibodies in diseased kidneys does not prove that the antibodies are pathogenic. For instance they may be trapped by the diseased glomerulus, or the anti-DNA antibodies may provide targets for anti-idiotype antibodies or soluble immune complexes (Goldman et al, 1982). Tsai et al (1992) have shown that polyclonal IgG anti-dsDNA antibodies can directly bind to mesangial cells and exert cytotoxic effects. Raz and colleagues demonstrated that mouse monoclonal anti-DNA antibodies and purified anti-DNA antibodies derived from patients with nephritis induced albuminuria in the isolated rat kidney (Raz et al, 1989). Interestingly, the human antibodies required complement to induce the effects whereas the murine monoclonal antibody did not. More persuasive is the observation that mouse monoclonal anti-DNA antibodies can cause renal disease when injected into normal mice (Vlahakos et al, 1992a). The study of monoclonal rather than polyclonal antibodies enables antibody specificity and pathogenicity to be precisely related. In addition, *in vivo* experiments are more likely to represent the pathological processes which occur in SLE and no equivalent *in vivo* human data exists for obvious ethical reasons. A significant minority of the anti-DNA antibodies studied by Madaio and colleagues (Vlahakos et al, 1992a) had no effect on the kidney, indicating that the

properties of anti-DNA antibodies other than its ability to bind to DNA may be important in pathogenicity. In regard to this last point some SLE patients, have high titres of anti-DNA antibodies, but no kidney disease. The anti-DNA antibodies that induced nephritis in the experiments by Vlahakos et al (1992a)had a wide range of isoelectric points challenging the notion that charge is an important factor in pathogenicity.

The mechanisms by which anti-DNA antibodies bind to the kidney has been the subject of much recent interest. There are a number of ways by which these antibodies can fix renal tissue. Immune complexes can be formed in the circulation and be trapped by the glomerulus, or the antibody can bind to antigens already present in the glomerulus or previously trapped by the kidney. DNA has been shown to be present in immune complexes in diseased renal tissue (Malide et al, 1993). Although DNA has been found in the circulation of patients with SLE it appears to be present in relatively small amounts. Anti-DNA antibodies may bind to a variety of renal antigens not directly but via histones. Brinkman and colleagues have suggested the concept of a bridge linking anti-DNA/histone antibodies to the glomerular basement membrane via heparan sulphate which is the major glycosaminoglycan constituent of the basement membrane (Brinkman et al, 1990). The binding of mouse monoclonal anti-DNA antibodies to glomerular mesangial cells is facilitated by histones (Chan et al, 1992). Mouse monoclonal anti-DNA antibodies also cross-react with other organ tissues such as skin and brain (Aiba et al, 1989; Amital et al, 1989).

Two relatively new mouse models, transgenic and SCID mice, have facilitated the further research of the pathogenicity of anti-DNA antibodies. Transgenic technology allows the introduction of genetic material coding for anti-DNA antibodies that are thought to be pathogenic in other mouse models. Thus Tsao and colleagues utilised the genomic DNA of the heavy and light

chain of an antibody derived from an NZB/WF1 mouse that can induce nephritis when injected into normal mice (Tsao et al, 1992). This gene was inserted into B cells of normal mice which did not result in the deletion of the B cells expressing the transgene. In addition, the mice had moderate levels of anti-DNA antibodies and a mild nephritis compared to (NZB/W) F1 mice of a similar age. Thus the expression of a pathogenic immunoglobulin is sufficient for pathology, although why the 'full blown disease' did not occur is not clear. Weigert and colleagues found that inserting DNA coding for the heavy chain of an IgM anti-DNA antibody did not result in expression of this immunoglobulin on B cells or secretion of the antibody (Erikson et al, 1991). This conflicts with the previous report only in terms of tolerance, not with respect to antibody pathogenicity since the IgM anti-DNA antibody was not shown to be pathogenic in the host mouse. An alternative explanation is that the isotype of the transgenic antibody is important. It may be that B cells that have moved beyond the IgM stage are resistant to toleragenic signals. B cells that never expressed IgM (because they possess an IgG transgene) are not encountered in the developing immune system.

The results of experiments involving transgenic mice can be difficult to interpret. In the paper by Tsao the surface expression of the transgenic antibody was low allowing these B cells to escape anergic mechanisms, while the serum level was comparatively high; moreover allelic exclusion did not occur as shown by.both the transgene and the endogenous heavy chain being expressed. The construction of transgenic mice can create a set of completely non-physiological conditions, which the investigator is obliged to study and thus becomes enmeshed in trying to understand the effects of the transgene. Moreover, all the transgenic experiments performed to date on anti-DNA antibodies have used a system where the "pathogenic" antibody was present in the primary repertoire of the host *ab initio*. This is not the case in SLE where

pathogenic antibodies arise at a later date and may be the consequence of antigenic selection. The tolerance mechanisms that work at this later stage may not be the same.

The severe combined immunodeficiency (SCID) mouse has been used recently to dissect further the pathogenesis of autoimmunity. This strain of mouse has no functioning B or T cells and thus is unable to reject foreign allografts. The immune deficiency is due to a defect in DNA repair resulting in the failure to correctly rearrange immunoglobulin and T cell receptor gene segments. It has been demonstrated that SCID mice can be successfully engrafted with human peripheral blood lymphocytes (Mosier et al, 1988). Although the occurrence of classical graft vs host disease has not been noted, more subtle graft vs host phenomena may occur. This complicates all experiments examining autoimmune disease since there are immunological and clinical similarities between autoimmune disease and graft vs host disease. This concern notwithstanding the SCID mouse does allow the precise study of lymphoid populations which may be involved in autoimmunity without interference by lymphocytes from the recipient.

The use of SCID mice allows for the analysis of both the mouse models of SLE but also human lymphocytes derived from lupus patients. Initial attempts have focused on the complete reproduction of SLE in these animals. However, when peripheral blood lymphocytes were injected into SCID mice an SLE like syndrome did not occur. Elkon and colleagues found that although the autoantibody profile was similar between the patient (donor) and the mouse (recipient), histological analysis of skin and renal tissue revealed no abnormality (Ashany et al, 1992a). However amongst the ten patients from whom the successful grafts were obtained none had renal disease associated with anti-DNA antibodies. Duchosal et al did not observe the histological changes of glomerulonephritis in SLE-SCID chimeras although they did detect

IgG and C3 deposition in the kidneys of 3/7 mice by immunofluorescence (Duchosal et al, 1990). The presence of anti-DNA antibodies in the patients or mice recipients was not unfortunately reported in this paper, relying instead on ANA results. Two (out of fifteen) of the mice in this latter study eventually developed a human lymphoma illustrating the artificial environment of the SCID mice and perhaps providing a link with graft vs host disease. Interestingly, when MRL/lpr splenocytes were injected into SCID mice, autoantibodies, acute graft vs host disease or a wasting syndrome was produced depending on the source of the cells (Ashany, 1992b). This suggests that graft vs host disease does not depend on functional B or T cells in the host. The induction of graft vs host disease is known to suppress spontaneous murine lupus (Ito et al, 1992) and this may explain the lack of success of reproducing lupus in SCID mice.

1.11 IMMUNOGLOBULIN GENES USED TO ENCODE ANTI-DNA ANTIBODIES

The genes encoding many murine and human monoclonal anti-DNA antibodies have been determined. There does not appear to be a unique germ line gene which encodes for anti-DNA antibodies. Further, there is little evidence for the anti-DNA response to be restricted to a few germ-line genes. Indeed, the only germ-line gene that appears to be over-utilised in an autoimmune response is the VH4-21 germline gene in cold agglutinin disease (Pascual and Capra, 1992). However, a number of germ-line genes appear to be overexpressed in the normal antibody repertoire and this should be considered when examining the autoimmune repertoire (Schwartz and Stollar, 1994). Amongst inbred strains of autoimmune mice in which one might expect to see a gene bias more clearly than in human SLE, the gene utilisation is roughly proportional to the expressed repertoire. Although there is an

increased expression of the J558 gene in the anti-DNA antibody population in SLE prone mice, this gene is also utilised in a number of other (auto)immune responses (Foster, 1991). This is not necessarily surprising since this is the largest family of VH genes in this animal. Shlomchik et al (1990) have found that some 15-20 VH genes dominate the anti-DNA response in MRL/lpr mice. This number is similar however to observations regarding the response to foreign antigens. More recently, Klinman and colleagues have bypassed the necessity to generate monoclonal antibodies, instead growing single B cells on nitrocellulose membranes and determining gene utilisation by hybridisation. Individual VH families contributed to anti-DNA production at a frequency roughly proportional to their representation in the expressed repertoire (Klinman et al, 1993). The fact that these B cells were stimulated with lipopolysaccarides may represent an artificial expansion of certain clones, but as no bias was observed this may not be relevant.

An alternative suggestion that certain combinations of VH, D and light chain genes are pathogenic has also been considered but these rearrangements are evidently random and the immune disorder in SLE may be the inability to down regulate these combinations once they occur (Diamond et al, 1992). Although the repertoire of anti-DNA antibodies may not be restricted, the number of V genes that encode the pathogenic subset may be. This is supported by the observations of Madaio and colleagues that an identical or highly related VH gene is used to encode a subgroup of murine lupus autoantibodies that share immune deposit forming properties (Katz et al, 1993).

Information regarding human gene bias in the production of anti-DNA antibodies is far more limited. There appears to be no restriction in the VH gene usage in the IgM monoclonal antibodies studied (Isenberg et al, 1994). There have been only a few reported sequences of IgG monoclonal anti-DNA antibodies. Only two of these studies have selected the antibodies on the basis

of antigen binding (van Es et al, 1991; Winkler et al, 1992), the other antibodies were generated on the basis of idiotypes thus introducing a bias into the system as some idiotypes may represent VH germ line genes. These two studies have produced one antibody that utilises a VH4 gene and six that utilise a VH3 gene. More IgG anti-DNA antibodies need to be generated to determine if a gene bias exists. All of these results are consistent with the fact that genetic susceptibility to lupus has not been linked to an Ig locus, and as yet no restriction fragment length polymorphism (RFLP) in the Ig locus has been definitively demonstrated to associate with lupus like disease in mice. There is a suggestion that a VH gene RFLP may associate with lupus and RA in humans (Olec et al, 1991). It is intriguing that in the unaffected relatives of SLE patients there is over expression of some anti-DNA associated idiotypes. Further information on immunoglobulin gene utilisation in humans comes from analysis of idiotypes.

1.12 IMMUNOGLOBULIN IDIOTYPES

As well as defining antibodies in terms of their antigenic specificity, antibodies can also be identified by other antibodies recognising the structures associated with the antigen binding region: the variable region of an antibody is antigenic (Figure 1.1). Individually these antigenic determinants are known as idiotopes and collectively on the antibody they comprise the idiotype. With the concept of idiotypes in mind, Niels Jerne put forward a network theory (Jerne, 1974). He reasoned that the idiotype on one lymphocyte (Id1) would recognise and react to the anti-idiotype on another lymphocyte (anti-Id1) which in turn could react with another antigen receptor anti- anti-Id1. The anti-anti-idiotype could be the internal image of the antigen. This process could in theory go on indefinitely forming a network of interactions that could up or down regulate the initial antigen response or initiate new immune responses. However there is

evidence that many of the idiotypes of an antibody response are shared or public and that the extent of the network is limited by these commonly occurring motifs. The fact that private idiotypes have seldom been reported may be due to a number of reasons. Although theoretically any combination of epitopes on an antibody molecule could make up an idiotype, the structures that are involved are those that are recognised by the immune system. Cross-reactive idiotypes may be immunodominant merely because a number of antibodies express the idiotype and thus act as a greater antigen stimulus during antigen development. The means by which idiotypes are detected i.e. through the xenogeneic immune response of an animal, may not correspond to those idiotypes recognised in an autologous immune response. It can not be discounted that anti-idiotypes recognising private idiotypes are not as "useful" reagents and thus have been less well studied. Some of the public idiotypes that occur represent germ line encoded antibodies and are observed most frequently in the fetal repertoire where the antibody repertoire is highly connected (Kearney et al, 1992). This is not wholly surprising since many of these immunoglobulins bind to self antigens, including immunoglobulin V regions, and are highly cross-reactive. In other words the self reactivity of these fetal antibodies includes the host immunoglobulin V regions. As the immune system matures the repertoire exhibits much less connectivity.

Jerne's theory raises a number of paradoxes which have led some authors to question the validity of the network theory (Langman and Cohn, 1986). For instance, Jerne proposed that some idiotypes bear "internal images" of antigens including foreign antigens, yet the idiotypes are not attacked in the same way as foreign antigens are. The immune response does not appear to eradicate the idiotypes as it would for foreign antigens, but to establish an equilibrium of activity between the idiotype bearing cells. How important is the idiotype network and the associated ideas relating to immune regulation when

compared to the overriding influence of antigen control? There would be little point in idiotypes and anti-idiotypes "squabbling" over control of the response after the antigen has been eliminated. This argument would apply in the case of foreign antigens, but self antigens are not eliminated. In addition, self antigens such as DNA are poorly immunogenic and a peptide, or, perhaps more likely, a group of peptides that possess an "internal image" may induce a stronger response than the self antigen.

The definition of an idiotype has been recently re-examined by Jefferis (1993). He maintains that some epitopes on the variable regions of antibodies are markers of germ-line V genes, thus these would be called isotypes rather than idiotypes (e.g. the 16/6 idiotype, section 1.13). As will be discussed later the correlation between germ-line genes and the idiotypes that are supposed to be their markers is not absolute. Previously, idiotypes such as 16/6 were identified as cross reactive idiotypes but Jefferis argues that every person would have these epitopes and thus he identifies them as isotypic markers. This raises a number of issues. For instance, would there be an antibody response to epitopes common to all individuals and, taking this point further, does the idiotype network involve the regulation of isotypes? In addition can isotypes be part of the antigen binding site? In contrast, Kazatchkine and Coutinho (1993) propose that the definition of idiotypes should not be based on structural considerations at all. Further, they suggest that individual idiotypes are of less importance than the complete idiotype network itself and that autoimmunity is due to the perturbation of the idiotype network. The data used to support this contention centres around the therapeutic use of infusing pooled human immunoglobulin. Their conclusion was that there is no difference between natural and pathogenic autoantibodies and that the only mechanism present to prevent all forms of pathogenic autoimmunity is the idiotype network. Their hypothesis does not take into account the contribution of antigen to the

regulation of autoantibodies.

Despite the controversies regarding the "definition" of idiotypes/idiotopes they are in reality defined by the reagents used to identify them i.e. anti-idiotypic antibodies. These reagents are commonly generated by injecting the antibodies carrying the idiotype into another species (in the case of human idiotypes) but may be produced from the same species when studying mice idiotypes. The different properties ascribed to idiotypes may be due to differences in the anti-idiotypes themselves. The immune responses initiated may not reflect those occurring when antigen is the inciting stimulus; only by studying natural anti-idiotypes can this hurdle be overcome. The idiotype associated with an antibody is not necessarily composed of the whole antigen combing site and that two idiotypes identified on the same antibody may not overlap at all (e.g Watts et al, 1991). This has important implications since the same antibody may be under the regulation of two anti-idiotypes. Another important consideration, particularly with regard to human idiotypes is the source of the idiotype itself. Affinity purified antibodies derived from serum and monoclonal antibodies have been utilised. The former have the advantage that the antibodies produced are relevant to the immune response of the particular antigen. The production of monoclonal antibodies, however, allows the precise delineation of the idiotype of a particular antibody in genetic and structural terms.

At least two types of idiotypes can be identified in relation to the antigen binding site. Idiotypes can be located at the antigen combining site or in the framework region. Idiotypes may represent aminoacid sequences on the heavy and light chain alone or in combination. Much research has been directed towards establishing the molecular structure of idiotypes (reviewed in Greenspan and Bona, 1993). Three approaches have been used: 1) Low resolution mapping such as competitive assays for idiotype expression; 2)

amino acid sequence analysis of Ig molecules reactive with anti-idiotypic antibodies; and (3) X-Ray crystallography of an Id Fab-anti-Id Fab complex. General conclusions from these studies are that 1) single amino acids can have dramatic effects on idiotype expression; 2) amino acid residues in both the framework and hypervariable regions can contribute to idiotypes; 3) residues that do not have an effect on idiotype expression alone may affect other residues that are involved in idiotype expression. One of the first reports regarding crystallographic studies of anti-Id/Id interaction is that of Fab D1.3 (specific for hen egg lysosyme) and Fab E255 (specific for D1.3) (Bentley GA et al, 1990). Thirteen residues form the contact between the two Fab fragments, all six CDRs of the anti-Id and five of the Id as well as one framework region from each Fab molecule are involved. There is some overlap between these residues and those which combine with antigen. Whether all three methods will yield the same results has yet to be confirmed. One confounding factor may be the T cell receptor, which is thought to be involved in idiotype interactions, and recognises processed peptides which may not correspond to structures on intact immunoglobulin molecules. Further, the T cell may stimulate production of anti-idiotypes that do not react with the intact immunoglobulin, but with linear peptides. These antibodies would be detected by epitope mapping of linear peptides, as has been used for the analysis of some anti-DNA antibody associated idiotypes (Staines et al,1993b), but not necessarily with intact Fab fragments.

1.13 ANTI-DNA ANTIBODY IDIOTYPES

Approximately 20 different anti-DNA associated idiotypes have been defined on human DNA binding antibodies, some of which are shown in Table 1.5. The majority of anti-Id reagents have been generated using monoclonal

Table 1.5 Common DNA antibody idiotypes

Id	Source	Found in tissue lesions	Location of Id	Special characteristics	% of SLE pts. +ve for Id	% of normals +ve for Id
GNs*	Glomerular eluate NZB/NZW mice	++ (G)	H chain	Dominant Id on glomeruli of patients with DPGN	67	13
16/6	MAb anti-DNA SLE pt.	++ (G,S)	H chain	VH-26 encoded	30	7
3I*	Polyclonal anti-DNA,SLE pt.	+	k chain	3I+ anti-DNA enriched in cationic IgG	50	7
F4*	Polyclonal anti-DNA,SLE pt.	?	H chain	Expressed only by IgG	35	?
BEG-2	MAb anti-DNA	No	lambda chain		8	7
PR-4	MAb anti-DNA leprosy pt.	?	confor - mational	Present in pts. with RA, leprosy and T.B.	70	3
9G4*	MAb cold agglutinin	+ (G)	H chain	VH-4.21 encoded idiotope	45	2

*= monoclonal rather than polyclonal anti-Id

anti-DNA antibodies, some have been produced using affinity purified antibodies from the serum of SLE patients. In addition, some murine anti-DNA antibody associated idiotypes have been detected on human anti-DNA antibodies. All of the human monoclonal antibodies used have been of the IgM isotype raising again the question of relevance to pathogenic antibodies in SLE. Few of these monoclonal antibodies have been specific for DNA, binding also to cardiolipin and other polynucleotides. Equally the affinity purified preparations may contain cross-reactive antibodies whose major specificity is not for DNA. Polyclonal anti-serum have been used to define most of these idiotypes though mouse monoclonal antibodies have been used to define 16/6, 32/15 and 8E7 and the affinity purified human anti-DNA antibodies 3I, 8.12, and F4.

The reasons for studying idiotype structure are several. Common DNA antibody idiotypes may be markers for, or unique to, pathogenic antibodies. They may provide clues to the stimulus for the production and genetic origins of these autoantibodies. As discussed earlier, it is by no means clear that DNA is the stimulus for the production of anti-DNA antibodies; idiotypes may provide links with other antibodies which bind to antigens that may be trigger factors. For instance, immunisation of normal and lupus prone mice with anti-Id antibodies carrying the internal image of a foreign antigen can activate self reactive antibodies bearing the same idiotype (Bailey, 1989). Idiotypes may also be useful targets for therapeutic intervention. Since SLE is such a diverse disease, or perhaps a number of diseases, there may be more than one, and probably many, disease associated idiotypes.

The assays used to detect the idiotypes have varied widely. In order to screen large number of sera ELISA systems are invariably used. Inherent in these detections systems is that an all or nothing response is very rarely observed, therefore a large number of controls have to be present in each

ELISA. Most ELISAs utilise the anti-Id coated directly onto the ELISA plate or the anti-Id is used to probe antibodies already bound to the plate. A close correlation between the two assays was found in a study of the PR4 idiotype (Williams et al, 1988). Additionally, Cairns and Rauch found little variation in results when the same ELISA method was used to quantify one idiotype in two laboratories (Cairns et al, 1992).

Quantitation of idiotypes in sera has been performed in a number of different ways. These have included comparing the readings to a serum positive control, to the purified idiotype (only possible where the idiotype was derived from a monoclonal antibody), to the purified idiotype diluted in normal serum, and a simple optical density reading. The "best" method is open to debate but it probably does not matter if the only issue is whether the idiotype is elevated compared to normal controls. Results by Mannheimer-Lory demonstrate that it may not be correct to believe that comparing serum ELISA readings to those derived from the monoclonal idiotype positive antibody would yield the exact "amount" of idiotype present in the serum (Manheimer-Lory et al, 1991a). They generated monoclonal antibodies which reacted with two idiotypes, 3I and F4, both of which were produced using polyclonal anti-DNA antibodies derived from lupus serum. The idiotype positive monoclonal antibodies diluted at the same immunoglobulin concentration yielded different optical densities when tested in the same idiotype ELISA. These conclusions can also be applied to monoclonal antibodies which carry the 16/6 idiotype. Out of nine monoclonal antibodies that were considered strongly 16/6 Id positive there was a 30 fold difference in their binding activity in the fluid phase as measured by ELISA (Young et al, 1990). Staines et al (1993) have looked at seven public mouse idiotypes and found that while each anti-Id reagent tended to be strongest with its homologous monoclonal antibody this was not always the case. Watts (1991) found very similar results when comparing serum

idiotype levels with a serum positive control and with the homologous monoclonal antibody (BEG-2) as a reference.

Examination of the many anti-DNA associated idiotypes yields a number of general conclusions. The 16/6 idiotype, one of the first anti-DNA associated idiotypes to be described, illustrates some of these well. Isenberg and colleagues demonstrated that the serum levels of this idiotype were higher in patients with active SLE compared to inactive disease and correlated in some patients with disease activity (Isenberg et al, 1984b). The 16/6 idiotype has been found in tissue lesions (Isenberg and Collins, 1985). It was also noted that the 16/6 idiotype was not disease specific being present in 25% of rheumatoid arthritis patients and 4% of controls. The 16/6 idiotype is present on conventional antibodies as well as on other autoantibodies (Kaburaki and Stollar, 1987). The 16/6 idiotype is not confined to a specific immunoglobulin class being present on both IgM and IgG antibodies (Shoenfeld et al, 1986). It has been proposed that the 16/6 idiotype represents a germ line encoded antibody being expressed by products of the VH26 germline V gene (Young et al, 1990). This study is based on EBV transformed cell lines which produced 16/6 idiotype positive IgM monoclonal antibodies. All but one of the 16/6 Id positive monoclonal antibodies were derived from the VH26 germline V gene indicating that the association of 16/6 idiotype with VH26 is not absolute (see below). Interestingly, whereas the interaction of the prototypic 16/6 positive antibody, 18/2, and anti-Id was inhibited by ssDNA, the interaction of another 16/6 positive antibody to anti-Id was not inhibited by antigen. This implies that, despite the fact that the antibodies have very similar binding curves to DNA, they bind to DNA in different ways. An alternative suggestion is that the 16/6 idiotype is not one entity and may have different structural correlates on different antibodies. Some of these structures may not even be observed as the use of EBV may bias the selection in favour of germ-line genes as suggested by the

fact that the majority of the 16/6 idiotype positive monoclonal antibodies isolated so far are of the IgM isotype (see section 1.13).

There are a number of idiotypes that differ from the 16/6 idiotype. Weisbert et al (1990) described a murine idiotype which appeared more specific for SLE and demonstrated a cross-species conserved idiotype associated with pathogenic anti-DNA antibodies. The idiotype is recognised by a monoclonal antibody (1C7) and was present in 9 out of 12 patients with anti-DNA antibodies who had nephritis, in 3/12 patients with anti-DNA antibodies without nephritis, in 1/53 SLE patients without DNA antibodies and 1/47 healthy controls. The 9G4 idiotope first described on cold agglutinins was significantly raised in patients with SLE but was rarely found in other autoimmune rheumatic disorders (Isenberg et al, 1993). The 9G4 idiotope appears to be a marker for the VH4-21 gene and maps to the FR1 region of this heavy chain (Potter et al, 1993). The F4 idiotype was found almost exclusively on IgG antibodies but was present on non-DNA binding antibodies as well as those that bind DNA (Davidson et al,1989). Idiotypes found purely on IgG antibodies may be composed of epitopes created by somatic mutation. Alternatively, these idiotypes may be associated with an IgG response, perhaps a V gene that is used late in the immune response. Seven anti-DNA antibody idiotypes including BEG-2 and PR4 were not found to be elevated in the sera of SLE patients and in a multicentre comparison of 18 different anti-DNA antibody idiotypes, the most common disease in which the idiotypes studied were raised was Sjogren's syndrome (Isenberg et al, 1990).

Taken together these observations refute the notion that anti-DNA antibodies derive from a separate set of variable genes to other antibodies, in agreement with the analysis of the actual VH gene bias. As mentioned above, the anti-DNA response does not seem to be distinguishable from any other antigen response in terms of its gene usage. When normal human B

lymphocytes were stimulated, 16/6 Id positive antibodies were produced but only a minority bound to DNA. In contrast, amongst the lupus patients in relapse that were studied, over 90% of the DNA antibodies were 16/6 idiotype positive (Datta et al., 1986.) The data from SLE relatives may support abnormal gene usage in immune response of SLE patients themselves. Twenty four percent of SLE relatives expressed the 16/6 idiotype as compared to 0-5% of healthy controls (Isenberg et al, 1988).

Recently, Schwartz and colleagues examined the distribution of the VH 18/2 gene (also called VH26) from cDNA libraries and found that it was equally elevated in two normal individuals as compared to one patient with SLE (Stewart et al, 1993). The SLE patient had raised levels of the 16/6 idiotype whereas presumably the normal individuals had normal levels. Schwartz concludes from this limited study that the elevated levels of 16/6 which have been observed in SLE patients can not be explained by the increased frequency of a VH gene that was thought to encode for it. Obviously these studies need to be extended but the simple equation of observed idiotype levels equalling VH gene expression does not in the 16/6 case seem to hold true. Further analysis of 16/6 idiotype monoclonal antibodies is needed to explain these contradictions. The simplistic explanation that the observed elevation of idiotype levels in patients with SLE is due to generalised polyclonal activation which accentuates the normal bias of VH-26 encoded antibodies is also unlikely to be correct. The original paper describing the 16/6 idiotype indicated that there was no correlation between immunoglobulin and idiotype levels (Isenberg et al, 1984b). Most subsequent papers have taken immunoglobulin levels into consideration, although whether the immunoglobulin levels were measured in the same serum sample from which the idiotype level was calculated has not always been clear. The elevation of idiotype levels may however be due to a more oligoclonal activation of B cells. It has been shown

that the hypergammaglobulinaemia in SLE is not truly polyclonal in that certain specificities have not been found in SLE sera (see section 1.7). It is possible for instance that B cells producing natural autoantibodies are selectively activated in SLE and this could explain the elevation of idiotype levels seen. To continue this argument to its logical conclusion would lead to the idiotype network theory, i.e. certain idiotypes are expanded in autoimmunity. Put another way, the expansion of cross-reactive idiotypes might represent a very limited oligoclonal activation of B cells.

In conclusion, not all the idiotypes described represent products of a single VH gene, some idiotypes can be generated from a number of VH genes and may represent a common structural motif that is used more frequently by anti-DNA antibodies. These may include the presence of arginine or asparagine that are thought to enhance the binding to DNA. Elevation of idiotypes do not necessarily reflect VH gene bias. Moreover, there has been a selection bias in the human idiotypes generated so far which would predispose to the identification of germ-line genes. All of the human monoclonal antibodies from which the idiotypes have been derived have been of the IgM isotype which are less likely to have extensive somatic mutations. It is not surprising that idiotypes that represent germ-line sequences are also found on IgG antibodies, e.g.16/6, 9G4, since the switch to IgG may very well maintain most of the epitopes of the variable region, particularly if the idiotype is situated in the framework region. The somatic mutations that do occur would most likely be in the hypervariable regions. However, as there is evidence that somatically mutated IgG antibodies are more likely to be pathogenic in lupus in contrast to natural autoantibodies, the idiotypes associated with mutated regions might be more relevant to the disease process and the initiating (or stimulating) antigen. Identifying idiotypes that represent mutated regions is more likely to be achieved by utilising monoclonal IgG anti-DNA antibodies. Whether this would

lead to the identification of private or unique idiotypes is not known.

Early hopes that idiotype measurements would add clinically useful information have in general not been realised. Only a few of the DNA associated idiotypes correlate with disease activity, e.g. 9G4, 16/6 and GN-2 idiotypes which do show some correlation with global disease activity (Isenberg et al, 1990; Isenberg et al, 1993). The fact that an idiotype may switch from a DNA binding antibody to another antibody of unknown specificity would be one reason why most idiotype levels do not correlate with disease activity. However, different idiotypes may reflect disease activity in different organs. Suenaga et al (1989) have demonstrated a spontaneous idiotype shift in the anti-DNA antibodies found in a single patient over a four year period. DNA antibodies associated with nephritis expressed a different idiotype compared to the idiotype found during an episode of cerebritis. Neither idiotype was present during a remission. Idiotypes may also be able to switch from IgM to IgG antibodies accompanied by a change in pathogenicity or association with disease activity. In a study of myeloma proteins the 3I idiotype was expressed on antibodies that bound DNA predominantly when the anti-DNA antibody isotype was IgG (Davidson et al, 1987) although this was not confirmed when examining monoclonal antibodies derived from lupus patients (Manheimer-Lory et al, 1991a).

One area where idiotypes are thought to be important in providing evidence of aetiology is the common idiotypes observed in patients with SLE and infection. Several examples exist of anti-DNA antibodies binding to a variety of bacterial antigens. However, the majority of these may represent natural autoantibodies which are thought to provide a first line of defence against foreign antigens. Thus, it is not surprising that they bind infectious agents. Some monoclonal antibodies against epitopes on *Mycobacterium tuberculosis* also react with DNA and carry the 16/6 Id (Shoenfeld et al,1986).

The PR4 monoclonal antibody derived from a patient with leprosy binds both ss and dsDNA and *M. leprae*, and the PR4 idiotype is detectable in leprosy and SLE (Williams et al, 1988; Zumla et al, 1991). Autoimmunity does occur in diseases such as leprosy and the elevation of these idiotypes may reflect this, rather than providing an aetiological link with autoimmune rheumatic diseases such as SLE. In other words these idiotypes may be elevated wherever autoimmunity occurs whether it be in diseases such as SLE or infection. Similarly idiotypes found on autoantibodies are also expressed on antibodies that bind foreign antigens without cross-reacting with an autoantigen (Grayzel et al, 1991). This may simply indicate that an autoimmune response uses the same antibody repertoire as any other antibody response.

1.14 MANIPULATION OF THE IDIOTYPE NETWORK IN SLE.

In the previous section the description of idiotypes has been based on observations regarding their distribution in the serum of individuals, and the genetics and specificity of idiotype positive antibodies. In addition some idiotypes such as the 16/6, GN-2 and 9G4 idiotype, have been detected in the diseased tissue of patients. These only provide limited evidence of pathogenicity i.e. that antibodies with one particular idiotype rather than another are causing disease, which is open to a number of criticisms as mentioned above. Ostensibly far more persuasive evidence was provided in experiments performed by Mozes and colleagues. Mice which were injected with an anti-DNA antibody bearing the 16/6 idiotype developed an SLE like disease and the full range of autoantibody specificities seen in SLE including anti-DNA antibodies (Mendelovic et al, 1988). Mice immunised with anti-DNA antibodies lacking the 16/6 idiotype remained healthy. Despite intensive efforts to

reproduce these results in several other laboratories experiments have failed to induce autoimmunity in normal mice using the same anti-DNA antibody (Isenberg et al, 1991; Williams and Isenberg 1994). One group did provide some independent support for the existence of the "16/6 model" by injecting BALB/c mice with affinity purified anti-DNA antibodies from the serum of an SLE patient [unfortunately it was not documented whether the patient had nephritis] (Tincani et al, 1993). Although the DNA binding fraction was 16/6 Id positive, the 16/6 positive antibodies were not further purified. Consequently, there may have been a whole variety of DNA associated idiotypes present which may have enhanced the autoimmune phenomena. In addition, this group did not use an adjuvant control. In one of the two experiments they performed they were unable to see immunoglobulins in the skin or kidney and they did not find the same range of autoantibodies in the sera of the animals reported in the original description of the lupus model. Surprisingly mice immunised with anti-tetanus toxoid antibody (as a control) did not show any DNA-binding activity but developed high titre anti-cardiolipin antibodies. The use of a polyclonal preparation complicates the interpretation since the antibodies are inevitably more heterogeneous than a monoclonal antibody.

Pucetti et al (1990) have shown that immunisation of normal mice with the light chain of an idiotype bearing antibody which shares sequence homology with the 70kD ribonucleoprotein can induce antibodies to this protein as well as ssDNA and dsDNA. No comment was made as to whether the animals remained healthy.

A number of environmental factors may be responsible for these discrepancies such as the food eaten by the mice or viral agents present in the animal house. There are a number of examples of gut floras influencing autoimmune diseases. For instance, colonies of transgenic experimental allergic encephalomyelitis mice also varied in their susceptibility to disease

depending on whether they were in a clean or conventional facility subject to infections, only the latter group developed spontaneous disease (Goverman et al, 1993).

A number of investigators have shown that manipulation of the idiotype network can slow disease progression in lupus susceptible mice. Weisbert et al (1990) has shown that immunisation of MRL/lpr mice with a monoclonal anti-DNA antibody leads to improved survival accompanied by a decrease in autoantibodies and the severity of nephritis. Injection of anti-idiotypes have also been performed to try and reduce the autoimmune response. Two important principles have emerged from these studies. Firstly the timing of the immunisations was important. An anti-idiotype administered to older lupus prone mice ameliorated the autoimmune disease, whilst the same anti-idiotype injected into younger mice increased the levels of autoantibodies (Ebling et al, 1988). Secondly, whilst it was relatively easy to produce short term modulation of autoantibody expression in experimental animals, long term suppression does not seem to occur. Hahn and colleagues (Hahn et al, 1983; Ebling et al, 1988) have shown that when one idiotype bearing autoantibody is suppressed by its homologous anti-idiotype the disease remits for a short time but subsequently another anti-DNA antibody bearing a different idiotype emerges and the mice rapidly die from accelerated nephritis. An alternative approach using anti-idiotypes linked to neocarzinostatin lead to suppression of disease in NZB/W mice but unfortunately the experiment was terminated before any potential idiotype escape mechanism could have occurred (Harata et al, 1990). Preliminary data suggested that the mice survived for an extra 12 weeks. A great deal more knowledge is needed regarding idiotype manipulation of networks before this approach could be tried in humans.

1.15 MONOCLONAL ANTIBODIES

The description of somatic cell hybridisation techniques to make antibody secreting B cells by Köhler and Milstein in 1975 (Köhler and Milstein, 1975) represents one of the great advances of technology in biomedical sciences. This process produced monoclonal antibodies which could be studied without the interference of other antibodies present. Monoclonal antibodies provide the only precise way of studying the specificity and structure of antibodies. Ideas regarding cross-reactivity and molecular mimicry are central to the aetiology and pathogenesis of autoimmune disease and the link with infectious agents. These ideas cannot be accurately investigated without monoclonal antibodies. Much of the information in this introduction has been based on work using monoclonal antibodies. The recent advances in molecular biology have opened up new avenues of research into monoclonal antibodies enabling immunoglobulin sequences to be derived. As mentioned earlier the Achilles' heel of the analysis of monoclonal antibodies is whether they are relevant to the disease process or reflect natural, physiological autoantibodies. To some extent this question is unanswerable since it is not known precisely what the "relevant" antibodies are in SLE. However, some data exist on whether the production of monoclonal antibodies represents a random or biased selection of B cells.

The first monoclonal antibodies were derived from rodents and the methodology became established quickly. The technique utilises an immortal cell line (fusion partner) which is fused with B lymphocytes (mortal) derived from a variety of sources using a 'fusogen' such as polyethylene glycol. The resulting hybridoma secretes the antibody specificity of the B cell continuously in culture. The unfused fusion partner is selected out by using medium

containing hypoxanthine, aminopterin and thymidine (HAT) to which it is sensitive. This sensitivity is due to the absence of the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) which prevents salvaging of nucleotide metabolites. The hybrid cells, using the metabolism of the B cell which has HGPRT, are able to bypass the otherwise lethal block imposed by the aminopterin which prevents the *de novo* synthesis of nucleotides. Whereas the fusion partner used in the original description secreted its own immunoglobulin chain, a number of non-secreting plasmocytomas have been utilised. Since the first description, mouse hybridomas have been successfully developed and used in a variety of situations including the production of monoclonal antibodies against immunogens, infective agents and autoantigens. The combination of a successful mouse fusion regime and a number of murine models of SLE has lead to the dissection of the anti-DNA response in these animals. However, the inadequacies of murine models of SLE are parallelled by the difficulties of generating human monoclonal antibodies.

1.16 HUMAN MONOCLONAL ANTIBODIES

The first reported production of human monoclonal antibodies did not use hybridoma technology. Steinitz et al (1977) used Epstein Barr Virus (EBV) to immortalise B cells and produce anti-tetanus toxoid antibodies. EBV binds to B cells via the receptor CR2 and immortalises a small percentage. Olsson and Kaplan (1980) produced the first human monoclonal antibody using the somatic cell hybridisation technique. Both methods have been beset by problems. EBV transformed cell lines are notoriously difficult to clone even with the use of feeder cells or conditioned medium. Further the resulting clones are often unstable and secrete low levels of immunoglobulin for only a few weeks. The use of human fusion partners has been hampered by the low fusion frequencies

yielding a small number of clones, and the slow growth and poor antibody secretion of the clones. Using one or other of these methods a number workers have managed to overcome these problems and produce monoclonal antibodies of sufficient quantities for their studies. However, the clones generated by either method have been predominantly of the IgM isotype. This point is particularly frustrating in autoimmune diseases such as SLE where the isotype associated with active disease is IgG. One cause of this may be that the source of the lymphocytes used for these studies is invariably peripheral blood. Apart from the fact that this source has many fewer B cells than the spleen, a number of other explanations have been offered to account for the poor yield from peripheral blood. These include insufficient numbers of the 'right type' of B cell, inappropriate state of B cell activation, and the presence of suppressor B cells and too many cytotoxic T cells. In one comparative study (Watts et al, 1990b) the fusion frequency of PBL was 0.1-5 clones/ 10^7 lymphocytes fused whereas the fusion of human splenocytes yielded 27 clones/ 10^7 cells.

A number of techniques have been developed to increase the number of monoclonal antibodies derived from peripheral blood and to enhance the likelihood of generating IgG monoclonal antibodies. One area that has received much attention is the search for a more productive human cell line as a fusion partner. However, despite intense efforts no laboratory has yet derived a successful purely human fusion partner. Investigators have therefore used mouse myelomas as fusion partners with human lymphocytes. It has been known for some time that this may lead to a preferential loss of human chromosomes, nevertheless the eventual yield of antibody secreting clones is greater than with purely human partners. Heteromyelomas have also been created by fusing a lymphoblastoid cell line with a mouse myeloma and then using the product as the partner for the human lymphocyte. Finally, the

combination of EBV transformation followed by somatic cell hybridisation increases the fusion frequency and the stability of the resulting cell line.

Simply increasing the fusion frequency may not overcome any biased selection of B cells which may be associated with EBV transformation or somatic cell hybridisation. On average, less than 5% of B cells are transformed with EBV when exposed to the virus (Tosato et al, 1985). EBV transformation cannot occur without the presence of the CR2 receptor to which the EBV virus binds and represents the first stage of transformation. Casali and colleagues (Casali and Notkins, 1989) found that a proportion of activated B cells, those in the S1 phase (i.e. dividing cells), lost this receptor and were thus resistant to EBV transformation as compared with cells in the G1 phase (activated, premitotic cells) and resting cells. However, the actual yields of IgM and IgG secreting EBV transformed lines reflected those found in the peripheral blood in normal individuals. Casali and colleagues conclude that the predominance of IgM secreting cells observed with this method is due to the increased number in the peripheral blood compared to IgG producing cells. Others do not concur with this. A much lower percentage of IgG+ B cells were transformed compared to IgM+ cells in studies by Åman et al (1985). Crain et al (1989) found that as well as possessing the CR2 receptor, B cells that were transformed by EBV expressed the early activation marker Bac-1. This marker is lost when surface IgD disappears which occurs when the B cell switches immunoglobulin class to IgG. As described in section 1.8 the number of circulating IgG and IgM secreting B cells are of the same order of magnitude in SLE patients and thus the preponderance of IgM producing clones would not be explained in this situation simply by a larger number of IgM secreting B cells. Casali and colleagues acknowledge that the "Lack of EBV binding to cells in S phase or plasma cells could explain the lower rate of EBV-induced immortalisation observed in cells spontaneously producing antibody in vivo, as is occasionally

the case for cells obtained from subjects with an active autoimmune disease." Binding by EBV to the CR2 receptor is only the first stage to transformation and other undetermined factors may govern whether the cells are actually transformed.

A comparison between the data obtained by EBV transformation and an ELISPOT assay using lymphocytes derived from lupus patients revealed that although similar results were obtained for IgM anti-DNA producing B cells; EBV transformation underestimated the number of IgG anti-DNA antibodies (Shibata et al, 1992). Unfortunately in this paper the data relating to anti-dsDNA antibodies as opposed to anti-ssDNA antibodies are not included and a comparison for this specificity cannot be made. In an analysis of rheumatoid factor producing lymphocytes in rheumatoid arthritis the authors found that there was no correlation between the B cells secreting RF in vitro and the number of EBV-activatable B cells secreting rheumatoid factors (RFs) (Moynier et al, 1991). The former, but not the latter, correlated with the serum RF titre. Equally large numbers of rheumatoid factors were produced by EBV transformation from rheumatoid patients and normal controls. From this study it appears that EBV was able to infect B cells producing natural autoantibodies, but not those B cells producing disease associated rheumatoid factor.

There is no doubt, however, that IgG anti-DNA antibodies are produced from EBV transformed B cells from SLE patients (Manheimer-Lory et al, 1991a), even if in smaller numbers than the number of IgG secreting cells present in the peripheral blood would suggest. The question then arises as to whether these IgG antibodies are relevant to the autoimmune response. Natural IgG autoantibodies are also known to occur and may be increased by the oligoclonal activation seen in SLE. If the conclusion that EBV does not infect actively dividing cells and plasma cells is correct, then this implies that the IgG antibodies derived may not reflect the set of "disease associated" anti-DNA

antibodies known to occur in SLE.

In contrast to EBV transformation, fusion of B cells with myeloma cells is thought to select for activated cells and thus may generate hybridomas from a pool of proliferating B cells which become plasma cells producing pathogenic antibodies (Olsson et al, 1983). In-vitro stimulation of peripheral blood B cells from normal individuals by a variety of methods increases the efficiency of hybridoma generation. This would explain the increased fusion frequency observed with EBV transformed cells which have become activated by the virus. However, which B cell population are most susceptible to fusion has not been determined.

1.17 HUMAN MONOCLONAL ANTI-DNA ANTIBODIES

The first human hybridoma derived anti-DNA antibodies were described in 1982 (Shoenfeld et al, 1982) using peripheral blood lymphocytes from a patient with SLE. This paved the way for the precise delineation of human anti-DNA antibody specificity, VH gene usage, the study of anti-DNA antibody associated idiotypes and the analyses of the antibody structure and function. The majority of human monoclonal DNA antibodies produced have been of the IgM isotype and polyreactive. Interestingly, IgM anti-DNA antibodies have also been derived from normal individuals (Cairns et al, 1984) and are indistinguishable (by specificity) from the anti-DNA antibodies derived from individuals from SLE. Thus the majority of monoclonal anti-DNA antibodies produced to date may be more representative of the natural autoantibody repertoire. It is likely that this population would be increased in SLE due to the observed limited polyclonal activation that occurs.

The first human monoclonal IgG anti-DNA antibody (van Es et al, 1991)

was produced from an EBV transformed cell line derived from lymphocytes from a patient with SLE. The sequence of the antibody and analysis of the donor patient's germ-line gene demonstrated that it was likely to have arisen through somatic mutation and bound to dsDNA. Subsequently, six IgG anti-DNA antibodies were generated from a heteromyeloma cell line known as CB-F7. These were derived from three patients with active disease, two of whom had nephritis and one with active skin disease (Winkler et al, 1991). These antibodies had high affinity for DNA and were not polyreactive. These monoclonal antibodies were only obtained because the patients had undergone leucophoresis, a technique that yields a large number of peripheral blood lymphocytes. No IgG anti-DNA antibodies were obtained by using conventional venesection to obtain peripheral blood lymphocytes, though IgM anti-DNA antibodies were produced using this technique. Interestingly, IgM anti-DNA antibodies used in that study were not derived from the patients with active disease that yielded IgG anti-DNA antibodies and it is unknown whether IgM anti-DNA antibodies are obtained using newer fusion partners from patients with active disease. In conclusion, from the limited number of IgG antibodies derived from patients with SLE it does appear that the monoclonal antibodies do partially reflect the antibodies thought to be involved in the pathogenesis of disease.

OBJECTIVES

The hypothesis underlying the work described in this thesis is that human IgG anti-DNA antibodies are relevant to the pathogenesis of systemic lupus erythematosus. The diversity of antibodies found in SLE prevents the precise study of these antibodies without producing monoclonal antibodies. The objective therefore was to generate and analyse human monoclonal IgG anti-DNA antibodies in terms of their specificity, idiotypes and biological properties. The specific objectives of this thesis were as follows:

1) To determine the most effective way of generating human monoclonal IgG antibodies from SLE patients.

2) To use the method derived from (1) to produce human monoclonal IgG anti-DNA antibodies.

3) To compare the IgG monoclonal anti-DNA antibodies produced with monoclonal IgM anti-DNA antibodies and those found in the patient's serum.

4) To analyse the structure of the idiotypes associated with these human monoclonal IgG anti-DNA antibodies and to analyse their distribution in sera and tissues from SLE patients.

5) To develop a system whereby human monoclonal antibodies can be studied in vivo and to test the biological properties of human monoclonal IgG anti-DNA antibodies by transfer in vivo.

6) To determine whether a human monoclonal IgG anti-DNA antibody can induce disease when immunised into normal mice.

CHAPTER 2: PATIENTS, MATERIALS AND METHODS

2.1 PATIENTS

There are three pertinent factors that relate to the SLE patient population used for this thesis. Firstly, did the patients fulfil the criteria for SLE, secondly what type of lupus involvement did they have and, finally, how active was their lupus at the time of venesection in each individual organ / system. All the patients studied attended the Bloomsbury Rheumatology Unit's SLE Clinic. Data regarding these patients disease activity and organ involvement had been collected by the attending clinicians and entered into a database run on an Apple Macintosh Computer as part of an ongoing clinical research project. All of the SLE patients met the American Rheumatism Association's revised criteria for the classification of the disease (Tan et al, 1982). Disease activity was assessed by the British Isles lupus activity group (BILAG) activity index (Hay et al, 1993a). This index based on the physician's intention to treat, assesses the activity of the disease in eight major organs or systems. These are haematological, general, cardiorespiratory, musculoskeletal, renal, neurological, dermatological and vasculitis. The patient is given a score which reflects the physician's intention to treat (section 5.5). The index also yields a global activity score derived from the individual organ scores which has been validated against other activity indices (Gladman et al, 1992b). Patients whose global score is above six are considered to have moderately or severely active disease. The HLA data of the patients was also documented. This information was kindly provided by Professor J.R. Batchelor (Royal Postgraduate Medical School) according to a previously described protocol (Fielder et al, 1983). In addition, the routine DNA binding and C3 levels were recorded. The former was performed by the routine immunology laboratory (Middlesex Hospital) by ELISA (Cambridge Life Sciences, Cambridge, UK) and the latter by laser

nephelometry (Biochemistry Department, Middlesex Hospital).

The period for this study was two years. The SLE patients who were selected for fusion were identified in the first eight months of this study, as the generation of monoclonals was the first goal of the work. The patients whose sera were analysed for the presence of the idiotypes were taken over a two year period. Some sera were examined from outside this period when serial studies were undertaken. All the sera were obtained from patients attending the Bloomsbury Rheumatology Unit unless otherwise stated.

Patient selection for fusion

During the first six months of this study SLE patients who attended the SLE clinic were selected by the senior clinician. Patients with the most active disease and/or those with elevated DNA binding levels were chosen.

Patient selection for idiotype screening.

SLE patients.

Sera from SLE patients that were tested for idiotype expression were selected on the basis of their BILAG scores. Sera were identified which were derived from patients who were active (only an A or a B score) in only one of the following organ or systems at a particular time: musculoskeletal (M/S), skin, renal, central nervous system (CNS), cardiovascular/respiratory (CVS/Resp) and vasculitis. These sera were identified using a database on an Apple Macintosh Computer using FoxBase. The database had been running for 2 years when the idiotype analysis was undertaken. Lists of patients were produced indicating which patients were active in one system at a particular time. From these lists sera were chosen from patients who were was active in only one system on a given date. Thus, if on one particular date both musculoskeletal (M/S) and CNS systems were active this sample was excluded. The sera were taken from patients during the two year period and

deposited in a -20°C freezer under the patient's name. This sera was being used for studies by other laboratory workers and for this reason and due to the fact that the sera were not always collected on the date required for this study, it was not always possible to analyse a particular serum sample. Separate from this analysis, serial samples from a number of patients were studied.

Rheumatoid arthritis. The sera of rheumatoid arthritis patients who met the revised criteria of the ARA (Arnett et al, 1988) were studied.

Sjogren's syndrome. The sera were from patients with primary Sjogren's syndrome who fulfilled the criteria for the disease (Isenberg et al, 1984a).

Myositis. The sera were from patients with idiopathic myositis who fulfilled the criteria of Bohan and Peter (1975).

Scleroderma. The sera were obtained from patients who fulfilled the preliminary criteria of the American Rheumatism Association (Subcommittee for Scleroderma Criteria, 1980).

Paraproteinaemia. Sera were obtained from patients with paraproteinaemia (kindly provided by Dr. A Norden, Chase Farm Hospital, Enfield).

The sera from 28 normal controls who were age and sex matched to the lupus patients derived from a number of sources (healthy volunteers).

2.2 GENERATION OF HUMAN MONOCLONAL IGG ANTI-DNA ANTIBODIES

The first objective of this thesis was to produce human IgG anti-DNA antibodies. The majority of human monoclonal antibodies have been derived from the peripheral blood lymphocytes of patients because of their availability. However, there is some evidence to suggest that other sources of lymphocytes might yield the desired clones more frequently. For instance, Watts et al (1990b) found the spleen to be the most efficient source of lymphocytes. Although splenic tissue would have been welcome none was forthcoming during the thesis period. Thus only peripheral blood lymphocytes were used for the generation of the monoclonal antibodies.

The PBL from three patients with similar BILAG global scores were used to compare three methods to generate monoclonal antibodies as outlined below i.e. direct fusion with the heteromyeloma cell line CB-F7, EBV transformation, and EBV transformation followed by fusion with CB-F7.

2.2.1 Separation of lymphocytes from peripheral blood.

Fifty-100 mls of peripheral blood were obtained by venesection from patients selected by the physician in charge of the SLE clinic and the blood was placed into heparinised tubes (10U/ml Sodium heparin, Monoparin, CP pharmaceuticals, Wrexham, UK). A few grains of sterile carbonyl iron (Sigma Chemical Company, Poole, Dorset) were placed into the tubes to remove the macrophages. After incubation at 37° C for 1 hour the tubes were placed on a magnet and the blood was pipetted off. The blood was then diluted with sterile PBS in a ratio of 1:1 and carefully layered on the surface of Ficoll-Hypaque (density: 1.077 ± 0.001 g/ml) (Nycomed, Oslo, Norway) in 50 ml Falcon tubes and centrifuged at 400g for 20 minutes. The ratio of diluted blood to Ficoll-Hypaque was 2:1. The lymphocytes were carefully harvested from the interface

between the Ficoll and the serum in the minimum possible volume, diluted with RPMI and washed in RPMI three times.

The resulting cells were suspended in 1 ml of RPMI and counted using a haemocytometer. 20 µl of the cell suspension was mixed with 20 µl of a 1:1 mixture of ethidium bromide and acridine orange. The cells were then placed in a modified Neubauer counting chamber (Gallenkamp, Loughborough, UK.) and allowed to settle for 1 minute prior to counting. The cells were counted under ultraviolet light where live cells were viewed green and dead cells were orange. All the green cells in the triple hatched area were counted. The number of cells was calculated using the formula:

Number of cells = cell counted x 1/dilution x 10^4

2.2.2 EBV Transformation

EBV transformation was achieved by incubating the separated cells with the filtered (0.45 micron, Sartorius, Epsom, Surrey) supernatant of the marmoset cell line, B95.58 (kindly provided by Dr. G. Cambridge, Department of Medicine, University College London) in the presence of 50 ng/ml of cyclosporine. The transforming capacity of the EBV supernatant was calculated by plating lymphocytes in 96 well plates (Nunc, Roskilde, Denmark) at a concentration of $2x10^5$ and incubating these cells with varying concentration of the supernatant. A 1 in 10 dilution was the minimum concentration required to consistently transform > 50% of cells. Prior to transformation the cells were plated out a density of 10^5 cells/well in 96 well plates (Nunc). After addition of the EBV supernatant at double the appropriate concentration transformants grew in each well. Each well was tested for IgM and IgG production as well as IgG and IgM anti-ss and dsDNA activity. In addition, $5x10^6$ cells of 3 patients were also incubated in flasks with supernatant of the marmoset B95.58 cell line. These cells were fused 4 weeks later with the heteromyeloma cell line CB-F7

(see below).

Subcloning of desired specificities using a similar method as described in section 2.2.5 was performed within 4 weeks of transformation using sterile 96 well plates (round bottomed wells, Nunc). To enhance the cloning potential two methods were used. The first entailed supplementing the complete medium with 20% J774 conditioned medium. The mouse macrophage line J774 (kind gift of Dr. Janice Taverne, Department of Immunology, University College London) is derived from a reticulum cell sarcoma and has been shown to produce a variety of macrophage specific factors. The line was grown in complete medium and allowed to become confluent. Supernatant from a dense culture was collected and filtered before use. The second method of enhancing clones involved the use of human feeder cells separated from the peripheral blood of normal volunteers and irradiated using a cobalt 60 source for 20 minutes. These cells were plated at a concentration of $1x10^4$ cells/ml onto the 96 well plates a day before the EBV transformed cells were subcloned.

2.2.3 Fusion with CB-F7

After separation the PBL were directly fused with the mouse human heteromyeloma cell line CB-F7 (kind gift of Dr. Siegbert Jahn, Charité Hospital, Berlin, Germany), a HAT sensitive, ouabain resistant non-secreting cell line by PEG 1500 (Boehringer Mannheim, Germany). The CB-F7 cell line was stored in liquid nitrogen until required. Thawing was achieved by transferring the freezer vial from the liquid nitrogen to a water bath at 37°C. When only a small ice clump was visible the contents were transferred to a 50 ml Falcon tube (Becton Dickinson, Lincoln, New Jersey, USA) and diluted with precooled RPMI in a stepwise fashion. The cells were centrifuged at 200g for 5 minutes, resuspended in serum containing medium (section 5.1) and plated into 24 well plates. The cells were fed when the first dividing cells were seen by changing half the medium. The cells were grown up until sufficient numbers were

achieved for fusion. Sensitivity for HAT is checked by culturing the cells in HAT medium for 24 hours and viability determined. No cells were alive at the end of this procedure as assessed by acridine orange/ethidium bromide staining.

The separated PBLs and the CB-F7 cells were simultaneously washed in RPMI three times and the cell concentration adjusted to achieve a ratio of CB-F7:PBL of 1:1. The cells were then mixed and centrifuged again (200g for 10 minutes) in 50 mls of RPMI. The liquid was decanted and the cells disturbed by tapping the bottom of the tube. 1 ml of prewarmed 50% PEG was added dropwise to every $5x10^6$ cells over a period of 1 minute. After a further 90 seconds during which the tube was tapped gently, the contents of the tube were diluted slowly with prewarmed RPMI. The cells were centrifuged again (200g for 10 minutes), resuspended in post fusion medium (section 5.1), and seeded in flat bottomed 96 well microtitre plates at a cell concentration of 10^5 cells/well, 100µl/well. The peripheral wells of the 96 well plates were not used for culture because of the increased risk of drying out and infection in these wells. Sterile phosphate buffered saline (PBS) was placed in these wells instead. After 24 hours, 100µl/well of double concentrated HAT medium containing ouabain were added into the wells. Growth was usually observed 7-10 days later in some of the wells. When the culture medium was turning yellow it was replaced with fresh HAT medium. The culture was maintained for a maximum of six weeks.

Wells containing growing cells were checked for antibody secretion when no more than a quarter of the well contained dividing cells. Wells with cells secreting an antibody of interest were transferred to 24 well plates (Nunc) and the cells were allowed to divide (expand). Following another test for antibody secretion the cells were subcloned. Cells were transferred to HT medium at this stage and subsequently to GM medium after a further two weeks. All the IgM and IgG anti-DNA antibody secreting hybridomas were initially frozen at -80°C (section 5.1) and/or subcloned for further study.

2.2.4 Screening of clones for immunoglobulin secretion and antigen specificity

Supernatants derived from wells with growing cells were screened for IgM and IgG secretion using a capture ELISA as described in Section 2.3.2. The supernatants were also screened for DNA binding (Section 2.3.5). For some of the fusions the supernatants were pretreated with 1 μg/ml of DNAase 1 (Sigma) in the presence of 1 mM $MgCl_2$ and 0.02 mM $CaCl_2$ for the DNA binding assay. The reaction was allowed to proceed for 1 hour at 37°C before being terminated by the addition of EDTA (15mM). The resulting mixture was then placed into the ELISA wells.

2.2.5 Subcloning by limiting dilution

Monoclonality was ensured in the cells of interest by subcloning three times by limiting dilution using mouse peritoneal cells as feeders. Peritoneal cells were obtained using a pair of female BALB/c mice (Tuck, Battlesbridge, Essex). The mice were sacrificed by cervical dislocation and the skin was cleaned using 70% alcohol. The procedure was performed in a class 1 hood. The peritoneal membrane was exposed and 7mls of cold (4°C) RPMI was injected into the peritoneal cavity using a 19 gauge needle. Care was taken not to inject into any intraperitoneal organ to avoid contamination. The abdomen was gently massaged and the cells aspirated into the same syringe. The cells were centrifuged (120g) for 10 minutes, at 4°C which prevents the macrophages sticking to the plastic. The cells were washed twice, counted and resuspended into GM medium. The cells were plated out at $5x10^3$ cells / well in 96 well plates.

The technique of subcloning prevents overgrowth by faster growing non-secreting cell lines. If the cells are seeded at a low enough density the fraction of wells with growth should follow the Poisson distribution (Lefkovits and

Waldmann, 1979):

$$f(0) = e\lambda$$

where f(0) = fraction of cells with no growth

λ = average number of clones per well.

The hybridoma cells from selected wells were counted and then diluted in medium to a density of 5 cells/ml, 10 cells/ml and 50 cells /ml. 200 µl aliquots were placed in the central 60 wells of 96 well tissue culture plates. The peripheral wells were filled with sterile PBS. Plates where more than 37% of the wells grew cells were discarded. All wells with growing cells were checked for antibody secretion and specificity. Subcloning continued until all wells in which there were growing cells secreted immunoglobulin with the same specificity.

Monoclonality was further ensured by chromosome analysis using a method as previously described (Shoenfeld et al, 1983) which was performed in the haematology department (Dr. H Walker). Briefly, colchicine was added to growing cells which were then incubated for 2 hrs and harvested by centrifugation. After fixing in methanol/acetic acid, the cells were air dried on slides, banded with trypsin and stained with Giemsa. Monoclonal hybridomas all appear identical by this method.

2.2.6 Bulk culture in serum free medium.

Cell lines of interest were transferred into serum free (SF) medium because serum can interfere with the purification of antibodies and increases the cost of long term culture. In addition, the experience in the laboratory indicated that media in large stirrer flasks, which are used for bulk production, are more liable to become infected if serum is present. Previous experience in our laboratory, and by others (Ozturk et al, 1991), has suggested that a change to SF medium does not compromise antibody production or specificity in human

or mouse cell lines but no data on the effects of heteromyeloma cell lines such as CB/F7 was available.

The cell line to be expanded was passaged in steadily decreasing concentrations of serum whilst increasing the concentration of a serum free medium (SF-1, Northumbria Biologicals, Cramlington, UK) over a period of four weeks. On each occasion $5x10^6$ cells were transferred to tissue culture flasks (Nunc) containing an increased proportion of SF medium (25% SF medium in the first week, 50% SF in the second week, 75% SF in the third week and 100% SF in the fourth week). To assess whether there was a fall in concentration of immunoglobulin in the cell cultures, supernatant was extracted from the flasks three days after transfer to medium containing less FCS. The immunoglobulin concentration was measured using an immunoglobulin ELISA (Section 2.3.2). The cells were expanded into two 75 cm^2 flasks in serum free medium and then transferred into 500 ml continuously stirred flasks (Techne, Cambridge, UK). During bulk culture 200 ml of medium was removed when the medium was tinged yellow and replaced with an equal volume of fresh medium. The flasks were maintained for six weeks and then replaced to prevent the risk of infection and cell overgrowth. The aspirated supernatant was centrifuged at 200g for 10 minutes and stored at -20° C until further purification.

2.2.7 Affinity purification of antibodies

Supernatant was thawed, filtered and passed over a HiTrap™ Protein G column (Pharmacia, Herts, UK) and cycled overnight at 4°C. The effluent was monitored using a UV monitor connected to a graph recorder (Pharmacia) in order to reduce the minimum volume containing the antibody. The monitor indicates changes in protein concentration. After the antibody was loaded onto the protein G, the column was washed with 10 column volumes of PBS followed by 3M NaCl pH 7.4 and finally with glycine-HCL buffer pH 2.3 (section 5.3.4). The pH of the eluted antibody was adjusted to pH 7.4 by the addition of

saturated Tris.

The eluates obtained with the 3M NaCl and with the acid wash were collected separately and their absorbance was measured at two wavelengths, 260 and 280 nanometres. The absorbance at 260 and 280 gives an estimate of the amount of DNA and protein respectively; whilst the ratio of the two absorbance values yields an approximate guide to the purity of the sample. Thus a ratio of the absorbance values at 280/260 of greater than 1.8 indicates a relatively pure preparation of protein with little DNA contaminating the sample; whereas an absorbance value ratio of 260/280 > 1.6 indicates a pure preparation of DNA without significant protein contamination (Strauss, 1991).

The antibody which is eluted in the acid wash was stored in aliquots at 20°C at a concentration of 1 mg/ml.

2.2.8 Affinity purification of DNA binding antibodies from serum

Celullose coupled to ssDNA and dsDNA was purchased from Pharmacia and placed into a column. The column was equilibrated with 0.1M Tris - HCL pH 7.4 containing 0.1% BSA and 1 mM EDTA. The serum was diluted 1:1 with this buffer and cycled over the column for 1 hour; 3M NaCl was used to elute the column. The specificity and absorption capacity of the column was determined using anti-DNA positive and negative sera. All serum samples required only one absorption cycle to remove all DNA binding antibodies as detected by ELISA. The effluents and eluates were tested separately for idiotype activity.

2.2.9 Analysis of DNA present in the supernatant

To analyse the DNA present in the supernatant agarose gel electrophoresis was used. DNA was extracted from two sources: the concentrated supernatant and the eluate derived from the protein G column when washed with 3M NaCl.

2.2.9.1 Phenol chloroform extraction of DNA

DNA extraction was performed using the phenol chloroform extraction method. An equal volume of phenol (e.g. 50µl) was added to the material containing the DNA to be purified. The solution was vortexed for 10 seconds and centrifuged for four minutes. The aqueous layer was removed and transferred to a new tube. 25 µl of phenol and 25µl of chloroform were added to the new tube and the mixture vortexed and centrifuged again. 50 µl of chloroform was added to the decanted aqueous layer, vortexed and centrifuged for a third time. 5 µl (1/10 of the volume) of 3M sodium acetate, pH 5.2 was added and the tube vortexed, followed by 100µl of ice cold 100% ethanol. The mixture was placed in a -80°C freezer for 1 hour. The tube was centrifuged for 10 minutes and the liquid decanted. 100 µl of 70% ethanol was added, the tube vortexed and centrifuged again. The fluid was decanted and the pellet vacuum dried for 10 minutes and resuspended in 20µl of water.

2.2.9.2 Agarose gel electrophoresis

Reagents: (Section 5.3.2)

Electrophoresis buffer: TBE

Ethidium bromide solution

Electrophoresis grade agarose

10x loading buffer

DNA molecular weight markers: Lambda HindIII and 1 kilobase ladder (Gibco)

One litre of TBE was prepared with 0.5µg/ml of ethidium bromide. A 0.8% agarose gel was made by dissolving 0.8g of agarose into 100 ml of TBE and heating in a microwave oven and regular swirling. After the gel casting apparatus had been sealed, the cooled agarose/TBE mixture was poured into the gel apparatus to a thickness of approximately 0.5 cm. The gel comb was

inserted and the gel allowed to set.

After the gel had hardened the tape and comb was removed from the gel apparatus. The sample and the molecular weight markers were loaded into the wells in loading buffer. The gel was placed into the electrophoresis tank and sufficient buffer added so that the gel was covered to a depth of 1mm. The leads were attached so that the DNA migrated to the positive lead and the voltage set to 75 Volts. The power supply was turned off when the dye from the loading buffer had migrated a sufficient distance for the DNA fragments to have separated. The gel was then removed from the apparatus and placed on a UV light source and photographed under exposure to UV light using a bellows type camera equipped with a Polaroid film holder.

2.3 IMMUNOASSAY S

Enzyme linked immunosorbents assays (ELISA) were used to detect the presence of immunoglobulin and its specificity, isotype and idiotype. ELISAs are a rapid, sensitive, specific, quantitative (at least in relation to known standards) and reproducible method for detecting immunoglobulin and the associated specificity. Immunoglobulin and other proteins bind to plastic or polyvinyl chloride microtitre plates through poorly understood mechanisms. Charge and hydrophobic interactions are believed to be important. After coating the plate with the desired protein, excess binding capacity can be saturated using an excess of an irrelevant blocking protein such as bovine serum albumin. Capture ELISAs use an adsorbed antibody to capture an antigen. This system may be used if the antigen does not bind to the plastic well or if the antigen has a high degree of non-specific "stickiness", and does not require a purified antigen. The idiotype detection ELISA described later was constructed in this way. It is often more convenient and practical,

particularly when there is no preexisting specific antibody to use a direct binding ELISA for antigen specificity and for inhibition ELISAs. Inhibition or competitive ELISAs are used to determine the relative affinity of the antibody and define the specificity in the fluid phase. The ELISA is completed by the use of a second antibody conjugated to an enzyme which reacts with a substrate to form a coloured product. The coloured product can be read automatically. Non-specific binding can be further reduced by the addition of a non-ionic detergent such as Tween (polyoxyethylene sorbitan monolaurate, Sigma) to all stages of the ELISA after the blocking stage. Anti-species cross-reactivity can be reduced by the addition of 1-2% of serum from the 'offending species', by adsorbing out irrelevant specificities on an affinity column or, best of all, by using a monoclonal antibody specific for the immunoglobulin species.

2.3.1 General ELISA methods (see also section 5.3.1)

Flat bottomed 96 well polystyrene immunoassay plates (Maxisorp, Nunc) were used for all assays. Volumes of 80µl were added to each well except for the blocking stage when 150 µl was used. All washing stages were carried out three times. Alkaline phosphatase conjugates were used for all assays as follows. After washing the plates three times with PBS-0.05% Tween (PBS-T) and three times with bicarbonate (Bic) buffer pH 9.6, the colour was developed using p-nitrophenyl phosphate tablets (5mg, Sigma) as substrate. Two tablets were dissolved in 10 ml of Bic buffer to which 20 µl of 1M MgCl2 had been added. After the addition of the substrate the reaction was allowed to develop at 37°C and read at two time points which differed depending on the assay and conjugate used. The colour was read at 405nm with a reference filter at 490 nm using a Dynatech 4000 ELISA (Dynatech, Billinghurst, UK) reader with direct data acquisition by an Apple Macintosh computer using Dynaread II software (Dynatech). All samples were tested in triplicate (duplicate for the serum

idiotype assays) and appropriate positive and negative controls were included on every plate in each assay. The degree of variation between triplicates did not exceed 7% in any of the ELISA assays performed.

2.3.2 Detection and quantification of immunoglobulin

Three species of immunoglobulin were assayed by ELISA. A capture ELISA was used modified from the method of Shields and Turner (1986). For detection of human immunoglobulin, goat anti-human IgG F(ab')2 or goat anti-human IgM (Sigma) was coated to the plate in Bic buffer at a concentration of 1 μg/ml overnight at 4°C. The plate was washed and blocked with 2% bovine serum albumin (BSA) for 1 hour at 37°C and after another wash in PBS/T the samples were applied to the plate diluted in PBS/T to an appropriate dilution for detection of immunoglobulin. The range of detection was in the range 5 - 200ng/ml. All samples, as well as the control immunoglobulin, were diluted down the plate in 1:3 dilutions. The starting dilution for serum was 1/1000, whilst supernatant was used neat. The assay was completed using goat anti-human IgG or IgM F(ab')2 alkaline phosphatase conjugate (1:1000, Sigma) and the colour was developed in the usual way. For the rabbit and mouse ELISA goat anti-rabbit and goat anti-mouse IgG (both at 1μg/ml) were used to coat the plate respectively. The immunoglobulin was detected using goat anti-rabbit IgG alkaline phosphatase (Sigma) and goat anti-mouse IgG (Sigma) alkaline phosphatase conjugate as appropriate. The immunoglobulin concentrations were calculated by comparison of the absorbance of the test sample with the linear portion of the standard curve.

2.3.3 Determination of the light chain class

The human immunoglobulin assay was adapted to determine the light chain class. Microtitre plates were coated with goat anti-human λ light chain (1:2000, Sigma) and goat anti-human κ light chain (1:2000, Sigma). The

protocol then followed the immunoglobulin assay. The positive control used for the assay was pooled IgG and monoclonals that were known to be possess a κ or λ light chain.

2.3.4 IgG subclass ELISA

The human immunoglobulin assay was adapted to determine the IgG heavy chain subclass. Microtitre plates were coated with goat anti-human IgG F(ab')2 (Sigma) in Bic buffer overnight at 4°C. The plates were blocked with 2% BSA and the monoclonal antibodies were diluted down the plate. After washing the plate was incubated with sheep anti-human monospecific to IgG1 (1:1000), IgG2 (1: 1500), IgG3 (1: 200) and IgG4 (1:200) conjugated to alkaline phosphatase (The Binding Site, Birmingham, UK). Known references of purified IgG subclasses were supplied by The Binding Site.

2.3.5 DNA direct binding ELISA

An ELISA for detecting binding to ssDNA and dsDNA (Isenberg et al, 1987a) was modified. Immunoplates were coated with poly-l-lysine hydrobomide (molecular weight 15-30 Kd, Sigma) (50 μg/ml in distilled water) for 1 hr at 37°C. Coating the plates with poly-l-lysine increases the binding of non-protein antigens to the plate. The plates were washed in PBS, and ssDNA, dsDNA and water were coated in the wells so that the plate was divided into three. ssDNA and dsDNA (prepared as in section 5.4) were coated on to the plate at a concentration of 5 and 10 μg/ml respectively overnight at 4°C. The plates were washed with PBS and the plates charge neutralised with poly-l-glutamate (100μg/ml in distilled water, Sigma) for 1 hour at 37°C. After a further wash with PBS, non-specific binding was blocked with 2% casein dissolved in PBS. Samples were initially screened at a 1 in 2 dilution. Some supernatants were also treated with DNAase as described above in section 2.2.4. Appropriate positive and negative controls were included on each plate. The

bound anti-DNA antibody was detected using goat anti-human IgG and IgM alkaline phosphatase (Sigma) and the colour was developed in the usual way.

Binding activity was assessed by reference to the median absorbance of the supernatants tested: <2 x median -ve; 2-5 x median +/-; 5-10 x median +; >10 x median ++. Background binding to the poly-l-lysine only side of the plate was subtracted from the DNA side of the plate.

2.3.6 Competition ELISA for DNA binding

The ELISA plate was coated with DNA as described in section 2.3.5 and the concentration of mAb giving 50% maximal binding to DNA in the direct binding ELISA was established. Equal volumes of antibody and different dilutions of DNA were incubated together for 2 hours at room temperature before being placed in the antigen coated wells. Dilutions of the culture supernatant were included on the same plate. The assay was completed as described for the DNA ELISA. Data were expressed as percentage binding to the solid phase DNA compared with the binding in the absence of inhibitor (% absorbance).

The relative affinity of the antibodies was calculated by comparing the concentration of DNA required to achieve 50% inhibition of the binding of the different antibodies to the DNA coated on the plate.

2.3.7 Cardiolipin direct binding ELISA

A previously described method for detecting cardiolipin binding activity was modified (Isenberg et al, 1988). Microtitre plates were coated with cardiolipin (100μg/ml in absolute alcohol; Sigma) and the alcohol was allowed to evaporate overnight at 4°C. Non-specific binding was blocked with 10% FCS in PBS and incubated for 2 hours at room temperature. Culture supernatants were initially screened neat whereas serum samples were tested at a dilution of 1 in 100. The plates were washed with PBS and bound antibody was detected

as for the DNA ELISA. At no time during the ELISA was Tween used as this results in the cardiolipin becoming unbound from the plate.

2.3.8 Histone ELISA

Anti-histone activity was detected using an ELISA method as described elsewhere (Subiza et al, 1989). Histone type IIS (Sigma) was coated overnight at a concentration of 10 μg/ml at 4°C in PBS. The plate was then blocked with 2% casein and any DNA contaminating the histone was removed by treatment with DNAase 1(Sigma). The supernatants were added to the plate and the antibody bound to the plate was detected as for the DNA ELISA.

2.3.9 ELISA for the determination of specificity of rabbit anti-idiotypic antisera

Immunoplates were coated overnight with the immunising antigen, and pooled human IgG (Sigma) at equivalent concentrations in Bic buffer. The plates were washed three times and blocked with 2% BSA in Bic buffer for 1 hour at 37°C. After a further wash, rabbit sera were diluted in PBS/T in 1:3 dilutions (starting at 1 in 100) and incubated for 1 hour at 37°C. The plates were washed with PBS/T and bound antibody was detected with mouse monoclonal anti-rabbit alkaline phosphatase conjugate (1:4000, Sigma). The colour was developed in the usual way.

2.3.10 Competitive ELISA for idiotype expression

Using a previously described method (Watts et al, 1990a), affinity purified rabbit anti-idiotypic antibodies (at a concentration previously determined to give 50% maximal binding of anti-idiotype to its homologous idiotype) were incubated with varying concentrations of mAb and pooled IgG for 1 hour at 37°C. Reaction mixtures were then added to mAb (bearing the test idiotype)

coated wells (1μg/ml) for 1 hour at 37°C. A mouse monoclonal anti-rabbit alkaline phosphatase conjugate (Sigma) was used to determine the amount of anti-idiotype bound. Results were expressed as percentage binding to the solid phase homologous antibody compared with the binding in the absence of inhibitor.

2.3.11 Analysis of mouse monoclonal anti-idiotypic antibodies

The direct binding assay and the competition assay used for the analysis of the rabbit anti-idiotype were modified to determine the specificity of the putative murine monoclonal anti-idiotypic antibodies. Bound antibody was detected by the use of a goat anti-mouse IgG (Fc specific) alkaline phosphatase conjugate (Sigma).

2.3.12 Competition ELISA between mouse and rabbit anti-D5Id for D5

Mouse or rabbit anti-D5Id were adsorbed to the ELISA wells and mixtures of D5 and the other anti-Id were incubated together at 37°C for 1 hour before being transferred to the plate. The amount of D5 that had bound to the plate was determined as above. In addition the binding of one anti-Id to the other was also measured.

2.3.13 Competitive ELISA for antigen binding site idiotype expression

The method has been described (Watts et al, 1990a). Reaction mixtures of mAb at the same concentration and antigen at varying concentrations, in this case dsDNA and ssDNA, were incubated for 1 hour at 37°C before being transferred to anti-Id coated wells for 1 hour at 37°C. The amount of human monoclonal antibody that had bound to the plate was determined by using an

anti-human IgG alkaline phosphatase conjugate (Sigma, dilution: 1: 3000).

2.3.14 ELISA for detection of idiotype on immunoglobulins in sera

A capture ELISA was used to detect the idiotype in human sera where the anti-idiotype was coated onto the wells. The optimum coating concentration for rabbit (affinity purified) and mouse anti-idiotypic antibodies was determined using a chequerboard assay, in which anti-idiotypic reagent (0.05-5μg/ml) was coated onto a microtitre plate overnight at 4°C in Bic buffer. After washing with Bic buffer the plates were incubated with 2% BSA to block non-specific binding. Serial dilutions of the mAb bearing the homologous idiotype in PBS/T were applied to the plate in a chequerboard fashion. The plates were washed in PBS/T and bound antibody was detected using goat anti-human IgG alkaline phosphatase conjugate F(ab')2 (Sigma, 1:20,000) with 10% FCS. The optimum dilution of human sera for use in the assay was determined by serial dilution (1:100-1:3200) of sera from both normal individuals and SLE patients.

For the optimum concentration of coating anti-idiotype and serum see Results section. The capture ELISA proceeded as follows. A 96-well ELISA plate was divided into two. One half was coated with the affinity purified anti-idiotype whilst the other half of the plate was coated with the same concentration of the control rabbit or mouse immunoglobulin as appropriate. Human sera was applied to both sides of the plate and the binding of IgG and IgM was detected using an alkaline phosphatase goat anti-human IgM or IgG F(ab')2 alkaline phosphatase conjugate (Sigma). The absorbance readings obtained on the control immunoglobulin side were subtracted from those obtained on the rabbit anti-idiotypic side of the plate. Two methods of calculating the results were compared using sera from normal individuals and patients with SLE: 1) as a percentage of the absorbance values obtained using a standard positive reference serum and 2) as μg/ml equivalents of mAb by calculation from the linear portion of a standard mAb curve. Both the reference

serum and the standard curve were applied to each plate. A good correlation (see Results) was obtained between the two methods and it was therefore decided to use the reference serum for reasons of economy and simplicity. This control represents the value produced in the Id ELISA and does not represent the percentage of antibodies that bear the idiotype. We used this control to reduce the interassay variation by always comparing the absorbance values of the other sera to the absorbance reading of this sera. Four healthy controls were used on each plate. The upper limit of normal was set as the mean + 2 standard deviations of values obtained for the normal control sera.

2.3.15 Detection of idiotype expression on immunoglobuiins in culture supernatants

The assay used to detect idiotype expression in sera was modified to detect expression of idiotypes on monoclonal antibodies derived from supernatant. Supernatants were screened neat. Binding was detected using goat anti-human IgM or IgG F(ab')2 alkaline phosphatase conjugate (Sigma). Positivity was assessed in relation to a bank of supernatants tested: <5x median -ve; 5-10 x median +; >10 x median ++.

2.4 PREPARATION OF ANTI-IDIOTYPES

2.4.1 Rabbit polyclonal anti-idiotypes

2.4.1.1 Immunisation protocol

Rabbits were selected to be immunised because these animals had previously produced good quality anti-idiotype for IgM antibodies in our laboratory. New Zealand White rabbits (Foxfield Rabbit Co, Foxfield, Hants) were immunised using a modification of the protocol of Vaitukakis (Vaitukakis 1981). The animal was bled prior to immunisation to ensure that the antibody detected later was due to the immunisation. Affinity purified mAb (250μg in

PBS) prepared as described in section 2.2.7 was emulsified with an equal volume of incomplete Freunds adjuvant (IFA; Difco, Surrey, UK) in an eppendorf using a whirlimixer until the emulsion was stable. A stable oil in water emulsion should hold together as a droplet on the surface of water. The fur on one side of the animal was shaved and the skin sprayed with xylocaine. The immunisation was given in multiple intradermal sites. Booster doses of antigen (50μg/ml with IFA) were at weeks 2, 5, 7 and 10. Blood was collected at regular intervals and the rabbit was exsanguinated fourteen days after the last injection if the serum titres against the immunogen were high. The blood sample was allowed to stand at room temperature for 1 hour and then overnight at 4°C to allow the clot to contract. The blood was centrifuged at 2000g and the serum stored at -20°C. A control rabbit was immunised with adjuvant and PBS alone using the same protocol.

2.4.1.2 Affinity purification of rabbit anti-idiotypic sera

Five mls of rabbit anti-idiotypic sera were thawed and passed over a human IgM/IgG Sepharose column (section 5.2) to remove the non-idiotype specific anti-human immunoglobulin activity. The sera was cycled repeatedly (overnight, approximately three times) and eluted until there was no detectable anti-pooled human immunoglobulin remaining as measured by ELISA. The anti-idiotypic serum was further purified to remove irrelevant specificities by purification on a Sepharose column coupled to the homologous antibody, followed by acid elution of bound antibody. The eluate was monitored using a Pharmacia uv unit. The pH of the eluate was adjusted with Tris base. The purified anti-idiotype was stored at 4°C until use. At every stage the sera was tested against the immunising mAb and against pooled immunoglobulin as described in section 2.3.9. The serum from the control rabbit was purified in the same way except that it was not purified on an idiotype column.

2.4.2 Preparation of murine monoclonal anti-idiotypic antibodies

Murine monoclonal anti-idiotypic antibodies were generated by the immunisation of mice with the mAb and harvest of splenocytes for fusion to yield monoclonal antibodies.

2.4.2.1 Immunisation schedule

BALB/c mice were immunised using the same protocol as for the rabbits using IFA for all immunisations. The initial dose of mAb was 50μg given subcutaneously followed by three booster injections a week apart. The sera of the mouse was tested for response to the mAb three days after the last two immunisations by ELISA and the mouse was sacrificed three days after the final immunisation if anti-IgG titres were satisfactory. Two mice were used for each mAb.

2.4.2.2 Murine fusion procedure

The murine myeloma cell line P3-X63-Ag8 653; a non-secreting, HAT sensitive cell line was used as the fusion partner (Kearney et al, 1979). The cells were thawed out as described in section 5.1 and maintained in log phase growth in GM medium.

Two days before fusion, peritoneal macrophages were harvested from the peritoneum of BALB/c mice as described in section 2.2.5 The immunised mouse was sacrificed and the spleen removed. The spleen was dissaggregated by sterile forceps and by pipetting with RPMI. The cells were washed three times in RPMI simultaneously with the myeloma cells. Cell counts and viability tests were performed (section 2.2.1). The cells were fused at 5:1 ratio using 44%PEG 1500 (Boehringer Mannheim). Polyethylene glycol (1ml/10^8 cells) was added to the cells dropwise at 37°C over a 60 second period and the cells were allowed to stand for a further minute. The cell suspension was diluted with RPMI slowly over 10 minutes. The cells were

washed and resuspended in HAT medium and plated at $2x10^5$ cells/well in the wells previously coated with murine peritoneal macrophages. Colonies became visible to the naked eye within 2 weeks and the supernatants were screened for anti-idiotypic activity by ELISA as described in section 2.3.11. Those colonies that produced antibodies that bound solely to the immunising mAb and not to pooled IgG in both solid phase and fluid phase ELISAs were selected for subcloning by limiting dilution. The cells of interest were then expanded in 75 cm^2 flasks (Nunc).

2.4.2.3 Purification of murine monoclonal anti-idiotypic antibodies.

The culture supernatant was passed over a HiTrap™ Protein G and eluted as described in section 2.2.7. The concentrate was again checked for anti-idiotypic activity.

2.5 ANALYSIS OF IDIOTYPE LOCATION ON THE MONOCLONAL ANTIBODY

SDS PAGE followed by Western blotting was used to determine whether the idiotype/idiotope was present on the light or heavy chain of the immunoglobulin molecule. The monoclonal antibody was heat denatured and the light and heavy chain were separated electrophoretically on the SDS-PAGE gel. The two chains were then transferred onto nitrocellulose sheets and the anti-Id reagent was then applied together with anti-light and anti-heavy chain with appropriate controls to determine which chain the anti-idiotype bound. If the anti-idiotype did not bind to the denatured mAb, it was subjected to SDS-PAGE without heat denaturing.

2.5.1 Preparation of gels

SDS-PAGE was performed using a modification of the method of Laemmli (1970) using the Mini Protean™ minigel system (BioRad, Herts, UK). The minigel was assembled according to the manufacturer's instructions and tested for potential leaks using distilled water. After the apparatus was dried using filter paper a 10% polyacrilamide gel was prepared as follows.

Reagents (see also section 5.3.3):

Acrylamide 40% / bis acrylamide 2.6% (Acrylogel 2.6) from BDH (Poole, Dorset)

Tris buffer 3M pH 8.7

Tris buffer 1M pH 6.8

10% ammonium persulphate in distilled water made freshly each time.

10% sodium dodecyl sulphate (SDS) (BDH)

N,N,N',N'- Tetramethylethylene diamine (TEMED), (Sigma)

EDTA (BDH) 100mM in 1M Tris pH 6.8.

The composition of 10% separating gel and stacking gel were:-

	Separating gel	Stacking gel
Acrylogel 2.6	2.5 ml	.416 ml
3M Tris	1.25 ml	--------
1M Tris	--------	.416 ml
distilled water	6.7 ml	2.1 ml
10% ammonium persulphate	.067 ml	.033 ml
10% SDS	.1 ml	.067 ml
TEMED	.006 ml	.003 ml
100mM EDTA	---------	.33 ml

The separating gel was prepared by the mixture of the above reagents with the exception of TEMED, ammonium persulphate, and SDS. This mixture was degassed and the rest of the reagents were added, mixed gently and 'poured' into the gel casting apparatus using a syringe. The remaining mixture was left in a Universal tube until set. The surface of the gel was overlayed with water saturated butanol until set. The stacking gel was prepared and then overlaid on the separating gel after all the butanol had been washed away with running buffer. A comb was inserted into the stacking gel and the gel was allowed to set. The comb was removed carefully and the wells washed with running buffer to remove any unpolymerised gel. The gel was placed into the running apparatus which was filled with running buffer.

Sample preparation:-

Monoclonal antibody in PBS was prepared so that the final concentration was 1 μg/ml. The mAb was mixed with the sample buffer in a ratio of 2:1. The volume was determined by the number of lanes of sample to be run in the gel. The sample was then boiled for three minutes in a water bath and allowed to cool.

The samples were then loaded into the wells (10 or 15μl/well) using a Hamilton syringe. Low molecular weight markers (Sigma) were included in each gel. The running apparatus ran two gels simultaneously using a BioRad powerpack 3000Xi (BioRad, Hemel Hemsptead, Herts.) at a constant voltage of 100 Volts. The power was interrupted when the leading edge was 1 cm from the bottom of the gel. The gel was then carefully removed from the running apparatus and prepared for blotting.

2.5.2 Western blotting

The protein was transferred from the gel to nitrocellulose sheets by

electrophoresis using the method of Towbin (Towbin, Staehelin and Gordon, 1979). A semi-dry electroblotter (Biotech instruments Ltd, Beds.) was used to blot the separated mAb onto nitrocellulose. The lower graphite plate was used as the anode and the upper as the cathode. During the whole blotting procedure rubber gloves were worn to prevent the transfer of protein from hands to the nitrocellulose. The preparation of the buffers is described in section 5.3.3

The graphite plates were rinsed with distilled water. Six layers of filter paper (Whatman No. 1, Whatman Lab Sales Ltd, Kent, UK) soaked in an anode buffer plate was placed on the lower (anodic) plate. These were followed by three layers of filter paper soaked in anode buffer 2, the nitrocellulose sheet (0.45μm; Schleicher and Schuell, Anderman and Co., Kingston-Upon-Thames, Surrey, UK) soaked in distilled water and the gel was placed upon the nitrocellulose sheet. Three layers of filter paper soaked in the cathode buffer were placed on top of the gel followed by six layers of filter paper soaked in cathode buffer. At each stage a glass Universal was rolled along the top to ensure even transfer of protein and to eliminate air bubbles. The apparatus was covered with the lid and the apparatus attached to a power supply unit to run at 0.8 mA/cm^2 for 90 minutes.

After the allotted time the nitrocellulose sheet was removed and the blotted proteins visualised using 0.2% Ponceau S solution until bands could be visualised. Blotted proteins appear as red bands. The lanes were marked with a ball point pen and cut into strips with a scalpel.

2.5.3 Immunostaining

Previous experience in our laboratory with immunostaining had been in the localisation of the position of idiotypes on IgM monoclonal antibodies (Williams et al, 1988). Utilising this method, with appropriate changes for use with IgG monoclonals, resulted in staining both the heavy and the light chain

even if PBS rather than the anti-Id had been used. This problem was due to using a polyclonal anti-rabbit IgG alkaline phosphatase conjugate which bound to human IgG although no binding to human IgM occurred. This problem was resolved by using a mouse monoclonal anti-rabbit alkaline phosphatase conjugate (Sigma) which did not bind human IgG. The method of immunostaining was as follows.

The nitrocellulose strips were placed in a box containing 2% casein in PBS and gently rocked overnight at 4°C. The nitrocellulose strips were washed in PBS/T and the following anti-sera were applied to the strips for 1 hour at room temperature with constant rocking. For localisation of the idiotype with the rabbit anti-idiotypes the following were applied to the strips separately: rabbit anti-idiotype (1 μg/ml), normal rabbit serum (1 μg/ml), rabbit anti-human gamma chain (1: 4000, Dako, High Wycombe, Bucks), rabbit anti-human lambda chain (1:2000, Sigma), rabbit anti-human kappa chain (1: 1000, Sigma). The washing procedure was repeated and mouse anti-rabbit alkaline phosphatase conjugate (Sigma, 1:4000) were applied to all the strips for 1 hour at room temperature. The strips were again washed and 33μl of nitro blue tetrazolium (NBT) and 16.6 μl of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega, Southampton, UK) were added to 10 mls of the colour development solution (see section 5.3.3) which was applied to the strips. The strips were allowed to develop in the dark for a set period and the reaction was then stopped with colour stop solution. After two minutes the strips were dried and immediately photographed.

2.6 IMMUNOHISTOCHEMICAL STUDIES

Immunohistochemical studies were performed on human renal tissue to ascertain whether antibodies possessing the idiotypes described in this thesis were deposited in the glomeruli of kidneys from lupus patients. The rabbit and

mouse anti-idiotypic reagents were used to detect the presence of the idiotypes in the renal tissue. As a positive control and to determine the approximate concentration of anti-idiotype to use for tissue staining, the hybridoma cells that produced the idiotype positive mAb were used in cytospin preparations. The mAb antibodies themselves were studied to determine whether they bound kidneys in fixed sections and were also tested on HEP-2 cells and *Crithidia Luciliae.*

The presence of antibody in tissue sections from the SCID mice experiments (see section 2.7.1) was detected by immunohistochemical methods.

2.6.1 Staining of cytospin preparations for positive control for the presence of idiotype

Hybridoma cells growing in GM medium during an exponential phase i.e. dividing rapidly, were washed and diluted to a concentration of $1x10^4$ in PBS. The cells were then placed onto a slide using a cytospin centrifuge (Shandon, Runcorn, Cheshire) at 8000 RPMI for 5 minutes. The slides were air dried under a fan for ten minutes and fixed in acetone for a further five minutes. The slides were washed in Tris buffered saline (TBS) and incubated with different concentrations of the rabbit and mouse anti-Ids for 1 hour at room temperature. The slides were washed three times in TBS and developed as detailed below.

2.6.2 Detection of idiotypes in renal tissue

Frozen kidney sections were prepared (kindly donated by Dr. Barrie Hartley, Guys Hospital, London) from 10 SLE patients and 15 non-SLE patients with immunoglobulin deposited in their kidneys. The sections were on glass slides wrapped individually in silver foil and stored at -80°C until use. Prior to staining sections were warmed to room temperature and unwrapped. The

slides were fixed in acetone for 10 minutes and washed in TBS.

The biotin streptavidin technique was used to detect the presence of the idiotype as defined by the rabbit anti-idiotypic antibodies. Ten per cent goat serum was added to all reagents. The slides were incubated with the rabbit anti-idiotypic antibodies at the concentrations determined in section 2.6.1 for 1 hour using normal rabbit serum as a control. After washing with TBS the sections were incubated with biotinylated goat anti-rabbit IgG antibodies (Dako, 1:400) for 30 minutes and washed again prior to incubation with streptavidin alkaline phosphatase (Dako, 1:200) for 30 minutes. The chromagen was developed using the substrate Napthol-AS-phosphate in the presence of Fast red TR salt (Sigma) with 0.1M Levamisole to block endogenous alkaline phosphatase. The sections were counterstained after washing in tap water using haemotoxylin and mounted using Apathy's reagent (BDH).

The alkaline phosphatase anti-alkaline phosphatase (mouse preformed complexes, APAAP) system was used to stain for mouse anti-idiotypic reagent. Mouse anti-Id and negative control mouse monoclonal of the same isotype (Sigma) diluted in TBS containing 10% goat serum was applied to the sections for 1 hour at room temperature. After washing with TBS, rabbit anti-mouse immunoglobulin (Sigma, 1:20) was applied for 30 minutes and, after a further incubation and wash, APAAP (Sigma, 1:50) was placed on the sections for 30 minutes. After further washing, fast red was incubated on the sections for ten minutes. Sections were washed in tap water, counter stained with haemotoxylin and assessed independently/blind by Dr. Barrie Hartley.

Photographs were taken with a Nikon FX-2 camera attachment and Kodak ASA 160 film.

2.6.3 Staining of normal sections with monoclonal antibody

Frozen sections of 6μm thickness were cut on a Bright's (Huntingdon, Cambridgeshire) microtome and taken up onto glass slides. Sections were air

dried under a fan for 10 minutes and stored in aluminium foil at -80°C until required. Prior to staining the sections were thawed at room temperature, unwrapped and fixed in acetone for 10 minutes. The sections were washed in PBS and incubated with the mAb at a concentration of 20μg/ml for 1 hour. The slides were washed and then incubated for 40 minutes with fluoresceinated goat anti-human conjugate (Southern Biotechnology, Birmingham, Alabama, USA) with 10% goat serum. After further washing the slides were mounted in PBS glycerol (Citifluor) and viewed using a fluorescent microscope (Nikon) and photographed with a Kodak ASA 400 film.

2.6.4 Indirect immunofluorescence on Hep 2 cells and Crithidia

Slides with prefixed Hep 2 cells or *Crithidia luciliae* were purchased from SciMedX (Biodiagnostics, Upton-On-Severn, UK) and stored in dessicating conditions at -20°C until use. The sections were allowed to warm up to room temperature and washed in PBS. Non-specific binding was blocked with 20% goat serum for 30 minutes. After a further wash the slides were incubated with the antibody (20μg/ml) in PBS for 45 minutes. PBS was applied to one slide as a negative control. After washing the slides were incubated with goat anti-human IgG fluoresceinated conjugate (1:50; Southern Biotechnology). After further washing the slides were mounted in PBS glycerol (Citifluor) and viewed using a fluorescent microscope and photographed.

2.7 INVESTIGATION OF THE BIOLOGICAL PROPERTIES OF HUMAN MONOCLONAL ANTI-DNA ANTIBODIES

The biological properties of the two anti-DNA antibodies B3 and D5 were studied in two ways. In the first set of experiments the purified antibodies and the hybridomas that produced them were injected intravenously and

intraperitoneally respectively into severe combined immunodeficiency (SCID) mice (section 2.7.1). Thus the binding of the monoclonals was investigated in vivo. When the hybridomas are injected into the mice sustained concentrations of the monoclonal would allow any biological effects to be observed. The binding of the monoclonals was also studied in vitro by applying the monoclonals to fixed kidney sections and detecting the pattern of binding using an anti-human IgG fluorescence conjugated antibody as described in section 2.6.3. The second set of experiments involved the immunisation of B3 with or without DNA into normal mice (section 2.7.2).

2.7.1 Use of SCID mice to assess pathogenicity of human monoclonal antibodies

Nine week old CB-17 scid/scid mice (purchased from Charles River, Margate, Kent) were kept in sterile boxes covered by a filter and fed sterile water and food without any antibiotics. The mice were bled before the experiment to check for the presence of murine antibodies ('leakiness'). Only those with undetectable amounts of murine immunoglobulin were used.

The SCID mice were treated with 0.5ml sterile pristane (4,6,10,14-tetramethylpentadecane, Sigma) 10 days prior to intraperitoneal (i.p.) injection of hybridoma cells. This provokes an inflammatory response of activated macrophages which is thought to provide more 'nutrients' to allow the hybridomas to divide. Hybridoma cells producing the monoclonal antibodies were harvested from midlog phase cultures and resuspended in 0.5ml RPMI so that the total number of cells was $1x10^6$ cells. The cell lines were subcloned once prior to injection in order to ensure monoclonality (the original monoclonal had been subcloned three times). The cells were injected into the peritoneum under sterile conditions using a 21 gauge needle. Six mice were used for each antibody and four uninjected pristane primed mice and four mice injected with

the non-secreting fusion partner cell line were used for controls.

Ascites was allowed to develop in the mice and they were sacrificed when either their body weight had increased by 20% due to the ascites, or if they appeared unwell. The urine was checked for proteinuria using a dipstick (Albustix®, Bayer Diagnostics, Berkshire, UK) and a blood sample was taken by tail vein venesection. The peritoneum was opened and the ascites drained. The kidney, spleen, liver and skin were sampled and snap frozen in a hexane/dry ice bath. The tissue was stored at -80°C until use. The tissue was cut on a SLE TE microtome and taken up onto glass slides. Sections were air dried under a fan for 10 minutes and stored in aluminium foil at -80°C until required. Additional tissue was fixed in 4% buffered formalin and processed by routine histopathology (courtesy of Dr. Meryl Griffiths). The ascites and serum was assayed for the presence of human immunoglobulin by ELISA as described in section 2.3.2.

In an additional set of experiments four hybridomas (kind gift of Dr. T Winkler, Erlangen, Germany) secreting human IgG anti-DNA antibodies were injected into the peritoneum of SCID mice (five to six mice per antibody). The binding properties of these antibodies are shown below (data kindly provided by Dr. Winkler and confirmed in our laboratory). The purified antibodies were not available.

clone	isotype	anti-DNA reactivity			
		ELISA ssDNA	ELISA dsDNA	Crithidia	Farr
32.B9	IgG3, lambda	+	++	+	+
33.H11	IgG1, lambda	++	+	-	-
33.C9	IgG2, kappa	+	++	+	-
35.21	IgG2, lambda	-	++	+	+

Injection of the purified antibody into SCID mice

1 mg of purified antibody in 0.2 mls of PBS was injected into the tail vein of the mouse (3 mice/antibody). The mouse was warmed in a 37°C incubator for 20 minutes prior to the injection. The mouse was sacrificed 6 hours after the

injection and the same organs were taken as described in section 2.7.1. Purified IgG antibodies (1 mg) derived from pooled human normal sera (Sigma) were injected as a control.

Immunohistochemical studies

The sections from the mouse organs were prepared in the same was as described in section 2.6.3. Human immunoglobulin was detected using goat anti-human IgG fluoresceinated conjugate (1:50; Southern biotechnology). The slides were viewed with a Nikon microscope using UV light.

2.7.2 Immunisation of normal mice with anti-DNA/DNA complexes

One anti-DNA antibody (B3, see results section) that bound the DNA found in the supernatant was selected for immunisation of normal BALB/c mice. The antibody was injected subcutaneously and intraperitoneally into female BALB/c mice (Tuck) with and without the DNA purified from the supernatant. Two experiments were undertaken. In the first, four groups of five BALB/c mice were immunised via the intraperitoneal route. The first group received 5μg of B3 in 50 μl of IFA, the second received 1 μg of DNA in IFA, the third 5μg of B3 and 1 μg of DNA in IFA, the fourth IFA and saline alone. The same amount of IFA was used in all four groups and each group received four intraperitoneal injections regularly spaced over a period of 6 weeks. The mice were monitored for proteinuria and blood samples were taken at 6 weeks, 3 months and 6 months. Anti-DNA antibodies were detected by an ELISA method as described in section 2.3.5 with a serum derived from an lpr mouse used as a positive control.

In a second experiment, the mice were immunised subcutaneously. In this experiment, 9 groups of three mice were used. The groups were as follows:

group 1: PBS

group 2: 1μg DNA

group 3: 5μg DNA

group 4: 5μg B3

group 5: 10μg B3

group 6: 5μg B3 + 1μg DNA

group 7: 5μg B3 + 5μg DNA

group 8: 10μg B3 + 1 μg DNA

group 9: 10μg B3 + 5 μg DNA

All mice received 50μl of IFA at each immunisation. The mice were monitored in the same way as for experiment one. Both experiments were terminated at six months.

CHAPTER 3 RESULTS

3.1 GENERATION OF HUMAN IgG MONOCLONAL ANTI-DNA ANTIBODIES

3.1.1 Comparison of different methods for producing human IgG monoclonal antibodies of any specificity

The first objective of this project was to produce monoclonal IgG anti-DNA antibodies from patients with SLE. In order to identify which method of generating monoclonal IgG anti-DNA antibodies might be the most successful, three methods were compared for their ability to yield monoclonal IgG antibodies without regard for antigen specificity. It was not feasible to compare the three methods for their ability to generate monoclonal IgG anti-DNA antibodies as that event only occurred in one patient during the study. These methods, as described in section 2.2, were EBV transformation, fusion with the heteromyeloma cell line CB-F7, and EBV transformation followed by fusion with CB-F7. Three patients' PBL were used to compare the three methods. Each patient had a BILAG score of 5 or 6 indicating mild to moderately active disease. The individual scores, together with DNA binding are shown in Table 3.1 (patients named in bold and underlined were used for the comparison). Table 3.2 gives the HLA typing data on these patients. The fused or EBV-transformed cells were plated out into 120 wells, the number of wells that had dividing cells and the number that secreted IgG and/or IgM were recorded (mean of the 3 patients) (Figure 3.1). The exact fusion frequencies were not calculated. It is likely that there were more than one clone per well, as more than a third of the

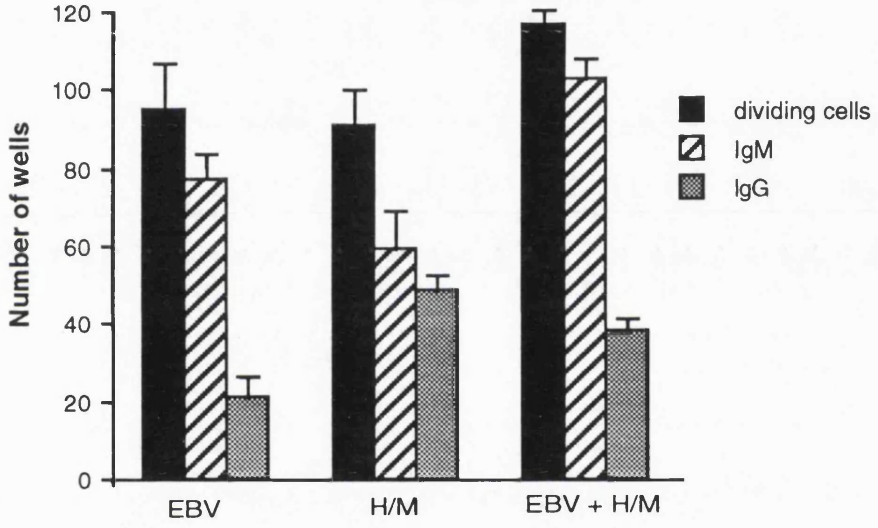
TABLE 3.1 Clinical data on the SLE patients from whom lymphocytes were obtained for use in the fusion experiments. The BILAG system was used to assess disease activity (see section 2.1). Patients printed in bold and underlined were used for comparing the three methods for generating monoclonal antibodies. M/S = musculoskeletal; VASC = vasculitis. DNA measured in international units; C3 measured in g/l.

SLE	AGE/	BILAG										
PATIENT	SEX	GENERAL	SKIN	CNS	M/S	CVS/RESP	VASC	RENAL	HAEM	GLOBAL	DNA	C3
ODO	28/F	C	C	C	C	B	C	D	D	8	418	0.61
CHR	29/F	B	D	D	C	D	D	D	C	5	612	0.9
SUM	31/F	C	D	C	C	B	D	D	D	6	173	0.94
NWA	39/F	B	D	D	D	D	D	D	C	4	50	0.73
HOB	50/F	C	D	B	D	B	D	D	D	7	242	0.96
WES	26/F	C	D	D	C	D	C	B	C	6	2284	0.6
SHA	30/F	D	D	D	D	D	D	B	B	6	240	0.77
MOR	41/F	D	D	D	D	D	C	D	B	4	1174	0.73
PUI	21/F	D	D	D	D	D	D	A	D	9	649	0.52
BOL	32/F	B	D	A	C	C	B	B	B	20	1780	0.54
ZEF	39/F	C	D	D	D	D	D	D	D	1	51	1.33
WIL	44/F	C	D	D	C	D	D	B	B	8	184	0.59
ASA	43/F	C	D	D	C	B	C	D	C	7	124	1.01
HAM-1	59/F	D	D	D	C	C	C	D	B	5	296	0.67
HAM-2	59/F	B	C	D	A	B	D	C	A	26	13140	-
MCQ	37/F	C	D	D	D	D	C	D	D	2	83	1.13
TSO	48/F	A	D	C	C	C	C	C	C	15	549	-

TABLE 3.2 HLA data on the SLE patients in Table 3.1. Gaps in the table are due to incomplete data

SLE PATIENT	HLA 'A'	HLA 'B'	HLA Cw	HLA 'D/Dr' (1)	HLA 'D/Dr' (2)	HLA 'D/Dr' (3)
ODO	2,3	35,51	Cw4	Dr4	Dr7	
CHR	3,11	7,8	Cw7	Dr2	Dr3	Drw52, Dq1, 2
SUM	2, 28	7, 27	Cw3	Dr2	Dr9	Dq1
NWA	3, 26	18,35	Cw3			
HOB	1,2	8,51	Cw2	Dr3	Dr5	
WES	1,10	8,22	Cw3	Dr3	Dr4	
SHA	2	8,12 (44)	Cw7	Dr3	Dr4	Drw52/53, Dqw2/3
MOR	2,32	44		Dr2, 5	Dq1, 3	Dw 52
PUI	2, 11	35	1			
BOL	1, 2	8		Dr3	Dr5	
ZEF	23, 32	18, 38	Cw3	Dr5		
WIL	1(10,25)	8	3	Drw52, Dqw2,3		
ASA	1, 11	51, 35	Cw4/2	Dr2	Dr6	Drw52, Dq1
HAM	2,11	22,44		Dr4	Dr9	
MCQ	23,52	18	Cw2	Dr5		
TSO	9,30	13	Cw4	Dr2	Dr7	Drw52,D9,1,7

Figure 3.1. Comparison of the three methods of producing human monoclonal IgG antibodies as described in section 3.1.1. Number of wells (60 wells used in each tissue culture plate, 2 plates used) with dividing cells, and number secreting IgG and IgM, generated from Epstein-Barr virus (EBV) transformation, direct fusion with CB-F7 (heteromyeloma, H/M), and EBV transformation followed by direct fusion (EBV+H/M). The peripheral blood lymphocytes from three SLE patients were used for the comparison and the mean and standard deviation shown. When the heteromyeloma was used alone the highest proportion of IgG to IgM containing wells was obtained.



wells in the plate contained dividing cells (see subcloning, section 2.2.5). The goal was to identify which method produced the highest number of IgG and the lowest number of IgM secreting hybridomas. A high fusion frequency which led to a substantial number of non-secretors was also undesirable since the hybridoma cells of interest would be overgrown more quickly.

The highest ratio of wells containing IgG to wells containing IgM immunoglobulin was obtained by direct fusion with CB-F7. EBV transformation increased the number of wells containing IgM. Fusion of EBV transformed cells appeared to increase further the number of IgM secretors, which may be due to an increased fusion frequency observed with this method. In order to compare the success of subcloning of the derived cell lines, two IgM anti-DNA antibodies obtained from the three different methods were subcloned once according to the method in section 2.2.5. Whereas the four cell lines derived from the fusion with CB/F7 and EBV transformation followed by fusion were subcloned successfully, the two cell lines derived from the EBV transformed cells did not survive subcloning. Subcloning the antibodies derived from the fusions with CB/F7 required a "feeder" layer for subcloning. This enabled cells plated out singly (or less) to divide. This feeder layer consisted of mouse peritoneal macrophages. Attempts to substitute this feeder layer with a commercially available 'feeder replacement' (Sigma) were unsuccessful in that no cells grew from any plate that had less than 10 cells/well which was no improvement on subcloning cells without any supplements. Irradiated human peripheral blood lymphocytes or medium from the macrophage line J774 were used to enhance the subcloning of EBV transformed cells on three occasions. The specific antibody was not recovered in any well that had less than 50 cells.

3.1.2 One patient's lymphocytes yielded IgG anti-DNA antibodies.

Following the comparison of the three methods for generating IgG monoclonal antibodies, direct fusion of the peripheral blood lymphocytes with CB/F7 was used in all further fusion experiments. Sixteen patients' lymphocytes were fused directly with CB/F7 (excluding the number that had infection rendering the plates unusable); 12 of the fusions yielded dividing cells. The clinical and HLA data of the 16 patients (seventeen fusions in all) are shown in Table 3.1 and 3.2 respectively. Six of the patients whose PBL were fused directly with CB-F7 had BILAG global scores of less than six i.e. mild or inactive disease. Nine had scores of between 6 and 15, whilst two had high scores of 20 and 26 i.e. very active disease. All of the fusions were screened for IgM and IgG DNA binding antibodies. Nine out of twelve fusions that generated dividing cells secreting immunoglobulin yielded IgM anti-DNA antibodies. The number of wells which contained IgM anti-DNA antibodies from each fusion is shown in Table 3.3 as well as the number of wells that contained IgG and IgM antibodies. Only one fusion yielded IgG anti-DNA antibodies. Interestingly, this patient had an exceptionally high BILAG score of 26 and high DNA binding [13140 U/ml] (Table 3.1, patient HAM-2). Six of the fusions were also screened using DNAase treatment of the supernatant to remove the presence of competing DNA in the supernatant. This did not alter the binding of any of the IgM anti-DNA antibodies produced but did result in an increase in DNA binding of two IgG anti-DNA antibodies (see below).

Table 3.3 Number of wells containing monoclonal antibodies generated from the fusion experiments.

Number of wells containing IgM and IgG; and IgM and IgG anti-DNA antibodies generated from the fusion experiments using the heteromyeloma cell line CB-F7. Note: Dividing cells were present in the fusions marked * but total IgG and IgM were not assayed.

SLE PATIENT	IgM ANTI-DNA	IgG anti-DNA	IgG Total	IgM Total
CDO	0	0	0	0
CHR	7	0	44	45
SUM	5	0	54	68
NWA	11	0	66	71
HOB *	0	0	N.D.	N.D.
WES	6	0	23	40
SHA	3	0	31	76
MOR	0	0	0	0
PUI	0	0	0	0
BOL	6	0	39	62
ZEF	0	0	45	98
WIL	3	0	42	59
ASA	0	0	0	0
HAM 1	5	0	50	65
HAM 2	0	5	75	32
MCQ	0	0	0	0
TSO *	3	0	N.D.	N.D.

3.1.3 Change in the isotype and specificity of monoclonal antibodies produced from patient HAM when disease was inactive to when disease was active.

Five IgG anti-DNA antibodies were produced from patient HAM-2 when her disease was active (BILAG score 26). Six months earlier when her disease had been less active five IgM anti-DNA antibodies were generated (HAM-1). As seen in Table 3.3, when the five IgG anti-DNA antibodies were produced no IgM anti-DNA antibodies were generated and, conversely, no IgG anti-DNA antibodies were generated in the first fusion. The BILAG scores and the DNA binding titre on the two occasions are shown in Table 3.1

The specificity of the two sets of monoclonal antibodies derived from patient HAM were also different (Table 3.4). Binding was expressed qualitatively as described in section 2.3.5, + and ++ values (i.e. at least five times the median value of the supernatants tested) were considered positive. [Median binding of the supernatants was between 0.00 and 0.050 for the antigen binding assays indicating the high degree of specificity when these assays are used to detect monoclonal antibodies]. The anti-DNA activity of the IgM antibodies was of relatively low affinity as demonstrated by the fact that none bound to the kinetoplast of *Crithidia luciliae*. This was reflected by the serum which was also negative in the *C. luciliae* assay. However, on the second occasion when the IgG fraction of the sera was positive in the *C. luciliae* assay, two IgG anti-DNA antibodies (B3 and D2) also bound to the kinetoplast of *C. luciliae*. Moreover, the ELISA reading of IgM anti-dsDNA antibodies in the sera reduced by almost four fold, whilst the IgG anti-dsDNA serum reading increased slightly, when the patient had active disease compared to the serum ELISA readings when the patient had inactive disease. (The ELISA readings of the sera are shown by optical density in Table 3.4).

TABLE 3.4 Binding characteristics of the five IgM and five IgG anti-DNA antibodies produced from patient HAM on two different occasions expressed qualitatively (see section 3.1.3). Also shown are the binding characteristics of the patient's serum taken at the same time (Optical density). ANA = immunofluorescent pattern on HEP-2 cells: cyto = cytoplasmic, homo = homogeneous.

	Monoclonal antibodies	ssDNA	dsDNA	cardiolipin	Histones	ANA	Crithidia
Fusion 1	IgM 1	+ +	-	+	-	cyto	-
Patient	2	+	+	+ +	-	cyto+nucleolar	-
inactive	3	+ +	-	+ +	-	cyto	-
	4	+ +	-	-	-	neg	-
	5	+ +	-	-	-	neg	-
Matched serum	IgM sera	0.519	0.402	0	0.386	cyto	-
results	IgG sera	0.457	0.379	0	0.381	cyto	-
Fusion 2	IgG B3	+ / -	+ +	+	+ +	homo + rim	+ +
Patient	D2	-	+ +	-	+ +	fine speckled	+ +
active	E7	+	-	-	-	cyto	-
	F8	+ +	-	-	-	cyto	-
	D5	+ +	+ / -	-	-	cyto+nucleolar	-
Matched serum	IgM sera	0.243	0.12	0	0.116	cyto	-
results	IgG sera	0.418	0.391	0	0.527	speckled	+ +

The DNA binding of two of the IgG antibodies by ELISA was increased by treatment with DNAase (Figure 3.2, B3 shown). These antibodies would not have been detected initially if the supernatant had not been treated by DNAase. This was due to the low concentration of B3 in the supernatant (0.23μg/ml) of the 96 well plate derived directly from the fusion. When the DNA binding activity of supernatants containing the antibody at concentrations above 1 μg/ml was measured, no DNAase treatment was necessary for a significant OD reading to be recorded. At concentrations above 5 μg/ml DNAase treatment had little effect on the binding. These two antibodies also bound histones, though in the case of B3 this binding was reduced by DNAase treatment of the supernatant (Figure 3.2). None of the IgM anti-DNA antibodies reacted with histones. Three of the IgM anti-DNA antibodies bound cardiolipin, two strongly (++); whereas only one IgG anti-DNA antibody reacted moderately (+) with cardiolipin. Interestingly, whereas on both occasions there was serum reactivity with histones, the patient did not have serum anti-cardiolipin antibodies at either time. Thus on the second occasion the monoclonal antibodies more closely resembled the serological findings than on the first.

3.1.4 Selection of antibodies for further study

Two IgG anti-DNA antibodies known as B3 and D5 were selected for further study. Out of the initial set of five IgG anti-DNA antibodies only these two survived three rounds of cloning and continued to secrete immunoglobulin. Both monoclonal antibodies were of the IgG1 isotype as determined by a subclass ELISA. B3 bound predominantly dsDNA but also weakly to ssDNA in the solid and fluid phase DNA ELISAs (Figure 3.3). The binding to histones was reduced by DNAase treatment and the addition of DNA to B3 (DNA concentration≤1 μg/ml) further increased the binding to histones (Figure 3.4).

Figure 3.2 The effect of DNAase on the binding of B3 in supernatant to histones and DNA. Whereas the binding of B3 to histones by ELISA was reduced by pretreating the supernatant with DNAase (first two columns), the binding to DNA by ELISA was increased (second two columns). Thus the DNA in the supernatant acted as a bridge for the B3 to bind to histones, but acted as an inhibitor of the binding of B3 to DNA.

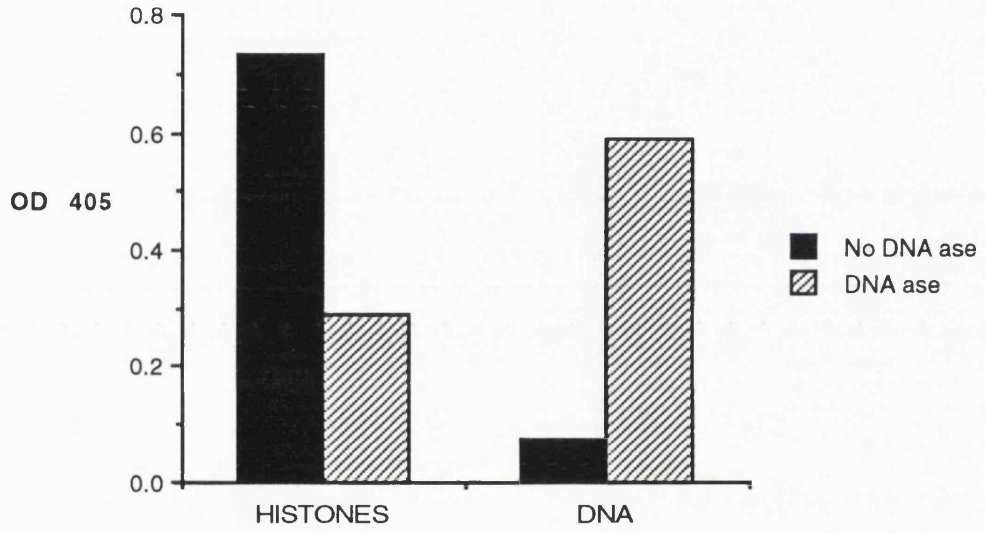
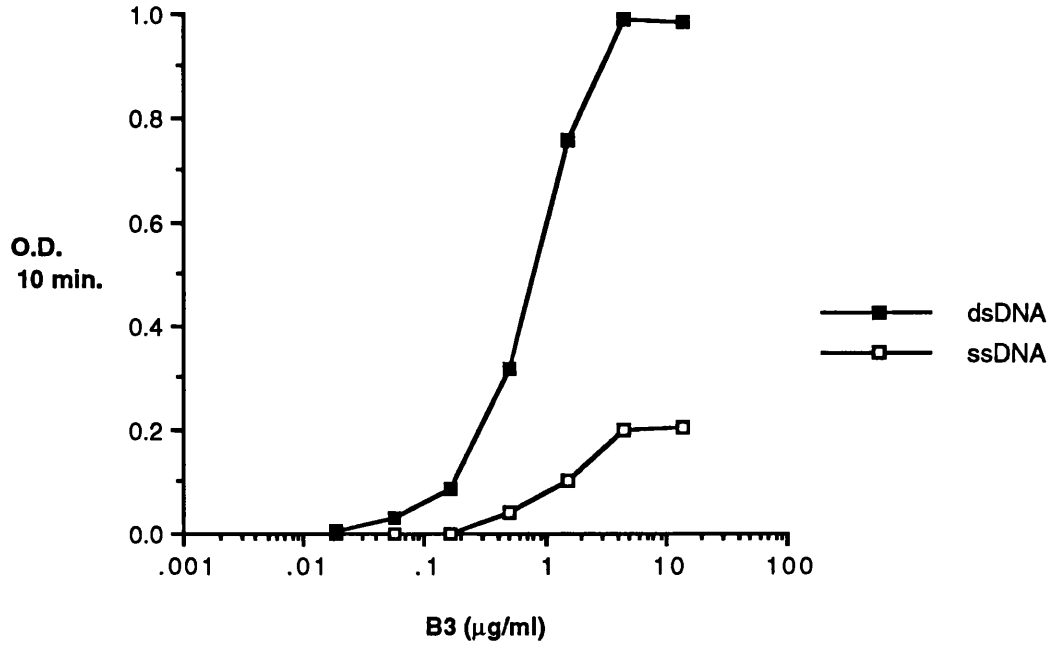


Figure 3.3 The binding of B3 to ss and dsDNA in solid (a) and fluid phase (b) ELISA. The fluid phase ELISA was performed using ss and dsDNA separately as inhibitors of B3 binding to dsDNA on the plate. .



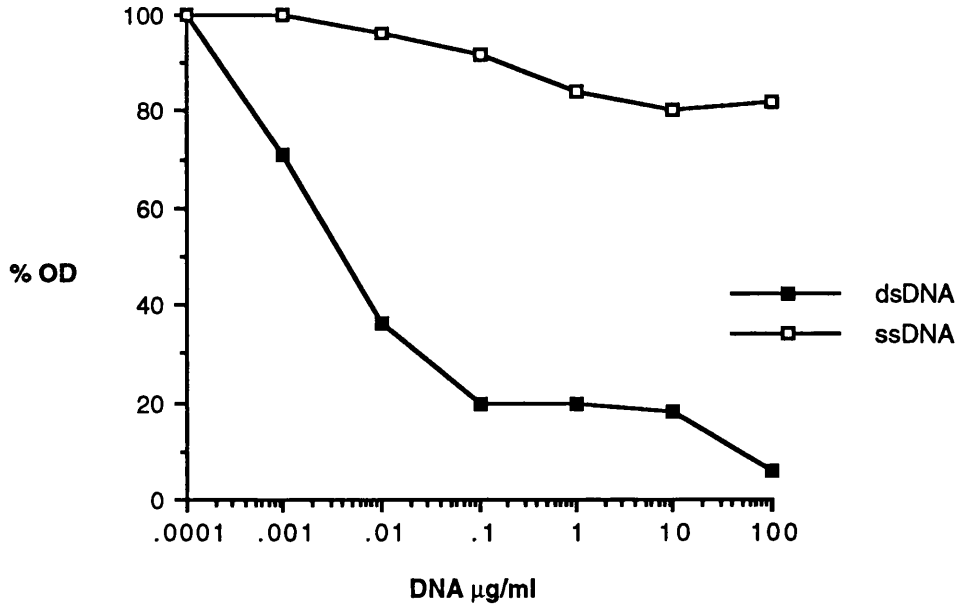
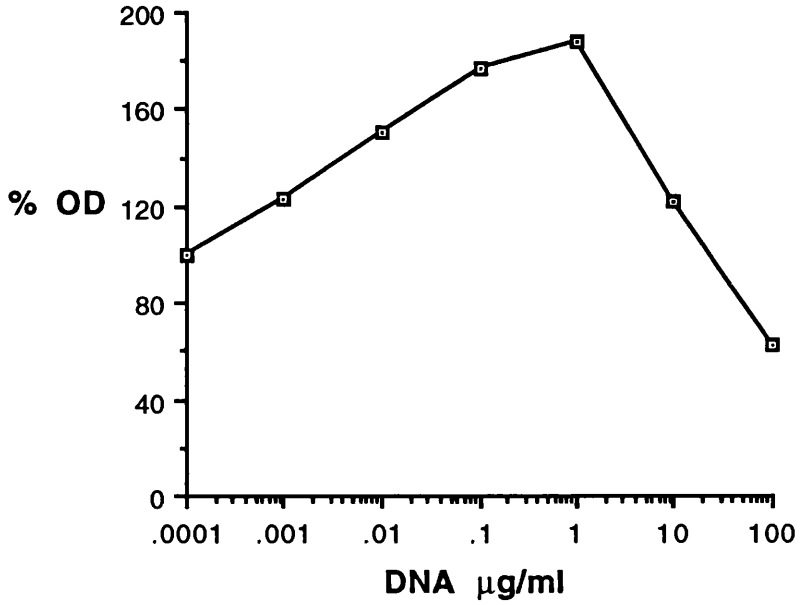


Figure 3.4 DNA in the fluid phase alters the binding of B3 to histones. In this ELISA DNA is in the fluid phase acting as an "inhibitor" of the binding of B3 to histones. Below a concentration of 1 μg/ml of DNA the addition of DNA increased the binding of B3 to histones.



The concentration of inhibitor required to give 50% inhibition of binding to ss and dsDNA is shown in Table 3.5. B3 bound to the kinetoplast of *C. luciliae* (Figure 3.5a) and gave a homogeneous and rim pattern on HEP-2 cells (Fig 3.6a).

D5 bound predominantly to ssDNA in solid and fluid phase ELISA but also weakly to dsDNA (Figure 3.7). D5 did not bind to histones (either alone or when D5 is preincubated with DNA) or cardiolipin, nor was its binding to DNA increased by DNAase treatment. The concentration of inhibitor required to give 50% inhibition of binding to ss and dsDNA is shown in Table3.5. D5 did not bind to the kinetoplast of *C. luciliae* (Figure 3.5b) but did bind to HEP-2 cells in a cytoplasmic and nucleolar pattern (Fig 3.6b).

Table 3.5 Concentration of inhibitor (μg/ml) required to give 50% inhibition of the binding of B3 and D5 to DNA. (mAb = monoclonal antibody)

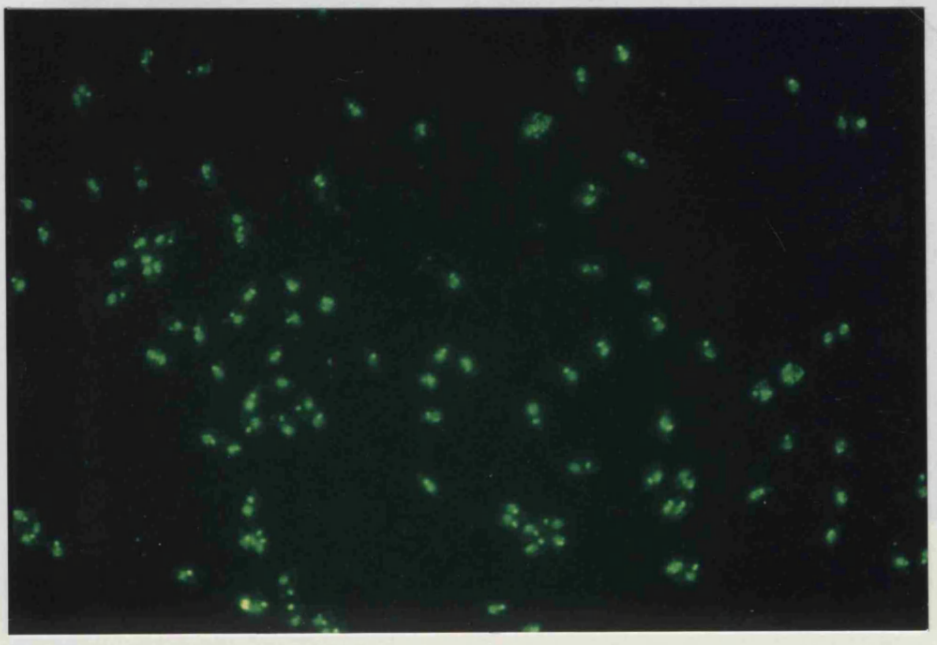
	Autoantigen	
mAb	dsDNA	ssDNA
B3	0.008	>100
D5	>100	0.9

3.1.5 Bulk culture and affinity purification

The monoclonality of B3 and D5 was assumed after each had been subcloned three times and subsequently confirmed by sequencing the DNA derived from the hybridomas. Only one light chain class was detected by ELISA. The light chain of B3 was lambda and of D5 kappa. The cells were

Figure 3.5 Immunofluorescence photograph showing the binding of (a) B3 and (b) D5 to *Crithidia luciliae.* Whereas B3 bound both to the kinetoplast (containing the dsDNA) and the nucleus, D5 bound neither. (Magnification x 40).

(a)



(b)

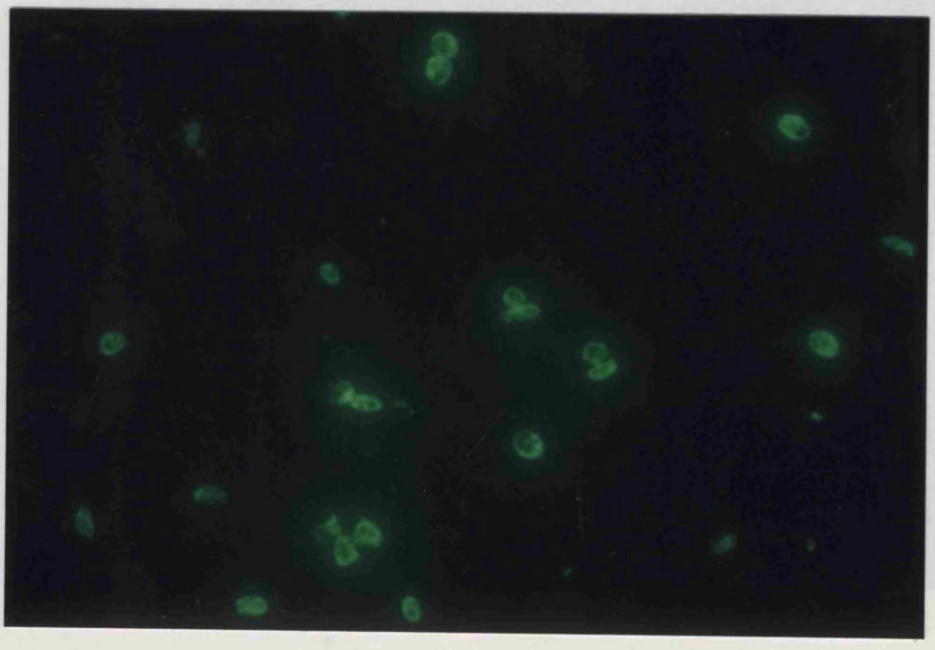
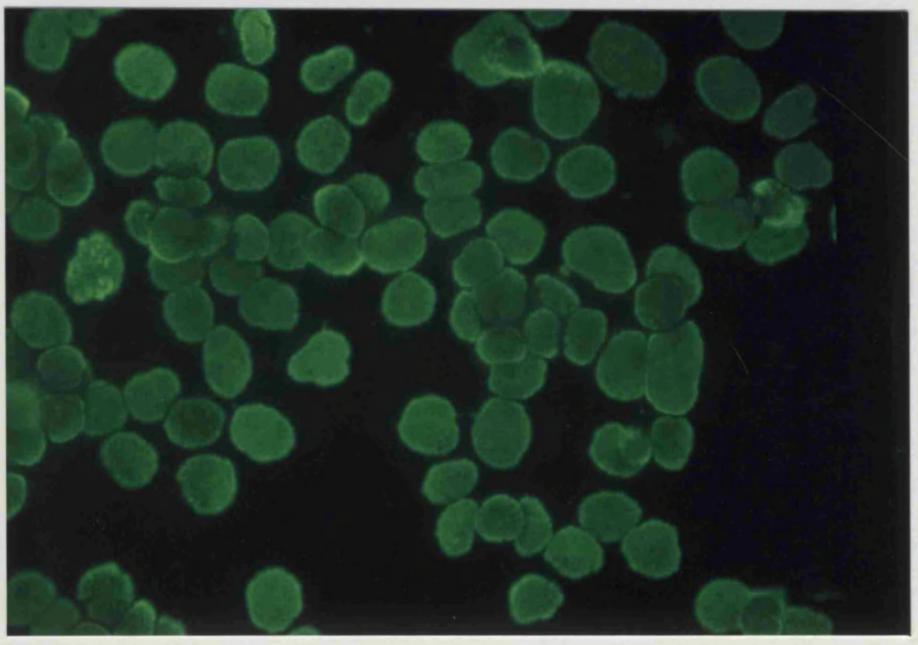


Figure 3.6 Immunofluorescence photograph showing the binding of (a) B3 and (b) D5 to HEP-2 cells. B3 gave a homogeneous and rim pattern whilst D5 bound to the cytoplasm and nucleoli. (Magnification x 40).

(a)



(b)

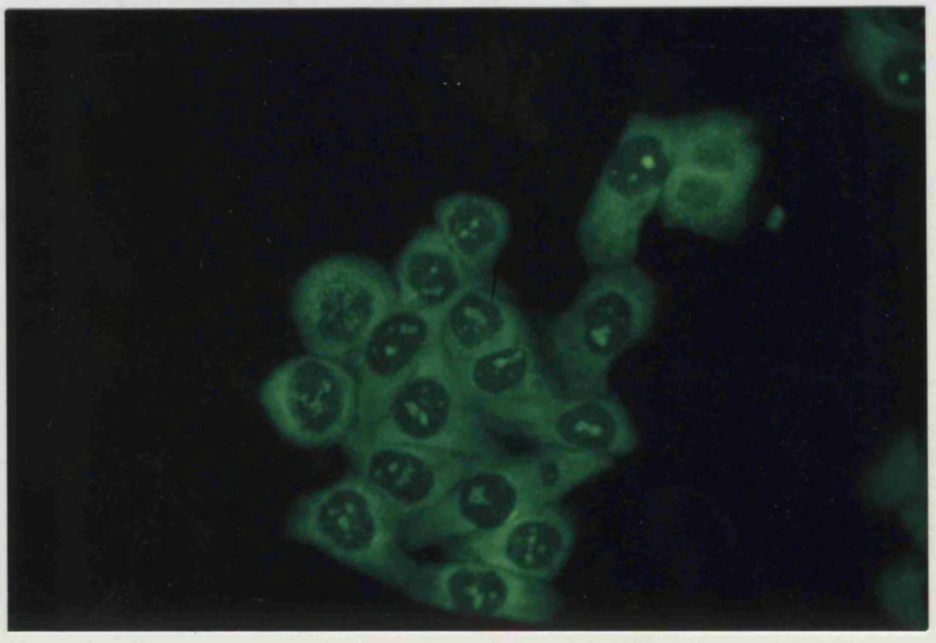
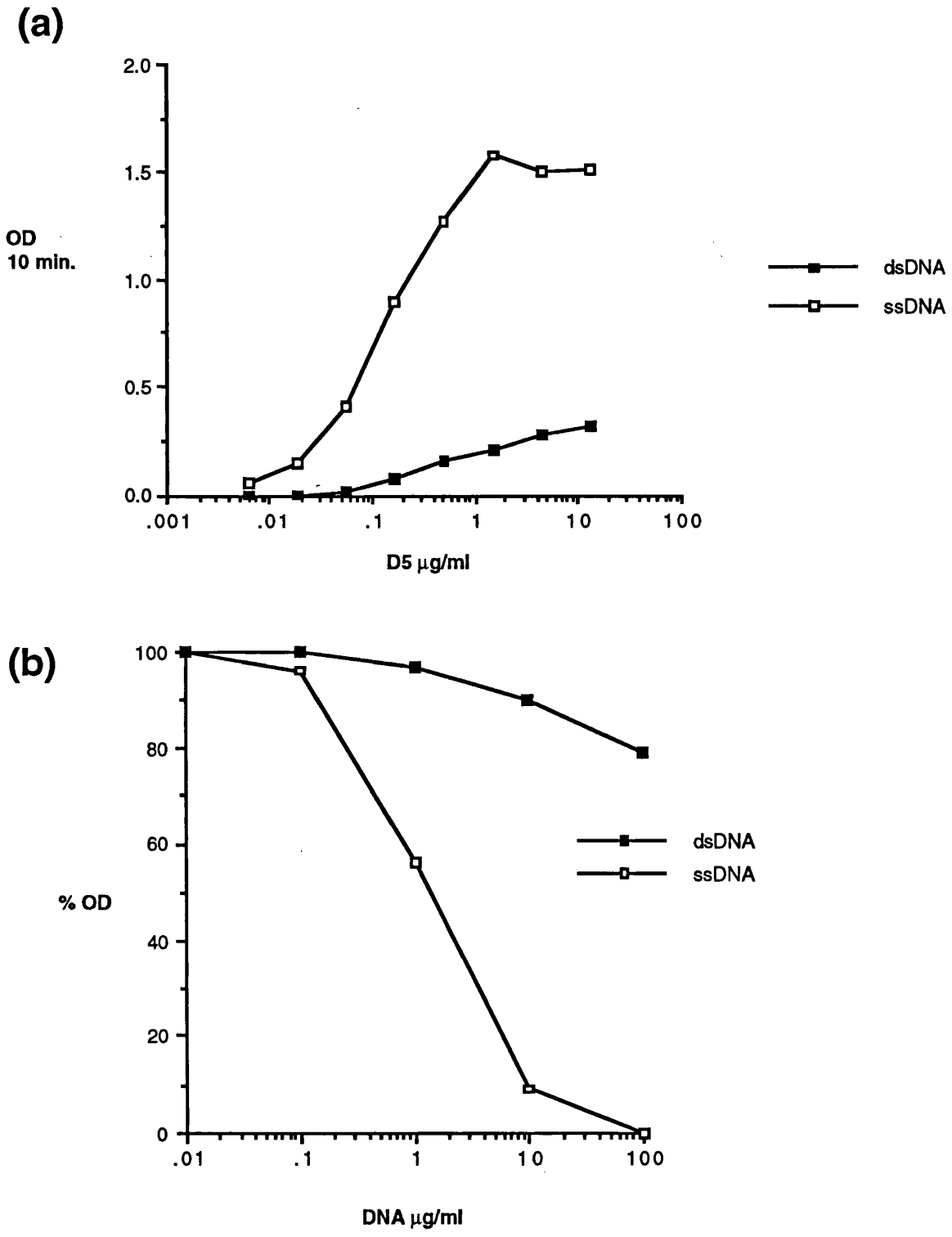


Figure 3.7 The binding of D5 to ss and dsDNA in solid (a) and fluid phase (b) ELISA. The fluid phase ELISA was performed using ss and dsDNA separately as inhibitors of D5 binding to ssDNA on the plate.



transferred to serum-free medium for bulk culture. Figure 3.8 shows the change of immunoglobulin concentration during this period and the immunoglobulin concentrations in the stirrer flasks.

Growth of cells in 500 ml continuous stirrer culture flasks resulted in an increased yield of immunoglobulin only for the D5 cell line, and by a factor of approximately 10. The D5 cell line was maintained in the stirrer flasks for 5 months whereas the B3 cell line stopped secreting after only six weeks.

The two monoclonal antibodies were purified using protein G column as described in section 2.2.6. The column was eluted sequentially with 3M NaCl and glycine-HCL, pH 2.7. Whereas B3 yielded a large peak for the 3M NaCl eluate equivalent to 1.197 mg of DNA, applying 3M NaCl to the protein G when D5 was bound yielded a much smaller peak (equivalent to 0.01 mg of DNA). Similar peaks were obtained when the acid elution step was performed for both B3 and D5 (for quantities see table 3.6). The optical densities of the 3M NaCl and acid eluate for one purification round using 1.5 litres of supernatant containing the two antibodies are shown in table 3.6. The capacity of the protein G column was never exceeded being in the region of 25 mg of human IgG (5 ml column, data provided by Pharmacia). All the Ig in the supernatant was removed by the column. The eluate corresponding to the 3M peak was subjected to agarose gel electrophoresis with ethidium bromide to look for the presence of DNA (Figure 3.9a). The DNA bands appeared in a ladder pattern on the gel, with the majority of DNA sized at approximately 200 kilodaltons. A similar pattern was obtained when the DNA in the supernatant of B3 (serum free) was isolated and examined by agarose gel electrophoresis confirming that the DNA derived from the column was obtained from the supernatant (Figure 3.9b).

Figure 3.8 Change in secretion of mAb by hybridoma cell lines during transfer from medium containing 10% fetal calf serum in standing flasks to serum free medium and into stirrer flasks. $5x10^6$ cells were transferred to tissue culture flasks (Nunc) containing an increased proportion of SF medium at regular intervals (25% SF medium in the 1st week, 50% SF in the second week, 75% SF in the third week and 100% SF in the fourth week). To assess the concentration of immunoglobulin in the cell cultures, supernatant was extracted from the flasks three days after each transfer. See section 2.2.6.

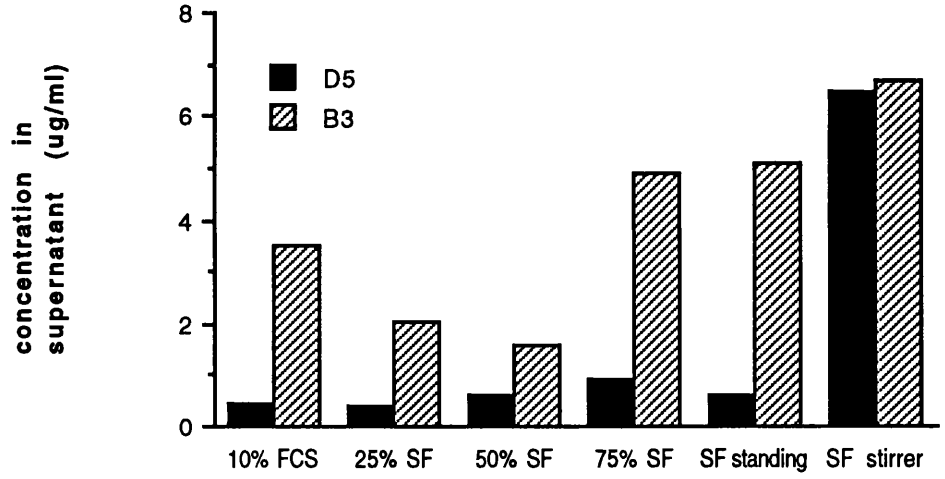
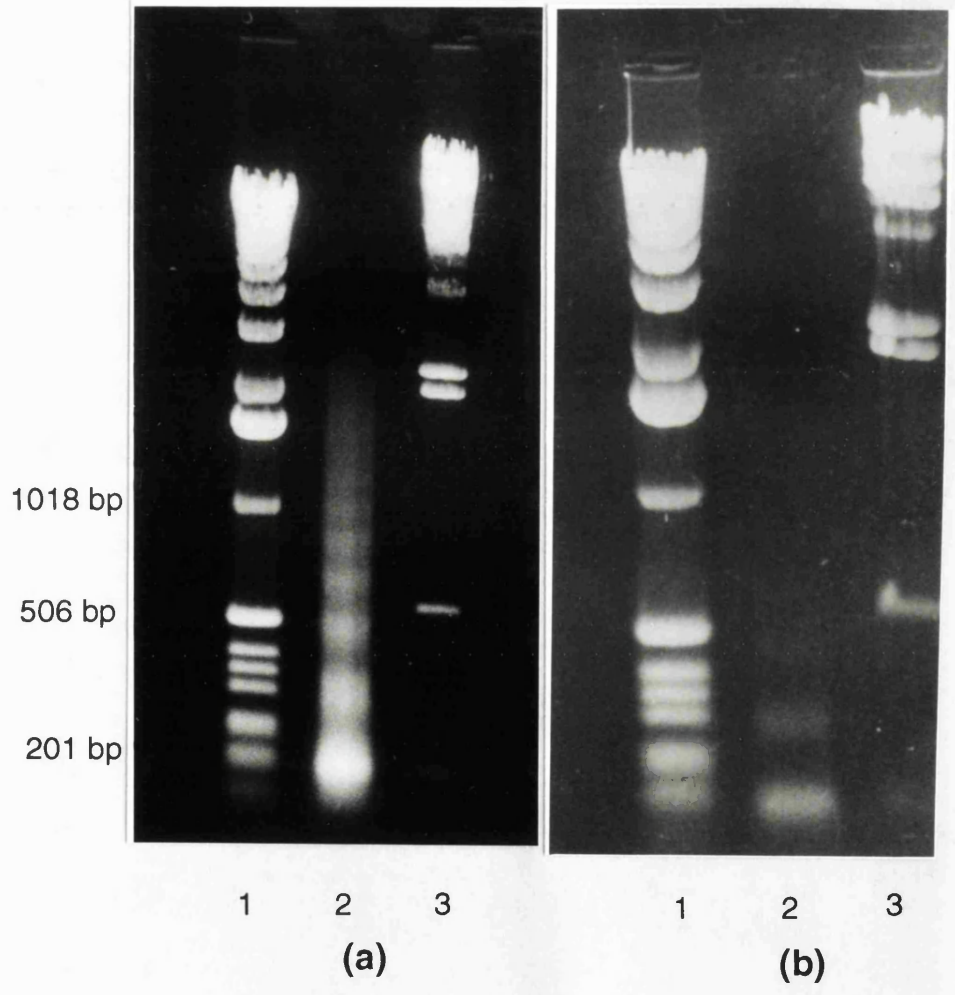


Table 3.6 Optical density of eluates from a protein G column loaded with 1.5 litres of supernatant (serum free) containing either B3 or D5 and washed with 3M NaCl followed by glycine-HCL, pH 2.7. Whereas there was a similar yield of immunoglobulin from the two supernatants, there was considerably more DNA obtained from the protein G column when loaded with supernatant containing B3 after elution with 3M NaCl. (Optical density at 160 nm of 1.0 = 50 µg DNA.)

Optical density	Volume (ml)	260 nm	280nm	Ab (mg)	DNA (mg)
3M eluate: B3	10	2.395	1.39	-	1.197
ACID eluate: B3	6	0.8	1.661	5.9	-
3M eluate: D5	3	0.23	0.208	-	0.010 (aprox)
ACID eluate: D5	15	0.344	0.684	6.2	-

Figure 3.9 Analysis of the DNA complexed to B3. An agarose gel is shown below indicating the size of (a) the DNA present in the eluate obtained when the protein G column (loaded with supernatant containing B3) was washed with 3MNaCl and (b) the DNA extracted from the serum free supernatant of hybridomas secreting B3. Lanes 1 and 3 are molecular weight markers: 1Kb DNA ladder and λ DNA/Hind III fragments respectively; lane 2 contains the sample DNA.



3.2 ANALYSIS OF IGG ANTI-DNA ANTIBODY IDIOTYPES

3.2.1 Production of two polyclonal anti-idiotype reagents

Two polyclonal anti-idiotypes were generated against B3 and D5 using two rabbits. Each rabbit was injected with purified antibody and incomplete Freund's adjuvant (IFA) according to the method described in section 2.4.1. The sera of these rabbits were collected throughout the immunisation course (0,2,5,7,10 and 12 [final bleed] weeks) and activity against pooled human IgG, the immunising antigen and DNA was assessed. Figure 3.10 shows the time course of the responses of the rabbits injected with D5 and B3 against the immunising mAb and pooled IgG. There was increased binding to the immunising antigen above that of pooled IgG only after the third boost of mAb. Interestingly, the rabbit injected with B3 developed DNA binding antibodies in the serum that reacted with both ssDNA and dsDNA (Figure 3.11). There was a transient peak in the antibody response to ssDNA in the rabbit injected with D5 but no anti-DNA antibodies were detected in the control rabbit injected with IFA alone. Of relevance is the fact that the anti-idiotypic antibodies purified from the rabbit injected with B3 did not bind to DNA indicating that the B3 anti-Id antibodies did not show epibody activity.

To purify the anti-idiotypic antibodies the rabbit serum (final bleed) was passed over a human IgG and IgM-sepharose column until no anti-pooled IgG or IgM activity was detectable. To remove irrelevant specificities the serum was then passed over its homologous mAb sepharose column. The anti-idiotypic antibodies derived from the rabbits immunised with B3 or D5 are designated anti-Id B3R and anti-Id D5R respectively which recognise the idiotypes B3-RId and D5-RId respectively.

Figure 3.10 Specificity of the unpurified sera derived from the rabbits injected with the monoclonal antibodies B3 and D5. Serum dilution = 1/40,000. Before use as an anti-idiotypic reagent the serum was passed over a pooled human immunoglobulin column until no further anti-human Ig could be detected. Further purification involved the use of a column coupled with the homologous monoclonal antibody.

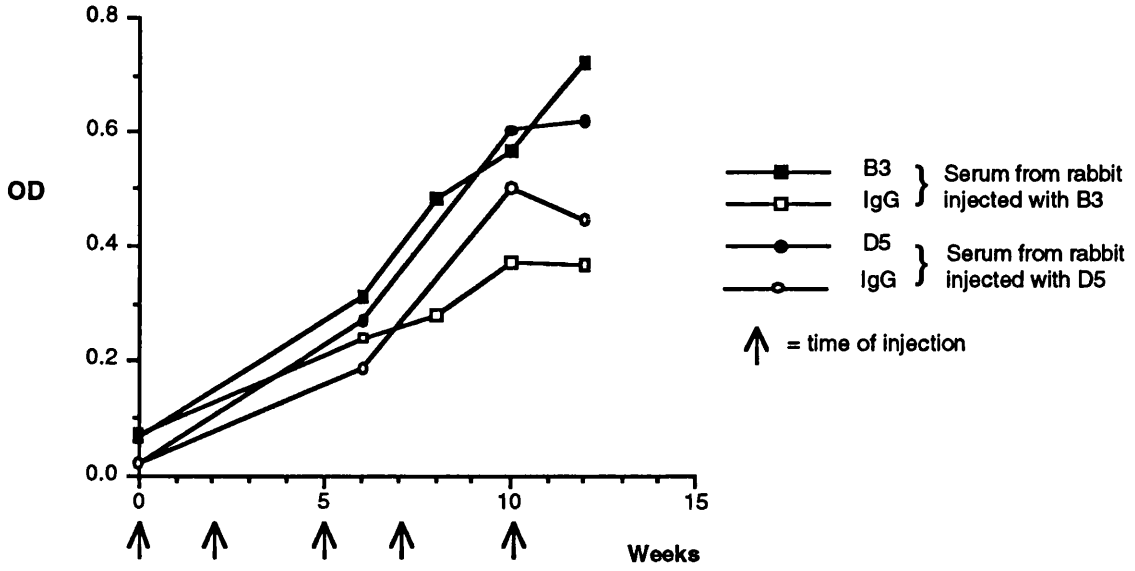
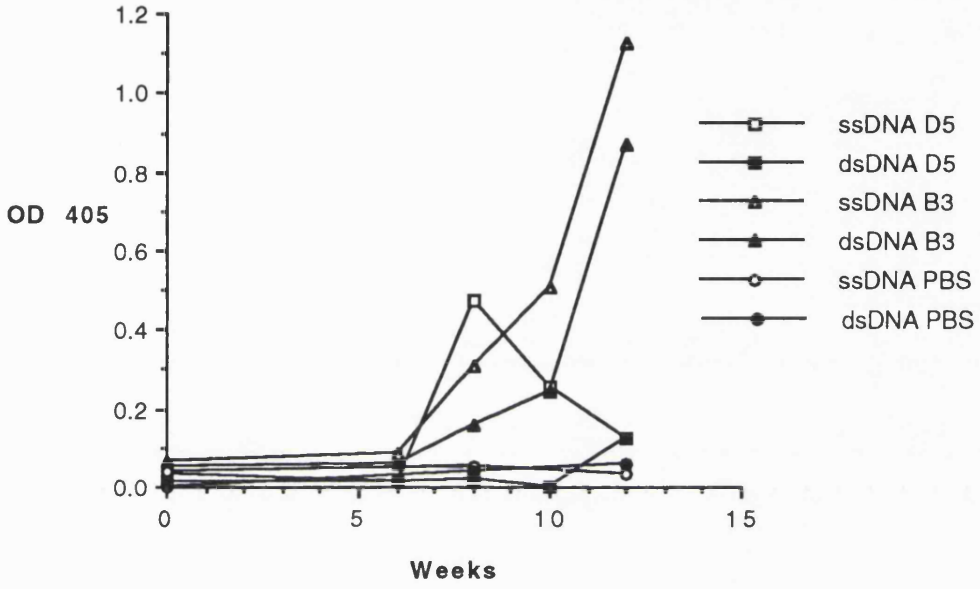


Figure 3.11. The binding of the unpurified sera (derived from the rabbits injected with B3, D5 and PBS) to DNA. Serum dilution = 1/100. The titre of antibody was much less than that seen for the anti-immunoglobulin response. ssDNA D5 = the binding of the rabbit sera injected with D5 against ssDNA etc.



3.2.2 Characterisation of D5-RId and B3-RId.

The specificities of anti-Id B3R and anti-Id D5R in solid and fluid phase ELISAs are shown in Figures 3.12 and 3.13 respectively. Both anti-Ids bound to their homologous mAb in solid phase ELISA, but not to pooled IgG (the binding of anti-Id B3 to D5 and vice-versa was similar to pooled IgG i.e. they did not bind to the other monoclonal antibody, data not shown). The concentration required to achieve 50% maximal binding for anti-Id B3R and anti-Id D5R in the solid phase ELISA was 0.08 μg/ml and 0.07 μg/ml respectively. In fluid phase assays the mAb inhibited the binding of its homologous anti-Id to solid phase mAb. The concentration of B3 and D5 that inhibited 50% of the binding of their homologous anti-Id to solid phase B3 and D5 was 0.4 μg/ml and 0.9 μg/ml respectively. Pooled IgG did not inhibit the binding of the anti-Ids to their homologous mAb.

The location of the idiotypes on the light chains of B3 and D5 were revealed by SDS-PAGE of the reduced MAb followed by Western blotting. Both idiotypes were found on the light chain of the antibody (Figure 3.14). In order to determine whether this method was able to locate the idiotype exclusively to one immunoglobulin chain, increased concentrations of B3 and anti-Id B3R were used. The intensity of staining was analysed using a densitometer (Figure 3.15). The legend indicates the concentrations used. No binding to the heavy chain was found with higher concentrations of the idiotype or anti-idiotype above that seen with the normal rabbit serum used as a control. Thus the heavy chain does not appear to contribute to the idiotypes.

In order to ascertain whether the idiotype was located at or near the antigen binding site for DNA a fluid phase inhibition ELISA was used as described in section 2.3.13. dsDNA was able to inhibit the binding of B3 to anti-Id B3R whereas ssDNA did not (Figure 3.16a). Conversely, ssDNA but not dsDNA inhibited the binding of D5 to its homologous anti-Id (Figure 3.16b). This

Figure 3.12 Specificity of anti-Id B3R. (a) Binding of anti-Id B3R to solid phase B3 and pooled human IgG as measured by ELISA. (b) Inhibition of the binding of anti-Id B3 to B3 in solid phase by fluid phase B3 and pooled IgG. %OD = % binding of anti-Id B3 to B3 compared to the binding without inhibiting antigen.

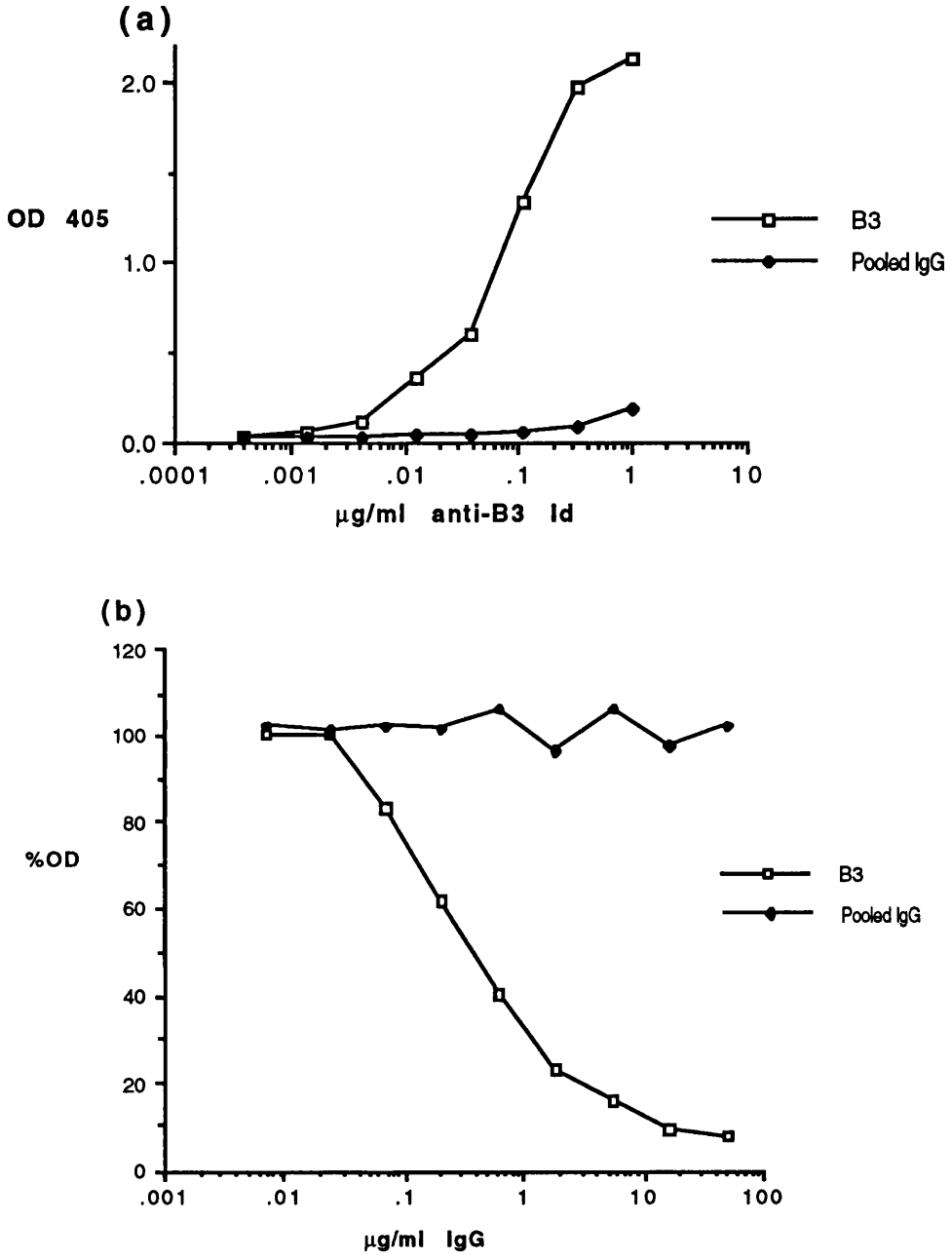


Figure 3.13. Specificity of anti-Id D5M and anti-Id D5R. (a) Binding of rabbit and mouse anti-Ids to D5 and pooled IgG in solid phase by ELISA. Results are expressed as optical density (OD) readings. (b) Inhibition of the binding of rabbit and mouse anti-Ids to D5 in solid phase, by fluid phase D5 and pooled IgG. %OD= % binding of anti-Id to D5 compared to the binding without inhibiting antigen.

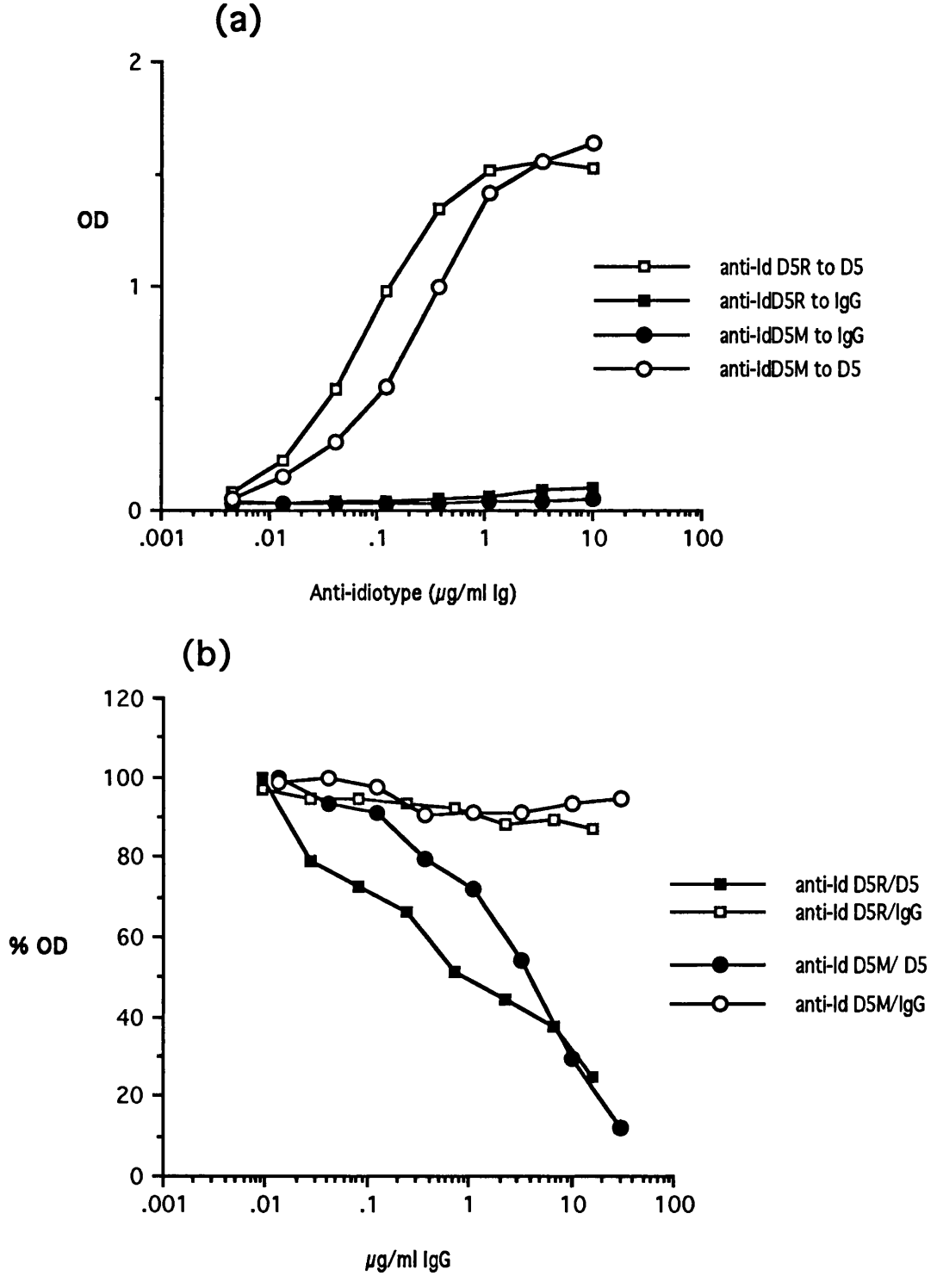


Figure 3.14 B3-RId and D5-RId are located on the light chain of their homologous mAb. (a) Western blots of reduced B3 (1 μg/lane) run on a 10% polyacrilamide gel, stained with anti-Id B3 (lane 1), rabbit anti-human λ (lane 2), rabbit anti-human γ (lane 3), normal rabbit serum (lane 4), PBS (lane 5).

(b) (page 141) Western blot of reduced D5 run on a 10% polyacrilamide gel, stained with rabbit anti-Id (lane A), rabbit anti-human κ (lane B), rabbit anti-human γ (lane C), normal rabbit serum (lane D), PBS (lane E).
Both B3-RId and D5-RId are situated on the light chain.

(a)

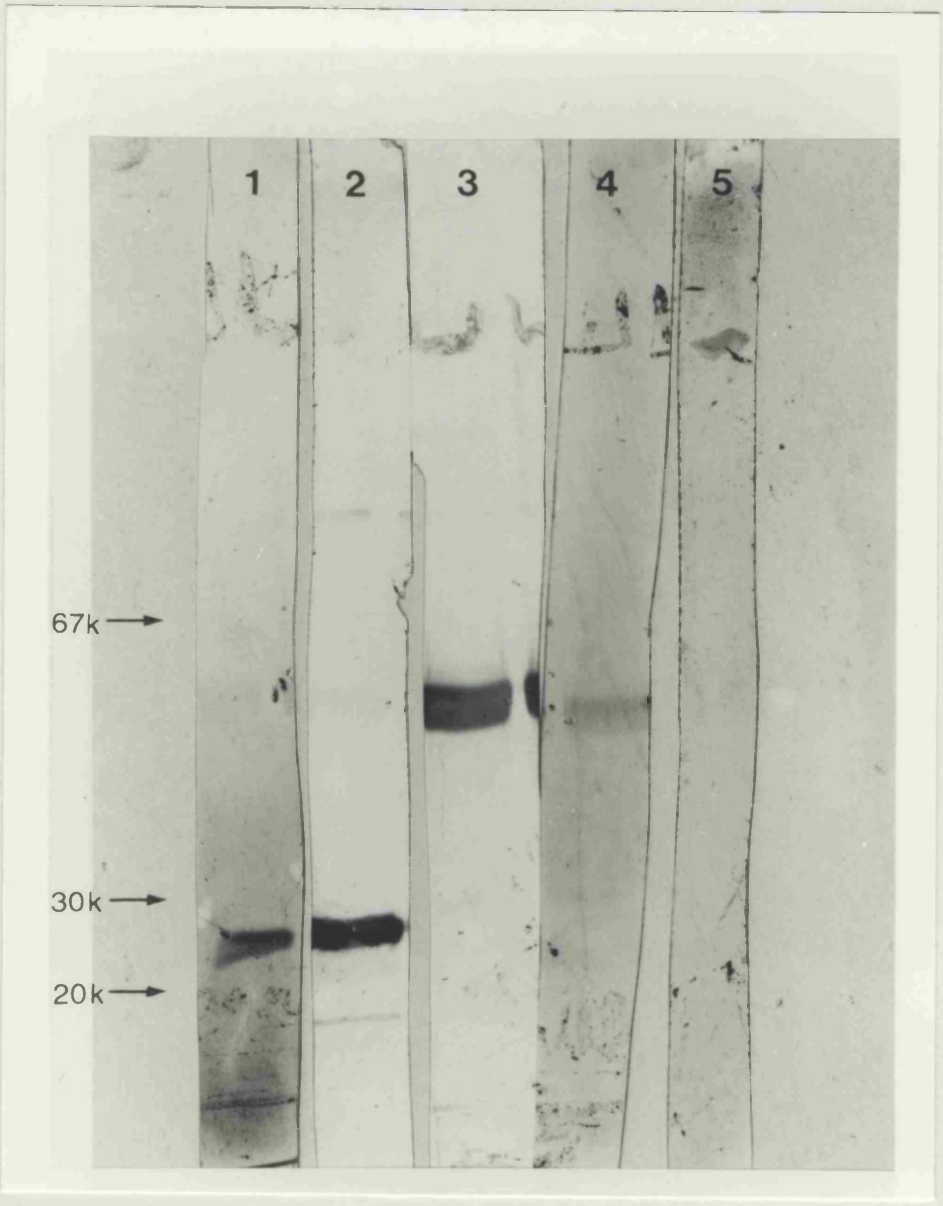


Figure 3.14 (continued)

(b)

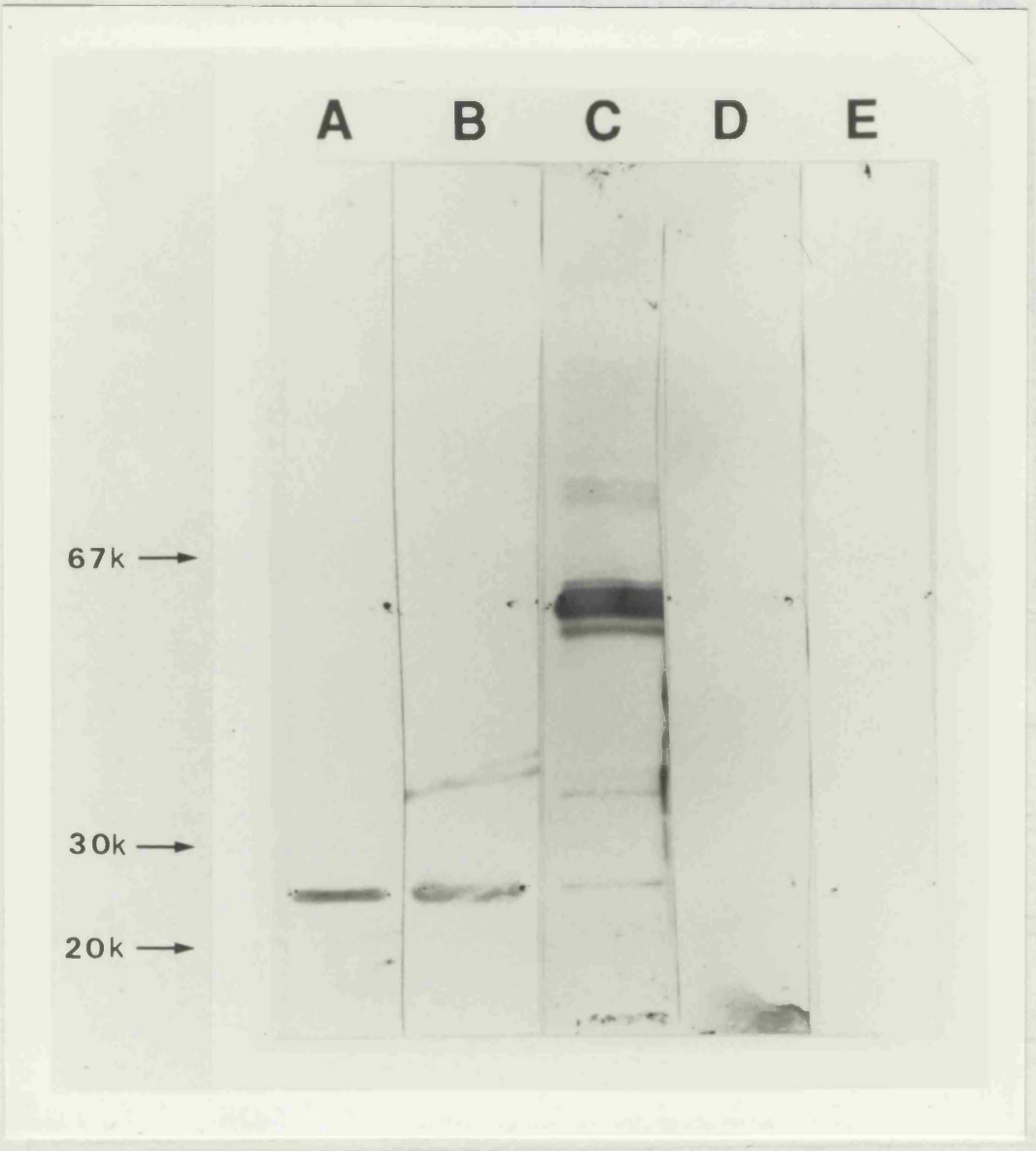


Figure 3.15. Analysis of Western blots by densitometry using higher concentrations of B3 and anti-Id B3R than in figure 3.14(a). Lanes 1-6 represent blots with 5 μg/lane of mAb B3 ; lanes 7-12 have 10 μg/lane of B3. Lanes 1-3 and 7-9 were incubated with increasing concentrations of anti-Id (0.5μg/ml, 2 μg/ml and 10μg/ml); lanes 4 and 10 were incubated with 2 μg/ml of normal rabbit serum; and lanes 5 and 11, and 6 and 12 were incubated with anti- λ and anti-γ chain respectively. There is no significant binding of the anti-Id to the heavy chain above that seen with the normal rabbit serum.

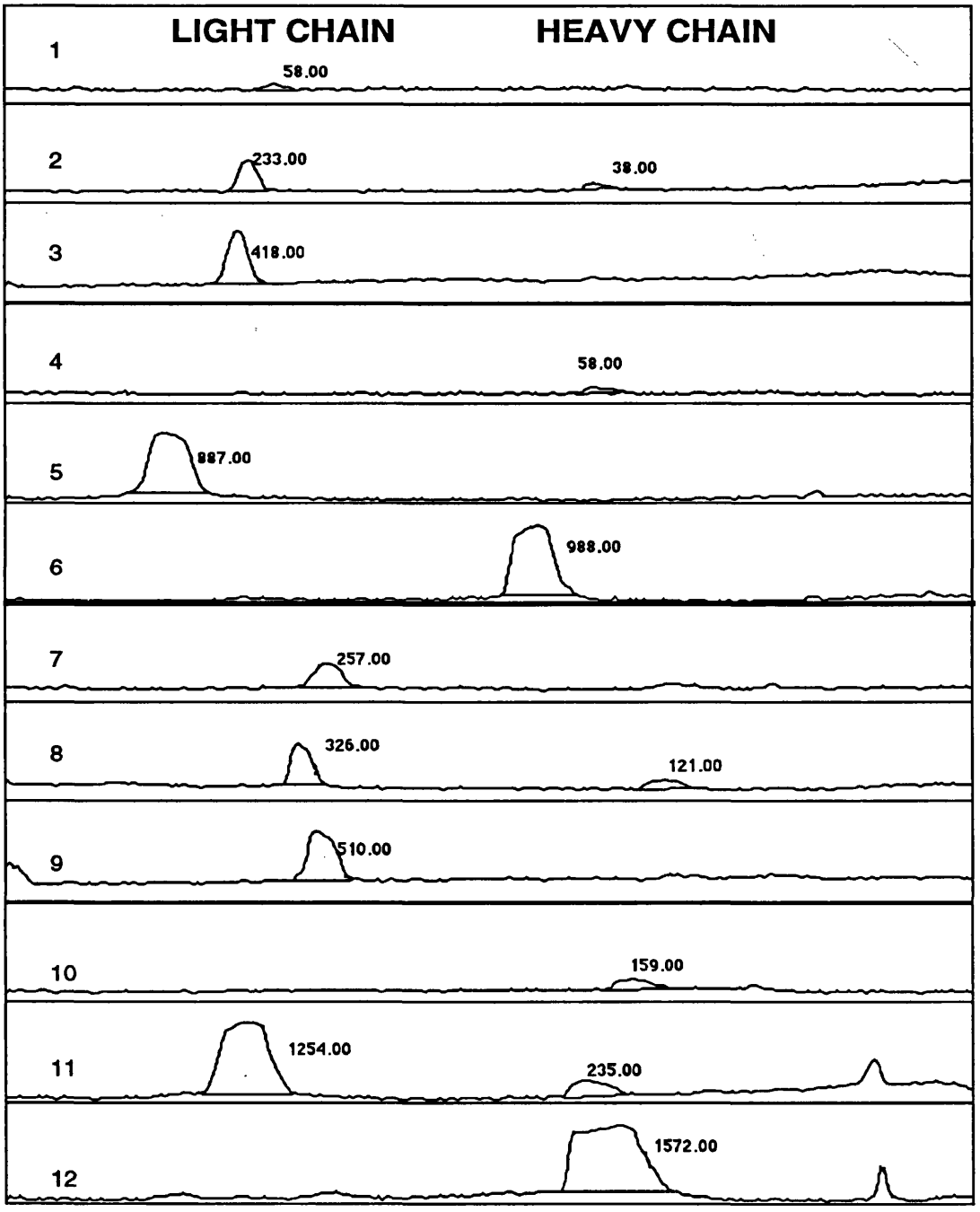
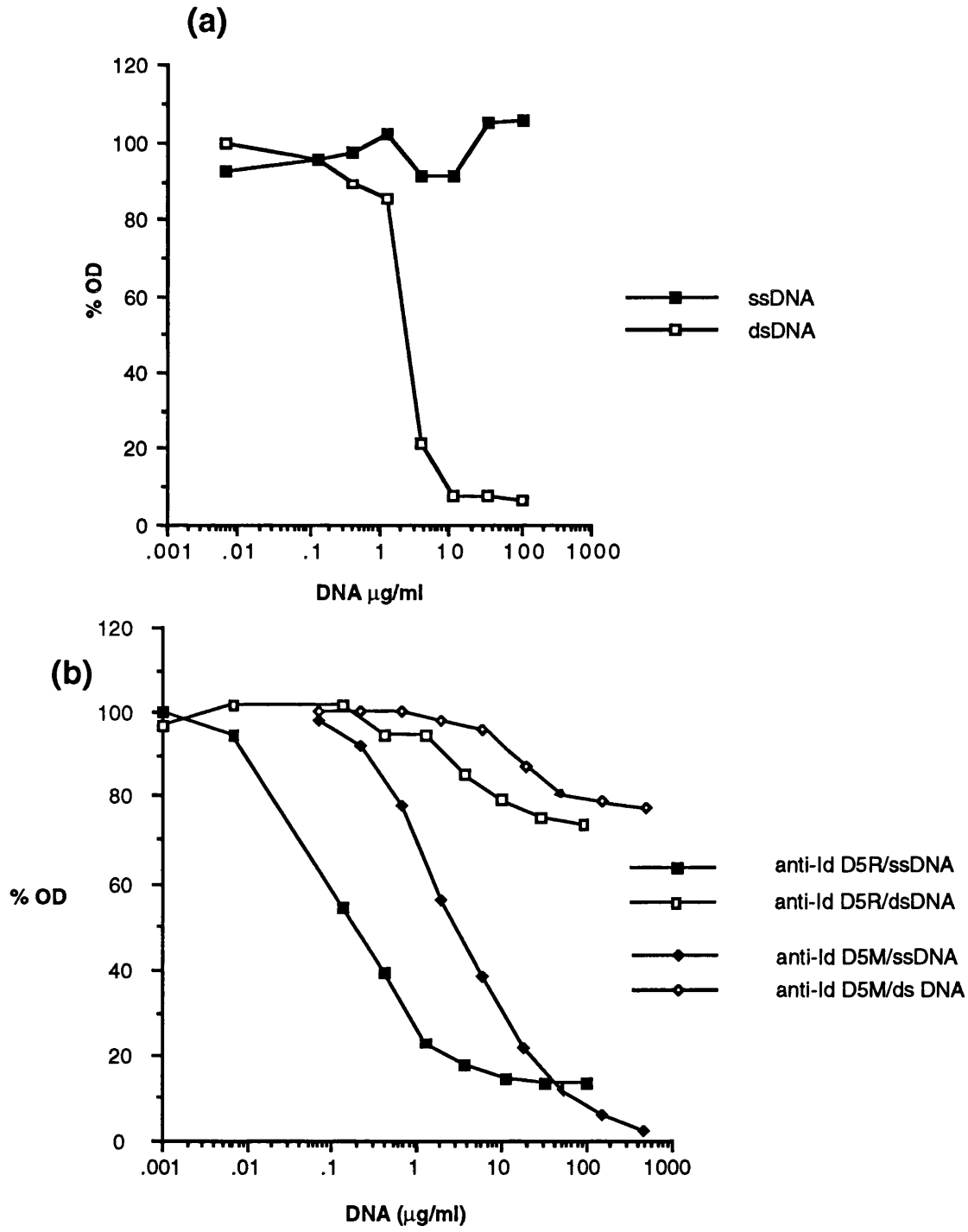


Figure 3.16 B3-RId, D5-RId and D5-MId are located at or near the binding site for DNA. Inhibition of the binding of (a)B3 to anti-Id B3R in solid phase by dsDNA and ssDNA and (b) D5 to anti-IdD5R and anti-Id D5M in solid phase by dsDNA and ssDNA. B3-RId, D5-RId and D5-MId are located at or near the binding site for DNA.



is in accordance with the specificity of the monoclonal antibodies and implies that the both B3-RId and D5-RId were both at or near the binding site for DNA.

3.2.3 Production of mouse monoclonal anti-idiotype antibodies

Two BALB/c mice were immunised with D5 and two mice with B3 in IFA as described in section 2.4.2. Culture supernatants derived from the splenic fusions of the immunised mice were screened against B3, D5 and human IgG each coated at the same concentration to identify any antibody that exclusively reacted with the immunising MAb. Table 3.7 shows the ODs of three antibodies that were selected as the most specific for the immunising MAb.

Table 3.7 Binding of three monoclonal antibodies raised by immunising mice with B3 and D5 measured by ELISA. (Optical density)

Reactivity against:	B3	D5	IgG
mAb:			
B3-1/G2	0.350	0.330	0.030
B3-5/G7	0.970	0.000	0.370
D5-6/G2	0.001	0.698	0.000

Only the monoclonal antibody that bound D5 appears anti-idiotypic. The B3-1/G2 monoclonal antibody may be anti-allotypic since it bound both monoclonal antibodies but only weakly to IgG. However, this could not be conclusively demonstrated since the monoclonal antibody was lost during subcloning. Neither monoclonal antibody that bound B3 was idiotype specific and could not be used for analysis of an idiotype associated with B3. D5-6/G2 mAb appeared to bind exclusively to D5 and was subcloned and expanded.

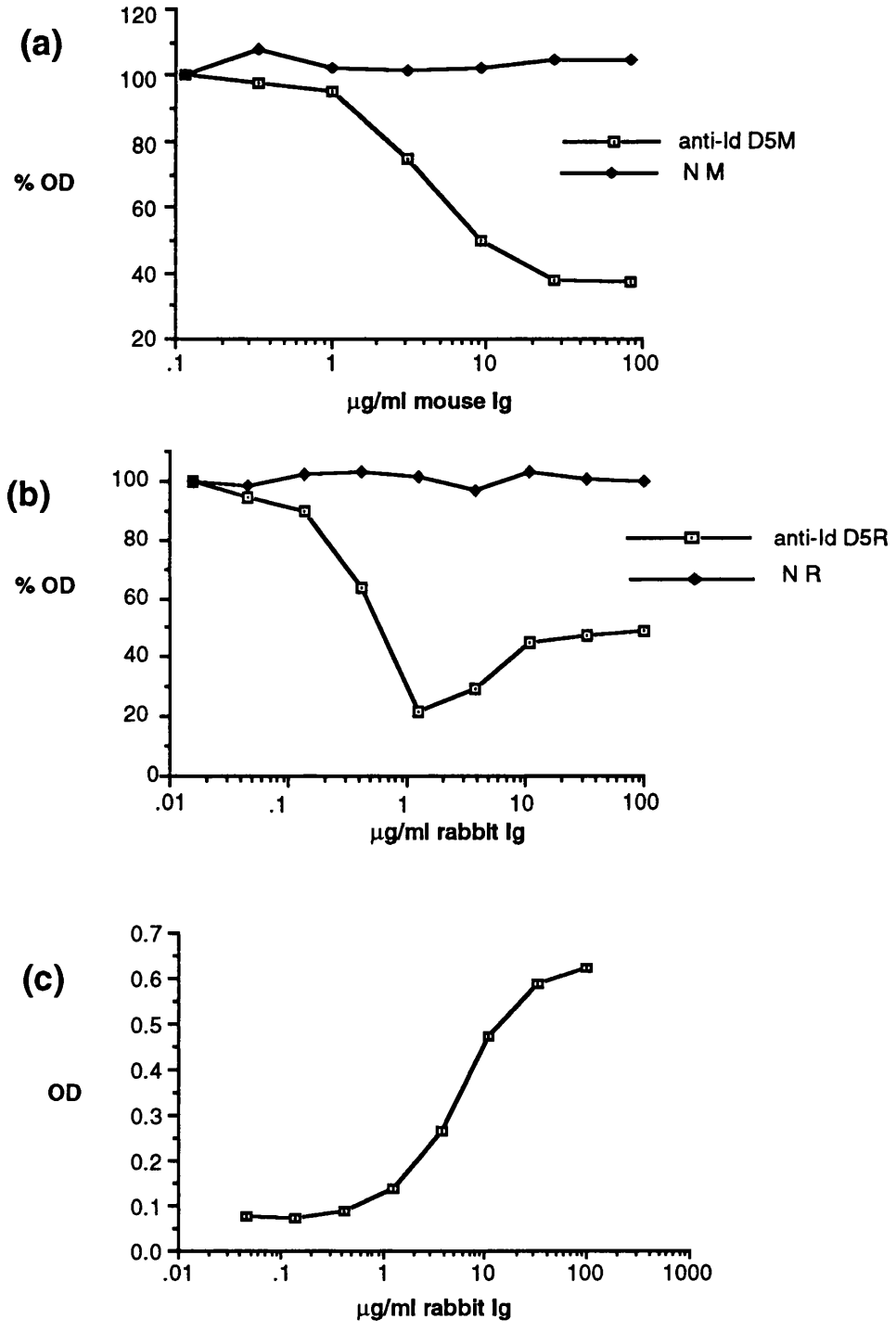
This monoclonal antibody is designated anti-Id D5M and recognises the idiotype D5-MId. The anti-Id D5M was affinity purified on a protein G column and stored at -20°C until use.

3.2.4 Characterisation of the D5-MId and its relationship with the D5-RId

The isotype of the mouse monoclonal anti-Id D5M was IgG1κ as determined by ELISA. The binding of anti-Id D5M to D5 in solid and fluid phase assays is shown in Figure 3.13 together with the binding of the anti-Id D5R. The concentration required to achieve 50% maximal binding for anti-Id D5M in the solid phase ELISA was 0.21 μg/ml. The concentration of D5 that inhibited 50% of the binding of anti-Id D5M to solid phase D5 was 3 μg/ml. Thus the affinity of the rabbit anti-IdD5R was approximately 4 times the affinity of the mouse anti-IdD5M. The D5-MId was shown to be at or near the binding site for ssDNA by inhibition ELISA (Figure 3.16b) . The amount of ssDNA that inhibited 50% of the binding of D5 to the rabbit anti-Id is approximately ten times less than that for the mouse anti-Id. Thus both the D5-MId and the D5-RId were at, or closely related to, the binding site but a higher proportion of the epitopes of the D5-RId that bound DNA were concealed when the DNA was bound to the mAb D5.

The relationship of D5-RId and D5-MId was further investigated by cross-inhibition ELISA as described in section 2.3.12. The mouse anti-Id antibody inhibited approximately 60% of the binding of D5 to the rabbit anti-Id reagent adsorbed onto the plate, but only at concentrations above 10 μg/ml, whereas the rabbit anti-Id reagent was able to inhibit up to 80% of the binding of D5 to the mouse anti-Id antibody at a concentrations around 2 μg/ml (Figure 3.17 a,b). However, at higher concentrations of the rabbit-anti-Id the observed inhibition of the binding of D5 to the mouse anti-Id actually decreased to 50%. This may be

Figure 3.17 D5-RId and D5-MId identify overlapping structures on D5. Inhibition of the binding of D5 to (a) the rabbit anti-Id D5R in solid phase by the mouse anti-Id D5M in fluid phase; and (b) the mouse anti-Id D5M in solid phase by the rabbit anti-Id D5R. (NR = normal rabbit; NM = normal mouse.) Figure 3c shows that the rabbit anti-Id D5R binds to the mouse anti-Id D5M at concentrations above 2 μg/ml.



explained by Figure 3.17c which shows that the rabbit anti-Id was able to bind to the mouse anti-Id adsorbed on the plate at concentrations above 1 μg/ml. The rabbit anti-Id did not bind to normal mouse immunoglobulin coated at the same concentration and neither anti-Id bound to DNA.

The position of the D5-MId on the D5 mAb was elucidated by Western blotting (Figure 3.18). The anti-Id D5M did not bind to either the light or heavy chain of D5 on the blot but when non-reduced D5 was present on the blot the anti-Id did bind indicating that the D5-MId is at least in part a conformational determinant involving both the heavy and the light chain.

3.2.5 Development of a capture ELISA for the detection of the idiotypes in serum and on monoclonal antibodies

The development of a detection system for the presence of idiotypes in serum and on monoclonal antibodies was similar for all three idiotypes: B3-RId, D5-RId and D5-MId. A capture ELISA was developed as described in section 2.3.14 incorporating a chequerboard pattern to determine the optimal coating concentration of the anti-idiotypes. The lowest concentration that gave a steep standard curve reflecting a high degree of sensitivity for both anti-D5R and anti-B3R was 0.3 μg/ml and for D5-MId was 2.5 μg/ml (Figure 3.19). These assays were specific for the homologous mAb as pooled IgG did not bind. The detection of idiotypes in serum introduces a reduction in specificity of the ELISA compared with its use to detect the presence of idiotype on monoclonal antibodies for a number of reasons. Serum may bind non-specifically to the plastic of the ELISA well, to the rabbit serum and to the anti-human immunoglobulin conjugate used to complete the ELISA. These problems would be further magnified if rheumatoid factors are present. Preliminary experiments with sera from SLE and normal patients were conducted to establish the

Figure 3.18 D5-MId is a conformational determinant involving both the heavy and the light chain. Western blots of (a) reduced D5 run on a 10% polyacrilamide gel stained with mouse anti-Id D5M (lane A), mouse control monoclonal (lane B), PBS (lane C), anti-human κ chain (lane D), anti- human γ and κ chain (lane E) and (b) (page 149) non-reduced D5 stained with mouse anti-Id D5M (lane A), anti-human κ chain (lane B), anti-human γ and κ (lane C), mouse control monoclonal (lane D), PBS (lane E).

(a)

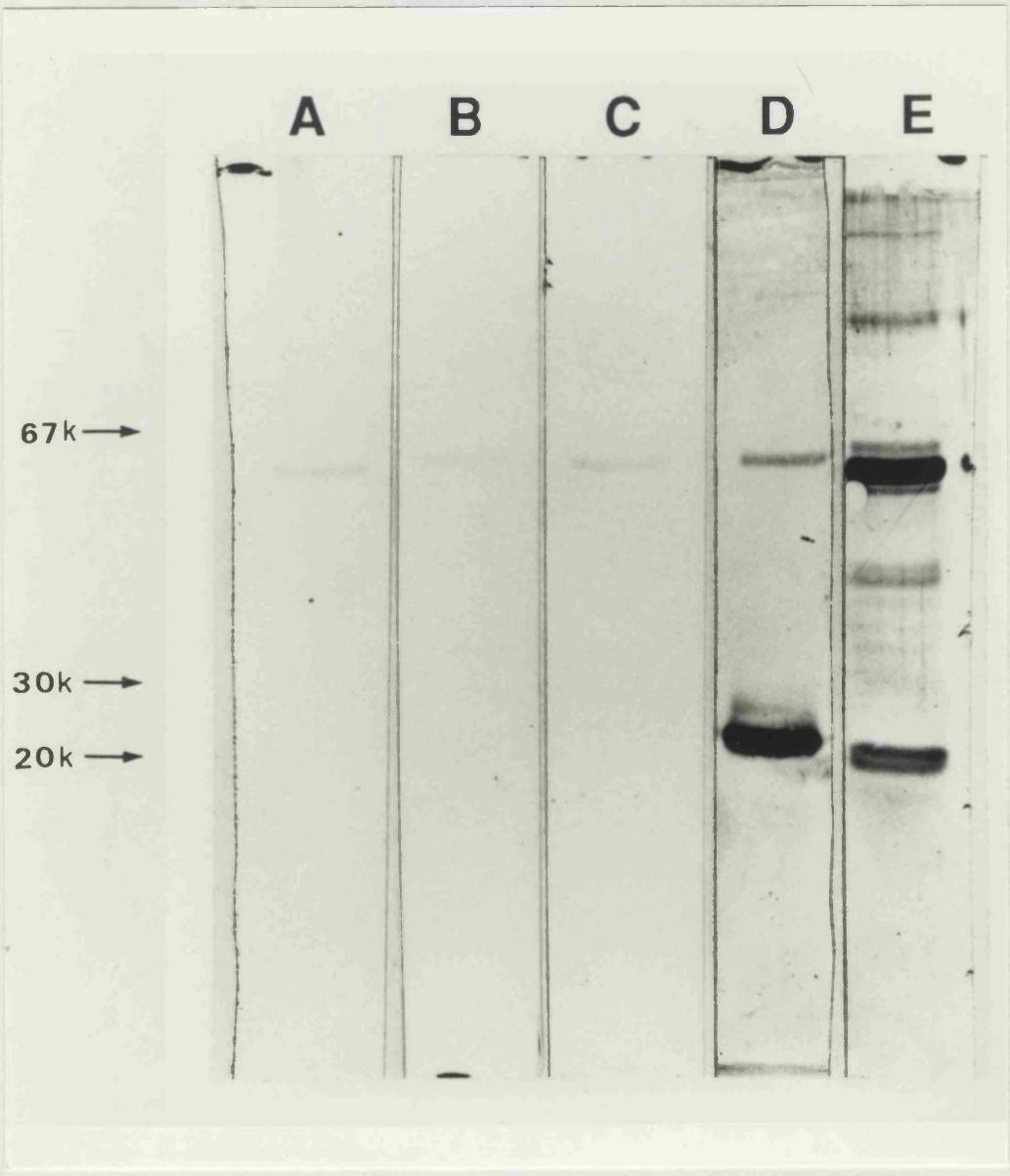


Figure 3.18 (continued)

(b)

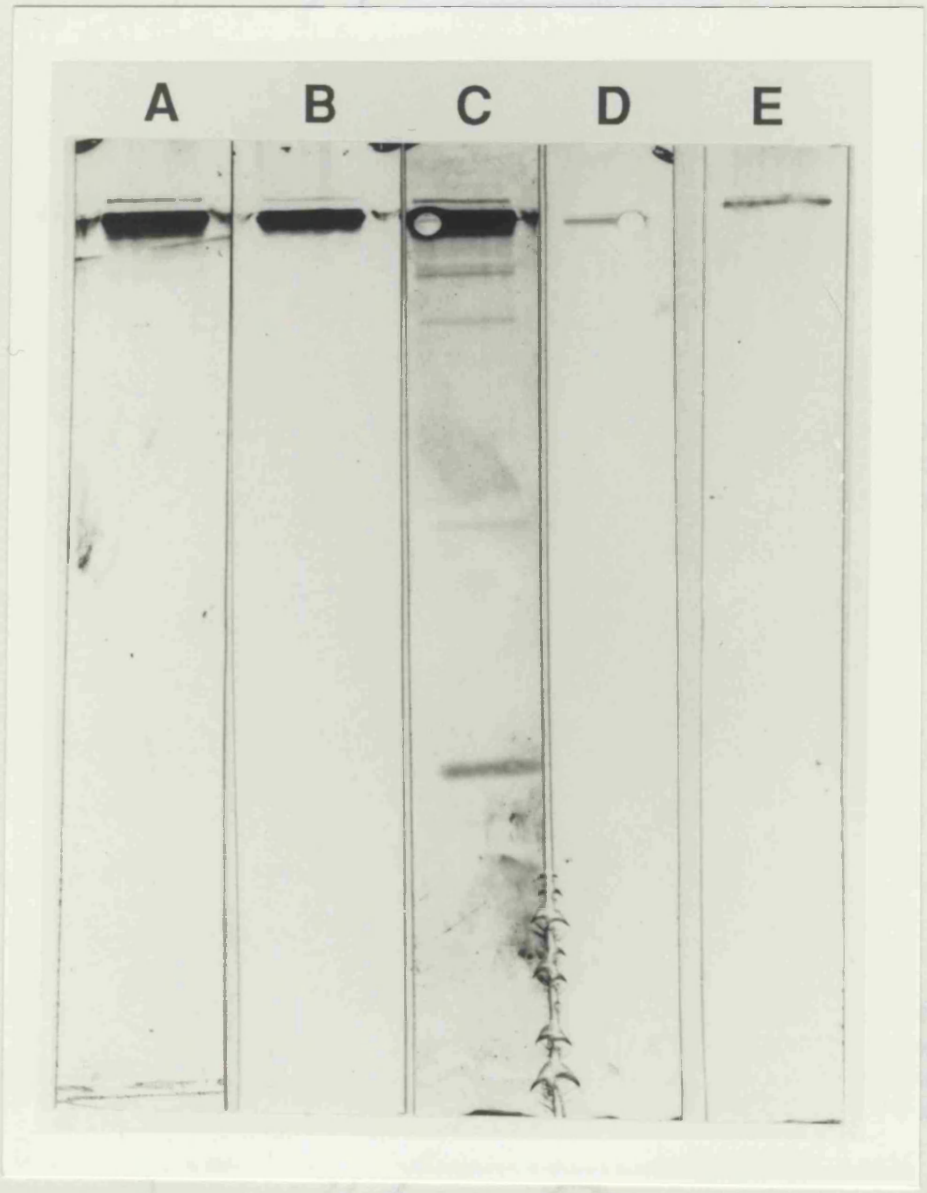
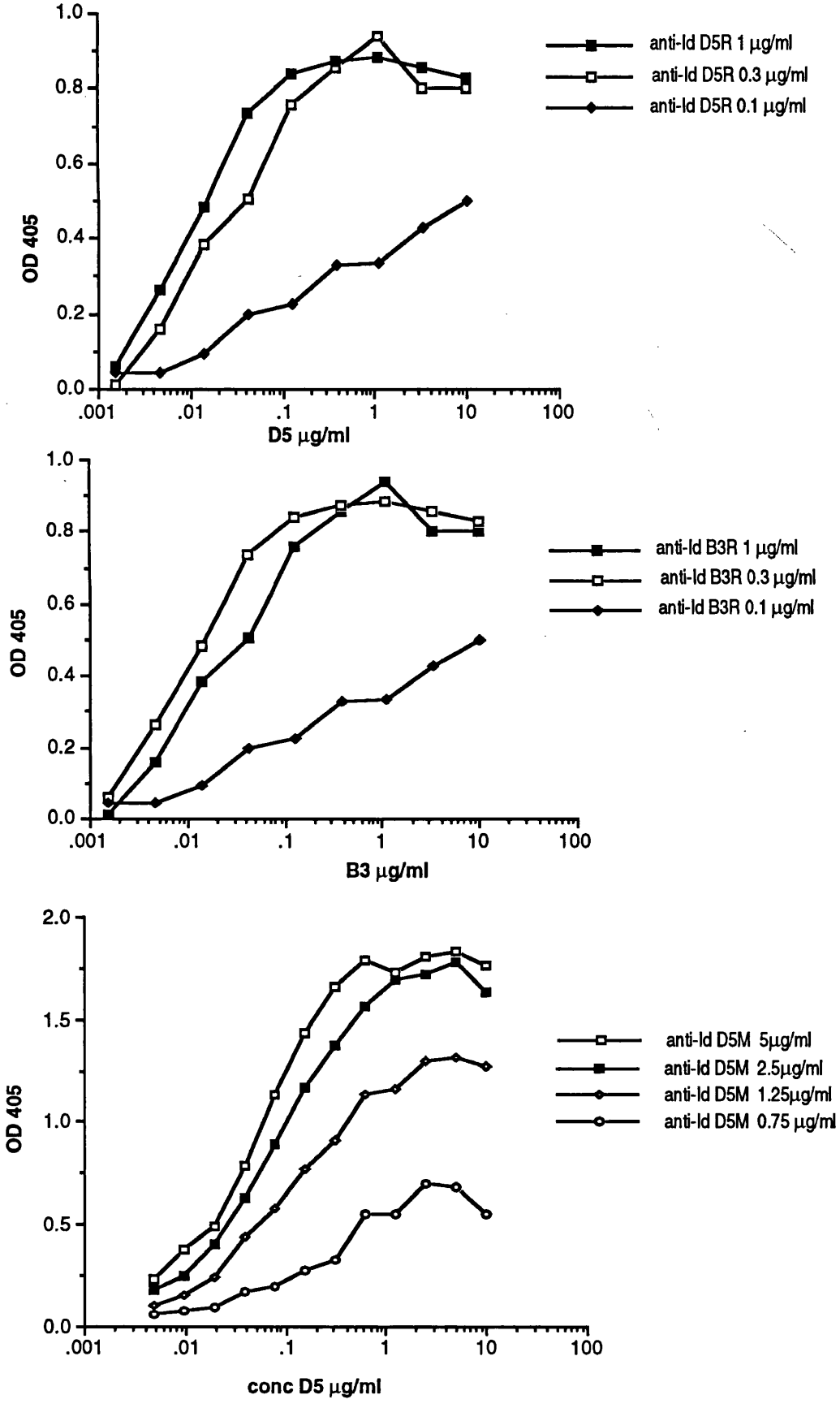


Figure 3.19. Determination of the optimal coating concentration of the anti-idiotypes for use in a capture ELISA to detect idiotype on immunoglobulins in sera and on monoclonal antibodies.



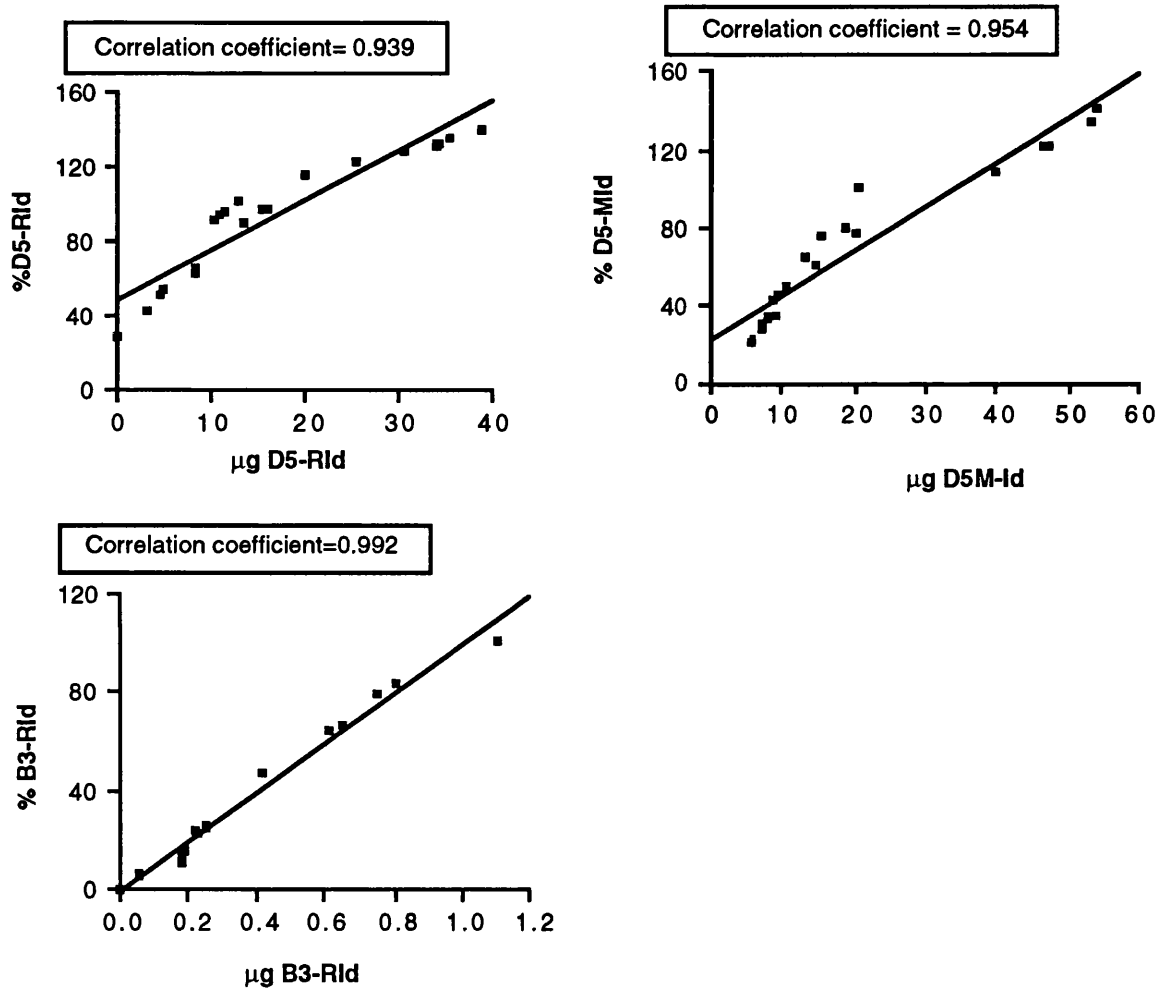
optimum coating of serum to use in the capture ELISA. Dilutions of sera between 1:100 and 1: 2700 were tested. A serum dilution of 1:300, 1:500 and 1:500 was used for the B3-RId assay, D5-RId and D5-MId respectively. These dilutions were chosen because they reduced background binding to the normal rabbit side of the plate whilst maintaining binding to the anti-Id in those sera that were 'positive'.

The Id levels were calculated by subtracting the mean OD values of the duplicate samples obtained in the control rabbit immunoglobulin half of the plate from the mean OD of the values obtained from the anti-Id antibody coated side of the plate (both coated at the same concentration). The values obtained were then expressed relative to a positive control serum. This method gave values comparable to those derived by expressing the subtracted ODs relatives to μg/ml of the homologous mAb in the three different idiotype assays. The two ways of quantifying the idiotypes were compared in ELISAs on both positive and negative sera (Figure 3.20).

3.2.6 B3-RId and D5-RId were not found expressed on other human monoclonal antibodies

A number of monoclonal antibodies available in the Bloomsbury Rheumatology Unit laboratory, and others obtained from elsewhere, were tested for the presence of the idiotypes under study using the ELISA developed for testing sera. The results were expressed as a multiple of the median OD 405 of the samples tested, and scored as (++) >10x median, (+) 5-10x median and (-) < 5x median. Anti-Id B3R did not recognise D5, anti-Id D5R and anti-Id D5M did not recognise B3. Due to the nature of human monoclonal antibody production, the majority of antibodies tested were of the IgM isotype. Thirty four IgM monoclonal antibodies (kindly provided by Dr. C. Ravirajan) derived from the

Figure 3.20 Comparison of two different methods of calculating the idiotype levels. Percentage of an arbitrary standard serum vs μg/ml equivalents. The correlation coefficients are shown for each graph. There is a high correlation between the two methods of expressing the idiotype levels.



fusion of spleen lymphocytes from two patients with SLE were all negative for the idiotypes.

In addition, two sets of monoclonal antibodies, whose amino acid sequence were known and were encoded by the variable region with the closest homology to the variable gene that was utilised by the light chain of B3 and D5, were tested for the presence of idiotype. Since both D5-RId and B3-RId are located on the light chain of their homologous monoclonal antibody, the idiotypes can be mapped more accurately by using monoclonal antibodies encoded by the same variable region. The sequences of B3 and D5 have been determined by Ms Celia Longhurst and are shown in section 3.4. Eight lambda monoclonal antibodies (kindly provided by Dr. Betty Diamond), four of which were encoded by the lambda 2.1 gene family, were tested for the presence of the B3-RId; none of these monoclonal antibodies were positive. Nine kappa monoclonal antibodies (kindly provided by Dr. Caroline Chapman) encoded by V kappa III were tested for the presence of the D5-RId; none of these were positive. In summary, no monoclonal antibody available for testing bound to any anti-Id except for the homologous monoclonal antibody. The significance of this will be discussed later in context with the sequences of B3 and D5 shown in section 3.4.

3.2.7 Detection of B3-RId, D5-RId and D5-MId on immunoglobulins in human sera

The capture ELISAs developed were first used to screen sera derived from normal individuals and patients with SLE, a variety of autoimmune rheumatic diseases, relatives of SLE patients and patients with paraproteinaemia. The upper limit of normal was set at 2 standard deviations above the mean for the normal individuals. The ELISAs were developed so that

each idiotype could be detected separately on IgG and IgM immunoglobulins. The sera from SLE patients were selected in order to differentiate activity in different organs. The method of selection, which is described in section 2.1, was designed so that sera were tested when only one organ was active.

Two important, potentially confounding, variables need to be considered when analysing serum for the presence of idiotypes. The first is the variation in immunoglobulin levels in the serum. Hypergammaglobulinaemia is a common manifestation of SLE and may lead to increased non-specific binding to the plastic as well as to the anti-idiotypic antibodies. These points were taken into consideration when measuring the idiotype levels since the binding to the normal rabbit serum was always subtracted from that to the anti-idiotypic antibodies. In addition, the concentration of serum IgG did not correlate significantly with the idiotype levels (Figure 3.21a, 3.22). The second potentially confounding factor occurs when rheumatoid factor is present in the serum. However, the increased binding to the rabbit anti-idiotypic antibodies would be mirrored by the increased binding to the normal rabbit serum. Rheumatoid factor was measured in some patients and shown plotted against B3-RId levels on IgM antibodies in Figure 3.21b.

3.2.8 B3-RId was elevated on IgG antibodies in SLE patients with active arthritis.

Based upon tests of sera from 28 healthy controls, the upper limit of normal of the B3-RId expression on IgG was set at 34% (% of the positive control) and the upper limit of normal of the B3-RId expression on IgM was set at 22% (upper limit of normal represents mean plus two standard deviations of the normal sera). Figure 3.23 shows the level of expression of the idiotypes on IgG and IgM in the different disease groups and normal controls (summarised in

table 3.8). When present, the idiotype was expressed more frequently on IgG than on IgM antibodies ($p<0.001$) in SLE patients whereas in the rheumatoid arthritis population it was expressed more frequently on the IgM population ($p<0.05$). Within the SLE cohort the B3-RId was present more frequently (42%) in those patients with active musculoskeletal disease compared to patients with the other manifestations (9%) ($p<0.001$).

Out of the 35 patients with musculoskeletal disease only three had myositis all of whom were negative for B3-RId, the other patients had arthritis. There was no difference in the clinical expression of the joint disease between those patients who were idiotype positive and idiotype negative. Seventy nine of the 101 SLE patients, in comparison with 16 out of 20 B3-RId positive patients, had anti-DNA antibodies indicating that there was no association between the presence of B3-RId and anti-DNA antibodies in the serum. The one patient with rheumatoid arthritis who had the B3-RId on the IgG population had detectable anti-DNA antibodies by ELISA (though not by *Crithidia luciliae* immunofluorescence). One out of the six SLE patients with B3-RId on the IgM population had an erosive arthritis as well. The level of expression of B3-RId on IgM was higher in the rheumatoid arthritis patients compared to the other groups.

In summary, the presence of B3-RId on IgG antibodies in serum was associated with SLE patients with active arthritis. In addition, B3-RId was also identified on IgM antibodies in sera from rheumatoid arthritis patients.

Figure 3.21. Lack of correlation between (a) IgG B3-RId levels and total IgG; (b) IgM B3-RId and rheumatoid factor respectively.

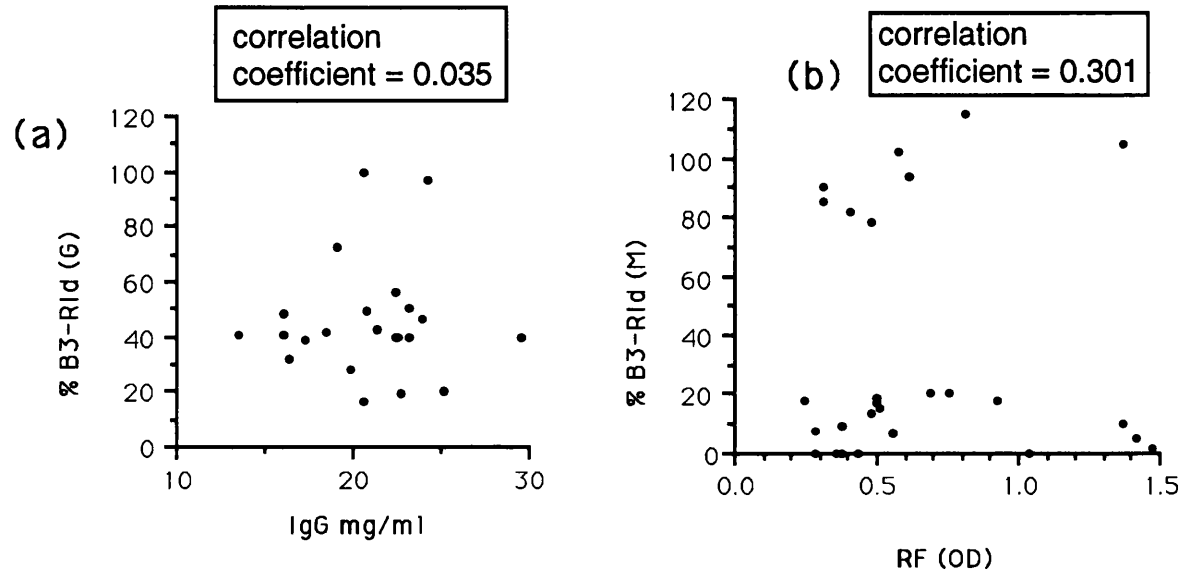


Figure 3.22 Lack of correlation between D5-RId and D5-MId levels and total IgG in a sample of patients' sera.

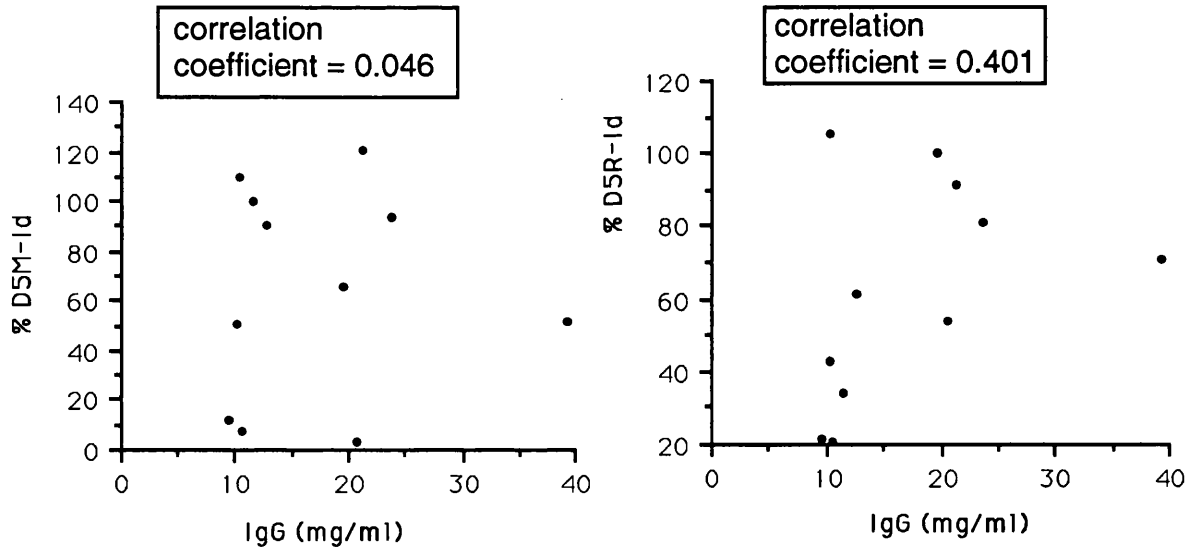


Figure 3.23. B3-RId is elevated on (1) IgG antibodies in a proportion of SLE patients with active arthritis and (2) IgM antibodies in a proportion of rheumatoid arthritis patients.

B3-RId levels on IgG (a,b) and IgM (c,d) in the sera of SLE patients classified according to organ involvement, normal controls and other patients groups as specified (see table 3.8). Horizontal line indicates mean plus two standard deviations of the normal group. (M/S = musculoskeletal, CNS = central nervous system, CVS = cardiovascular)

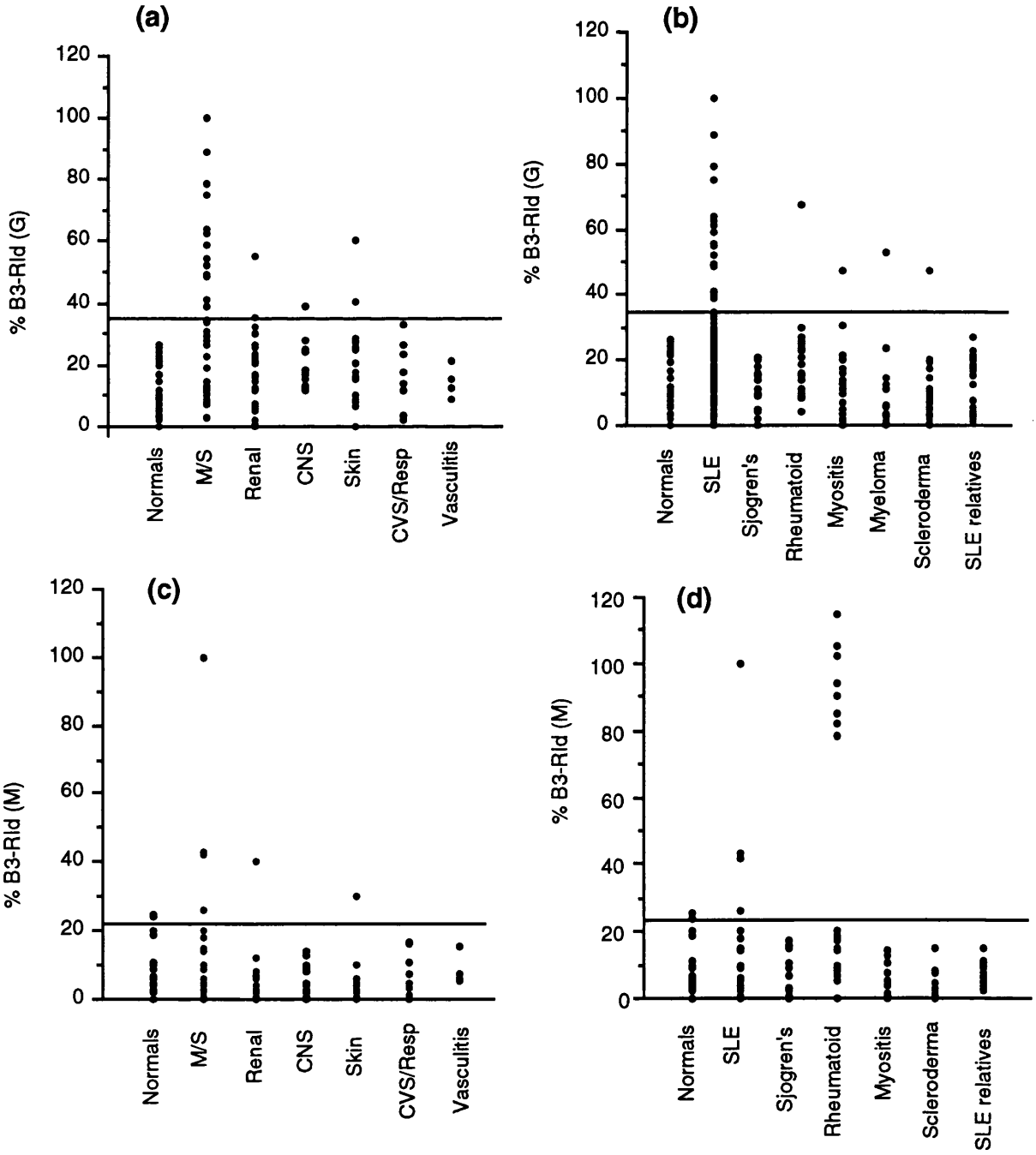


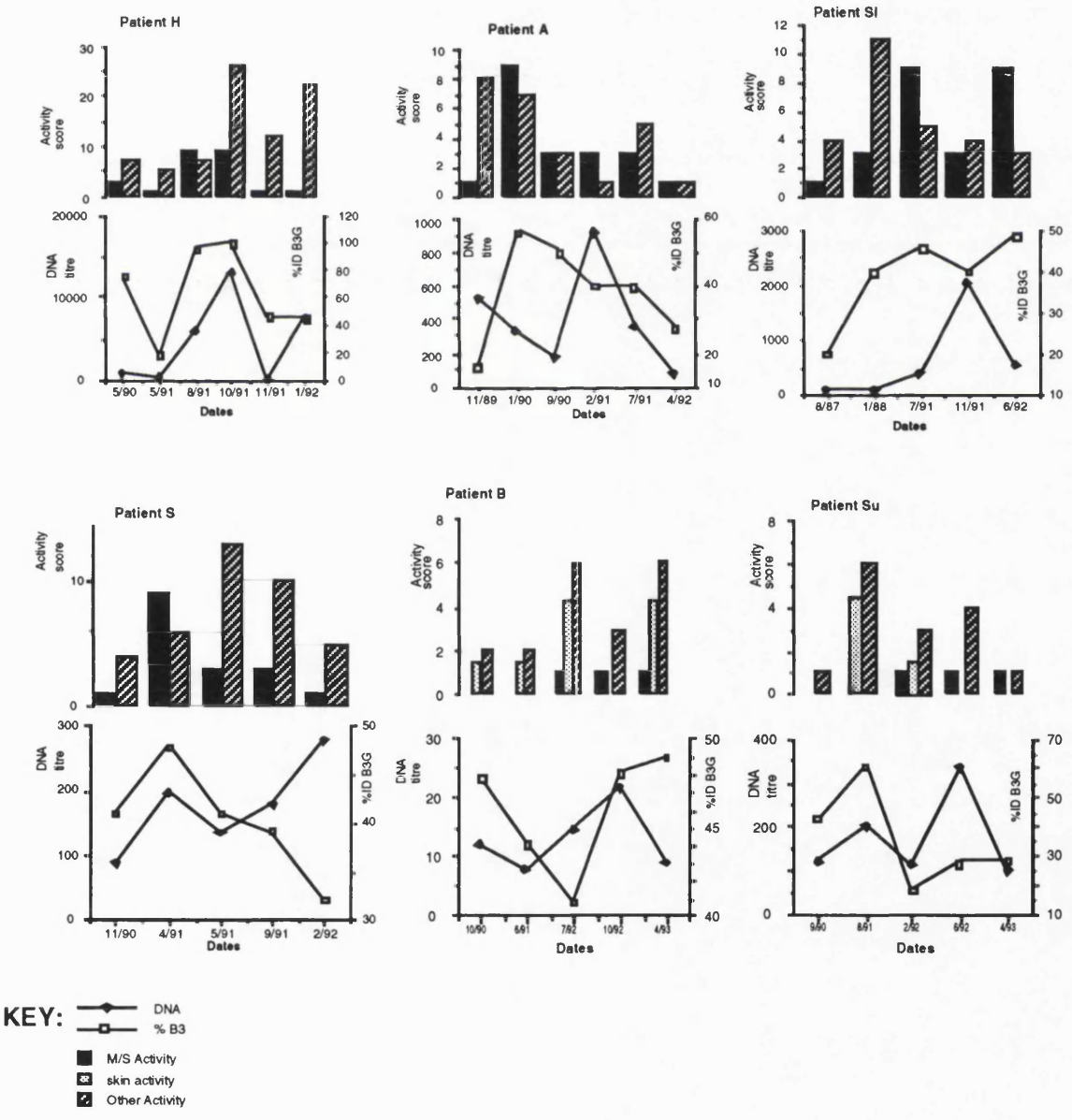
Table 3.8. Expression of B3-RId, D5-RId and D5-MId in the sera of different disease groups and normal controls. Both D5-RId and D5-MId were found only on IgG in the sera whereas B3-RId was found on both IgG and IgM. C/R = Cardiovascular/Respiratory, SCL = scleroderma, SLE Rel = SLE relatives.

	M/S	Renal	CNS	Skin	C/R	Vasc	SLE	RA	Sjogren's	Myositis	SCL	Myeloma	SLE Rel	Normals
Total	35	24	14	16	8	4	101	27	20	17	15	35	24	28
B3-RId (G)	15	2	1	2	0	0	20	1	0	1	1	1	0	0
B3-RId (M)	4	1	0	1	0	0	6	8	0	0	0	-	0	2
D5-RId (G)	9	7	6	7	2	2	33	2	4	0	1	1	4	0
D5-MId (G)	12	6	5	5	1	2	31	4	2	0	1	1	4	2

3.2.9 Correlation of the B3-RId with musculoskeletal disease activity

Serial samples were taken from six SLE patients who expressed B3-RId in their serum to analyse the variation of the idiotype level over time. In the four patients with active musculoskeletal disease the level of B3-RId reflected the musculoskeletal disease activity more closely than the overall disease activity without the musculoskeletal component (Kendall Rank Correlation $p< 0.05$, Figure 3.24). There was no association with skin or musculoskeletal disease activity in the other two patients whose elevated B3-RId level was associated with active skin disease. When the disease was inactive the idiotype was not found on IgM antibodies in these patients. There was no correlation between B3-RId and DNA binding.

Figure 3.24. Serial bleeds from six B3-RId positive SLE patients comparing levels of B3-RId (%Id B3G) to musculoskeletal (M/S) disease activity and overall activity without the musculoskeletal component. (Disease activity assessed by the BILAG system which can yield a global score, see section 2.1). In the four patients who had active musculoskeletal disease the correlation between the musculoskeletal disease activity and the idiotype level was significant (Patient SI p=0.004, Pt. A p=0.001, Pt H p=0.004, Pt. S p=0.05; Kendall Rank Correlation). There was no correlation between the B3-RId level and the overall disease activity without the M/S activity. The other two patients were the only patients that had active skin disease (Patient B, Patient Su) associated with a raised B3-RId level, but there was no correlation with disease activity. (Skin disease activity is shown separately for these two patients.) Also illustrated are the serial levels of DNA binding.



3.2.10 The D5-RId and D5-MId were raised in SLE irrespective of which organ system was involved

Both the D5-RId and D5-MId were expressed on IgG antibodies in serum. In contrast, the binding of sera to anti-D5R or anti-D5M, when assessed by an anti-human IgM conjugate, was not significantly higher than the binding to the normal immunoglobulin (rabbit or mouse as appropriate). Thus neither D5-RId nor D5M-Id were expressed on IgM antibodies from any serum.

Based upon tests of 28 healthy controls, the upper limit of normal of the D5-RId expression on IgG antibodies in sera was set at 44% of the positive control, and the upper limit of normal of the D5-MId was set at 17% (%s represent mean plus two standard deviations of the normal sera). Thus the specificity of the D5-MId ELISA was considerably increased as compared to the D5-RId ELISA. Table 3.8 shows the distribution of D5-MId and D5R-Id in SLE patients divided into different organ systems and the other disease groups; Figure 3.25 shows the level of idiotype expression in the different groups.

33% and 31% of SLE patients had raised levels of D5-RId or the D5-MId respectively. Twenty one percent of the SLE patients had elevated levels of both showing a considerable overlap in the expression of the D5-MId and the D5-RId. This overlap was also observed in the other disease groups though the number of patients expressing the idiotypes were less than in the SLE group. The expression of the D5-MId and the D5-RId was significantly increased in SLE patients compared with healthy controls and to some of the disease controls (see Figure 3.25). There was a significant correlation between the D5-MId and the D5-RId idiotype levels observed in all the SLE patients (Figure 3.26), the correlation coefficient (r) was 0.428 ($p<0.01$). For comparative purposes, and to further demonstrate that the elevation of idiotype levels are not due to polyclonal hypergammaglobulinaemia, the correlation between the

Figure 3.25. D5-RId and D5-MId levels in SLE and other disease groups. D5-RId and D5-MId levels were raised in the sera of SLE patients irrespective of which organ system was involved. D5-RId (a,b) and D5-MId (c,d) levels on IgG in the sera of SLE patients classified according to organ involvement, normal controls and other patients groups as specified (see table 3.8). Horizontal line indicates mean plus two standard deviations of the normal group.

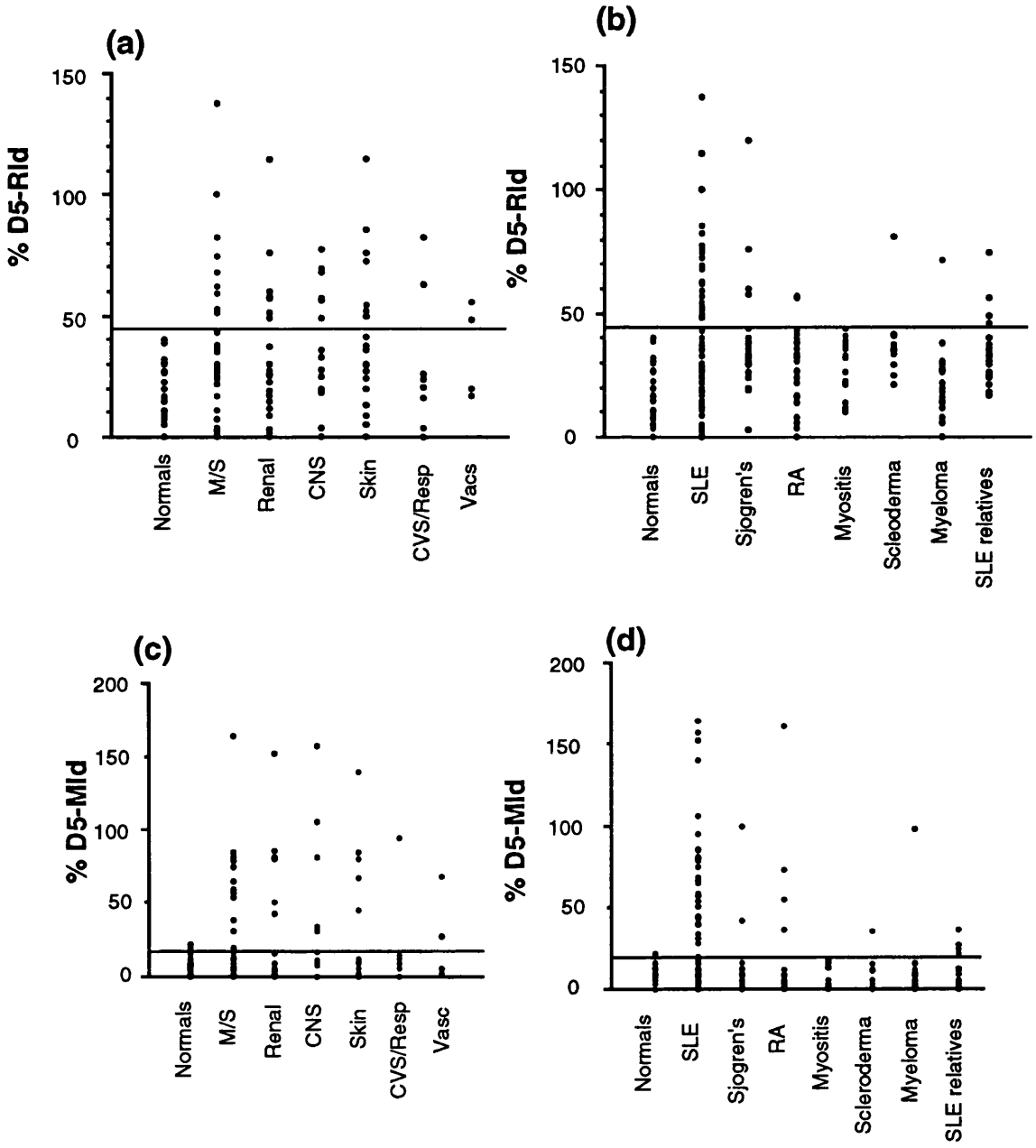
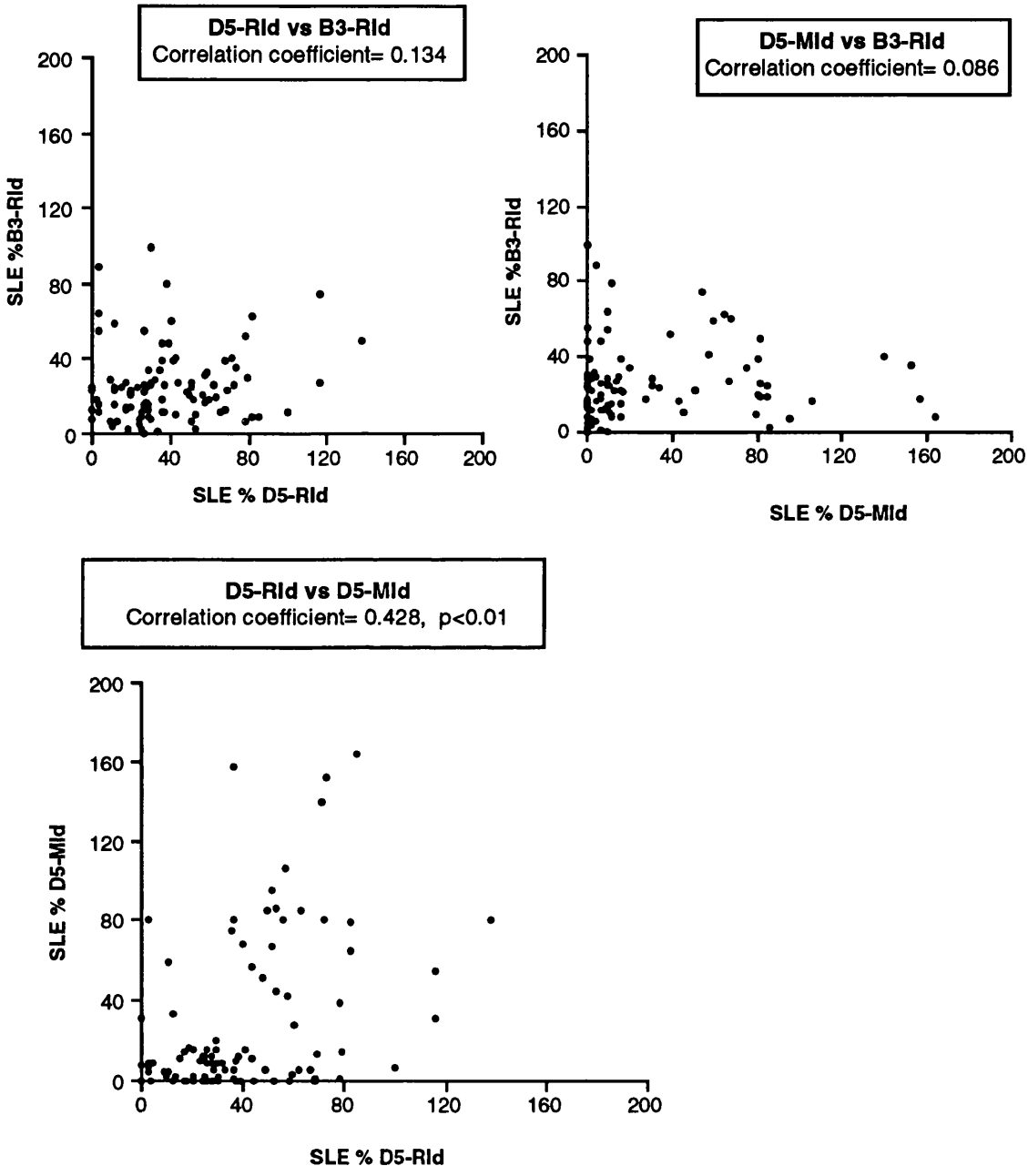


Figure 3.26. Correlation between the levels of the three idiotypes studied: D5-RId, D5-MId and B3-RId in 101 SLE patients. Correlation coefficients are indicated on the graphs. There was a significant correlation only between D5-RId and and D5-MId.



B3-RId levels and the D5-MId and D5-RId were calculated (figure 3.26). The correlations were not found to be significant. (B3-RId vs D5-RId, r= 0.134 ; B3-RId and D5-MId, r= 0.086).

Serial bleeds were taken from four SLE patients who had elevated levels of both D5-RId and D5-MId (Figure 3.27). The correlations between the two idiotype levels, and between the two idiotype levels and the BILAG activity scores were not significant. The contrast between figure 3.26 and figure 3.27 is explained by the much larger number of data points examined in the former. Thus although the correlation coefficients were of a similar order (in figure 3.27 the average r value is 0.458 compared to 0.428 in figure 3.26 for D5-RId vs D5-MId) the p value was not significant for the serial data (p= 0.07).

3.2.11 All the idiotypes are present on both DNA and non-DNA binding antibodies

After absorption of 10 individual DNA binding SLE serum samples, 9 of which were positive for the D5-MId and/or the D5-RId, on a DNA-cellulose column, anti-DNA activity was unmeasurable. Between 61-91% (the % is based on OD values and thus represents an approximation) of the D5-MId and 55-78% of the D5-RId was present in the DNA binding fraction of the nine D5-RId and/or D5-MId positive patients (Figure 3.28a). Thus a large fraction of the idiotype positive antibodies bound to DNA. Four B3-RId positive patients were also studied in a similar way. Between 46-64% of the B3-RId was present in the DNA binding fraction eluted from the DNA column (Figure 3.28b). Thus a smaller proportion of the B3-RId positive antibodies bound to DNA than the D5-Id positive antibodies.

Figure 3.27 Serial bleeds from four patients who had elevated levels of both idiotypes associated with D5: D5-MId and D5-RId. The level of correlation between the idiotype levels and the BILAG score or DNA binding levels was not significant.

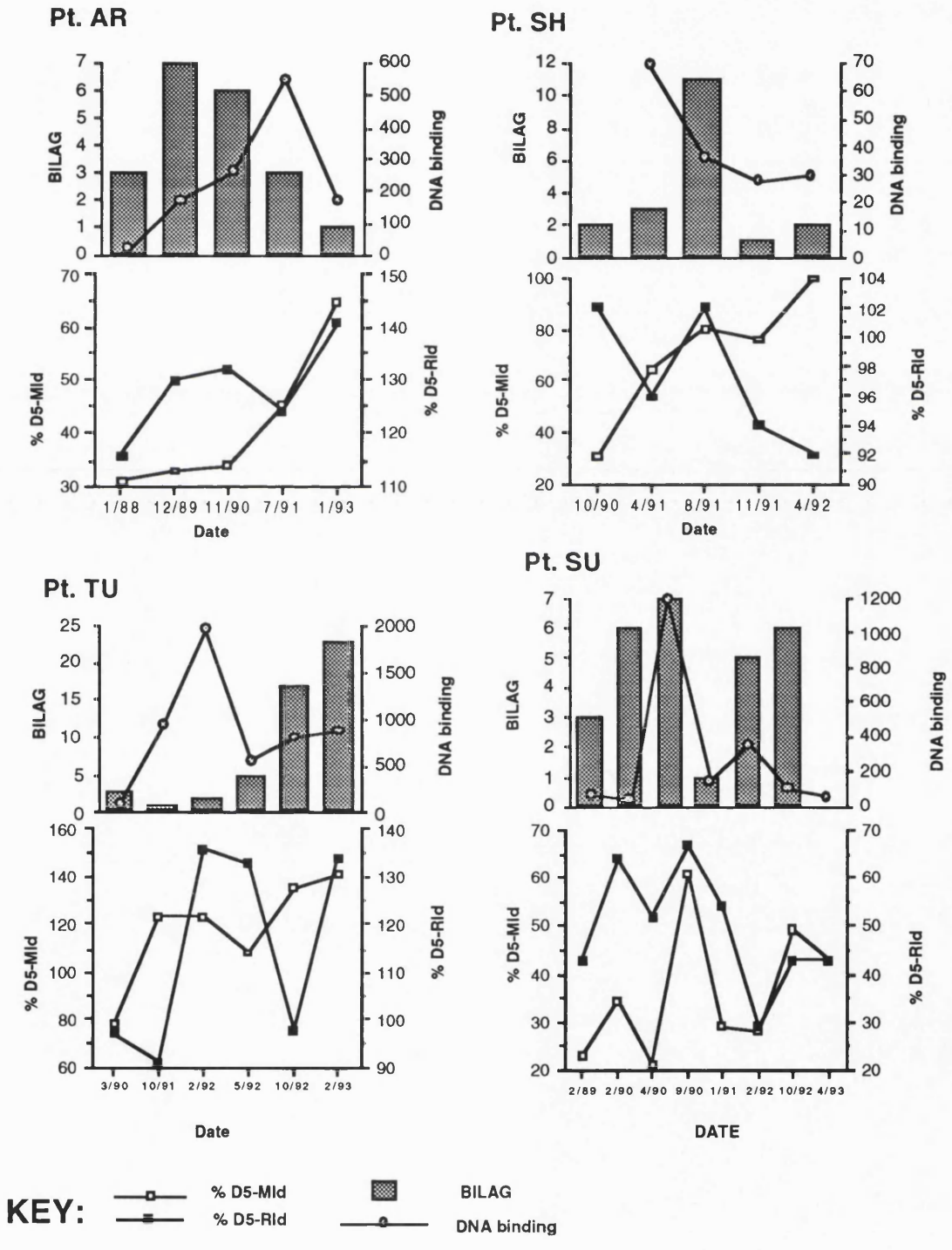
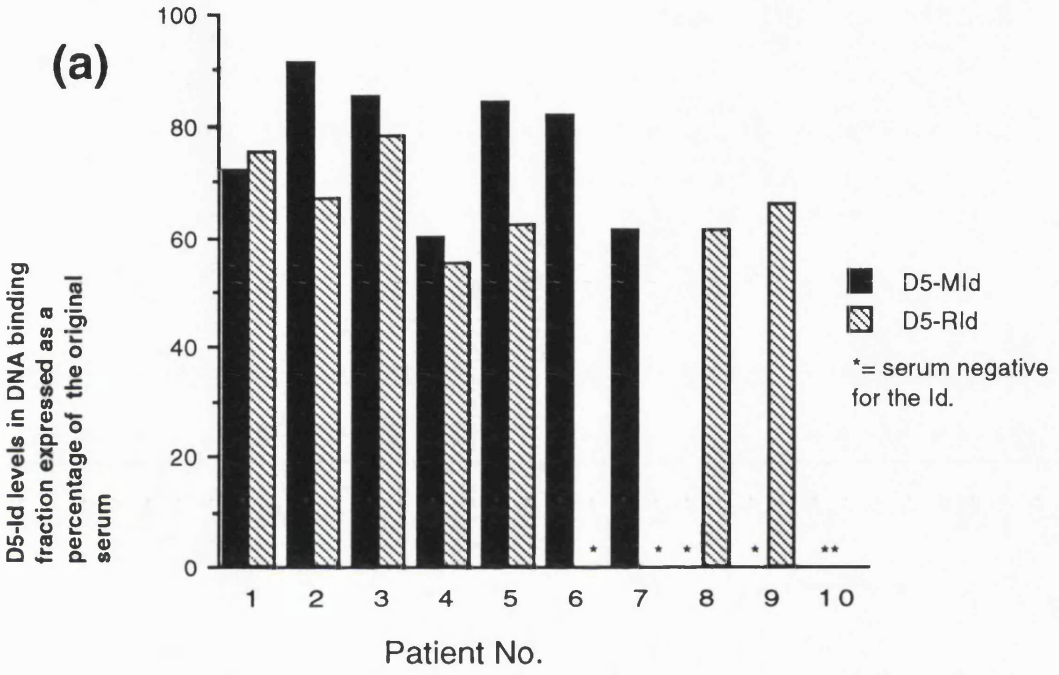
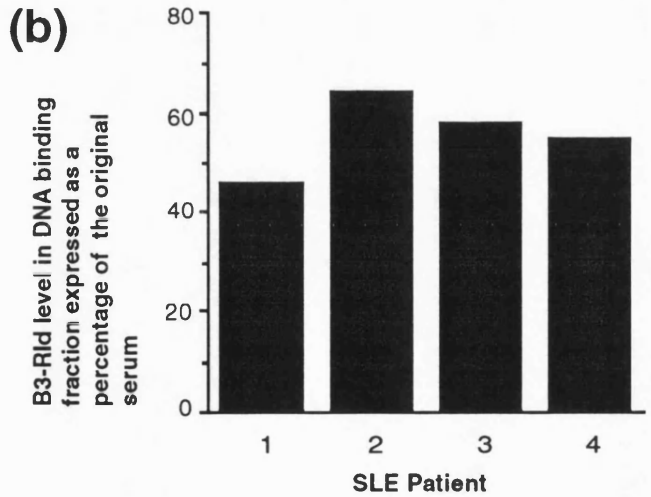


Figure 3.28. The idiotypes are present on both DNA and non-DNA binding antibodies. (a) D5-RId and D5-MId, and (b) B3-RId in the DNA binding fraction of SLE sera expressed as percentage of the idiotype level of the original serum sample. The percentages are calculated on OD values and are thus an approximation.





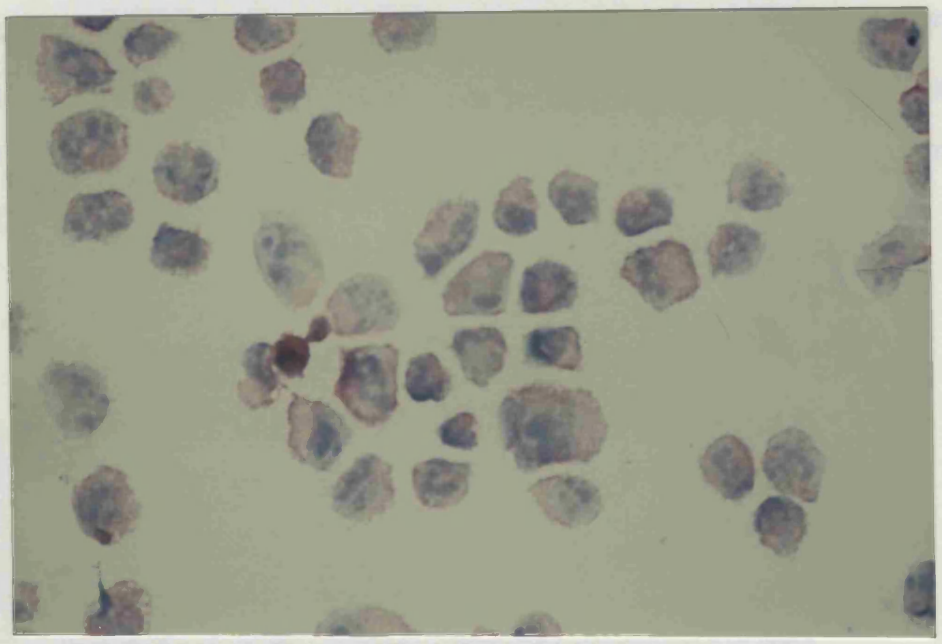
3.2.12 Only D5-MId was found present on immunoglobulins deposited in kidneys from SLE patients

The expression of the three idiotypes was investigated by immunohistochemical methods on renal tissue. In order to determine the optimal concentrations of the three anti-Ids for use in immunohistochemistry various dilutions of the anti-Ids were tested against cytospin preparations of the hybridomas producing the homologous mAb. The optimal concentrations were for anti-IdB3R: 20 μg/ml, for anti-Id D5R: 10 μg/ml, and for anti-Id D5M: 20 μg/ml. Figure 3.29 shows, as a positive control, the staining of hybridoma cells producing D5 stained with anti-Id D5M with a negative control (rabbit immunoglobulin) for comparison.

Out of ten SLE kidney tissue biopsies examined, 6 had immunoglobulin present which were positive for the D5-MId whereas 2 out of fifteen non-SLE disease control kidney tissue were positive for the D5-MId ($p<0.05$). The idiotype positive immunoglobulins were deposited on the capillary wall in the Id positive SLE biopsies. Figure 3.30a shows one of the kidney sections staining positive for the D5-MId with a negative control for comparison (Figure 3.30b). There was no particular pathological diagnosis associated with the presence of D5-MId in deposits in kidney sections. The D5-RId and the B3-RId were not found in any of the biopsies tested.

Figure 3.29. Hybridoma cells producing D5 were stained with (a) anti-Id D5M and (b) a negative control (rabbit immunoglobulin) for comparison.

(a)



(b)

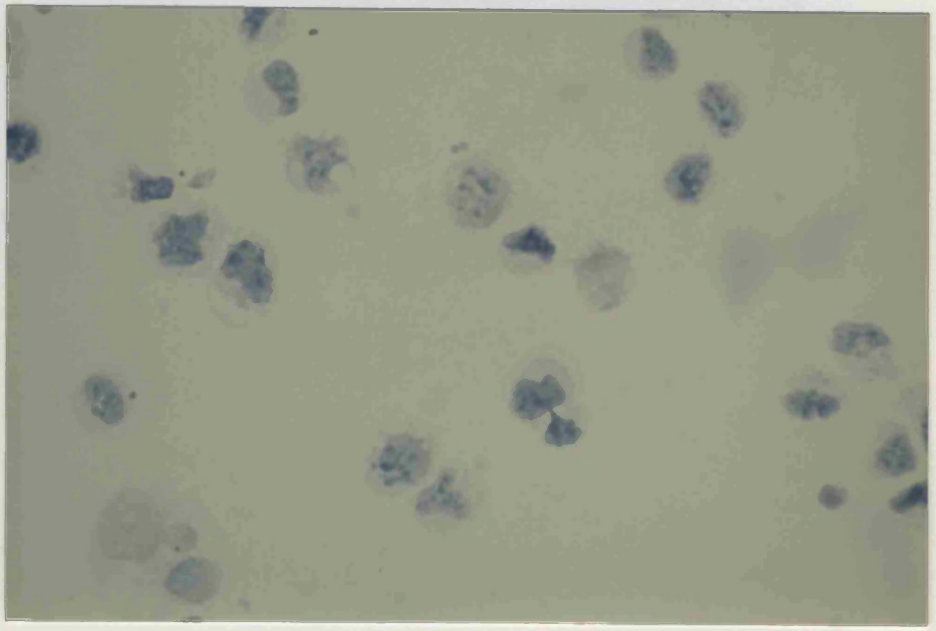


Figure 3.30. D5-MId was present on immunoglobulins deposited in kidney sections from SLE patients. In figure (a) red staining pattern indicates binding of the mouse anti-Id D5M to the glomerulus of a kidney section of a lupus patient. The distribution of the staining is capillary and mesangial. Figure (b) (page 169) shows the absence of staining of a mouse monoclonal antibody of identical isotype at a similar concentration tested on a renal section from the same patient. (Magnification *20).

(a)

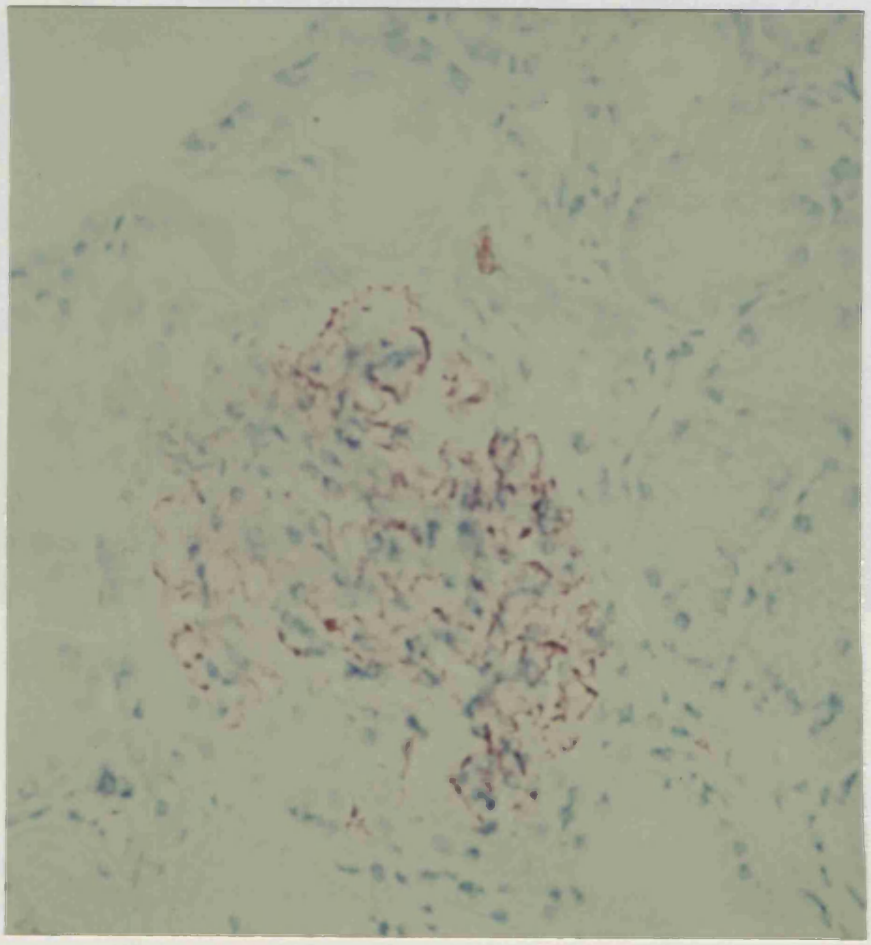
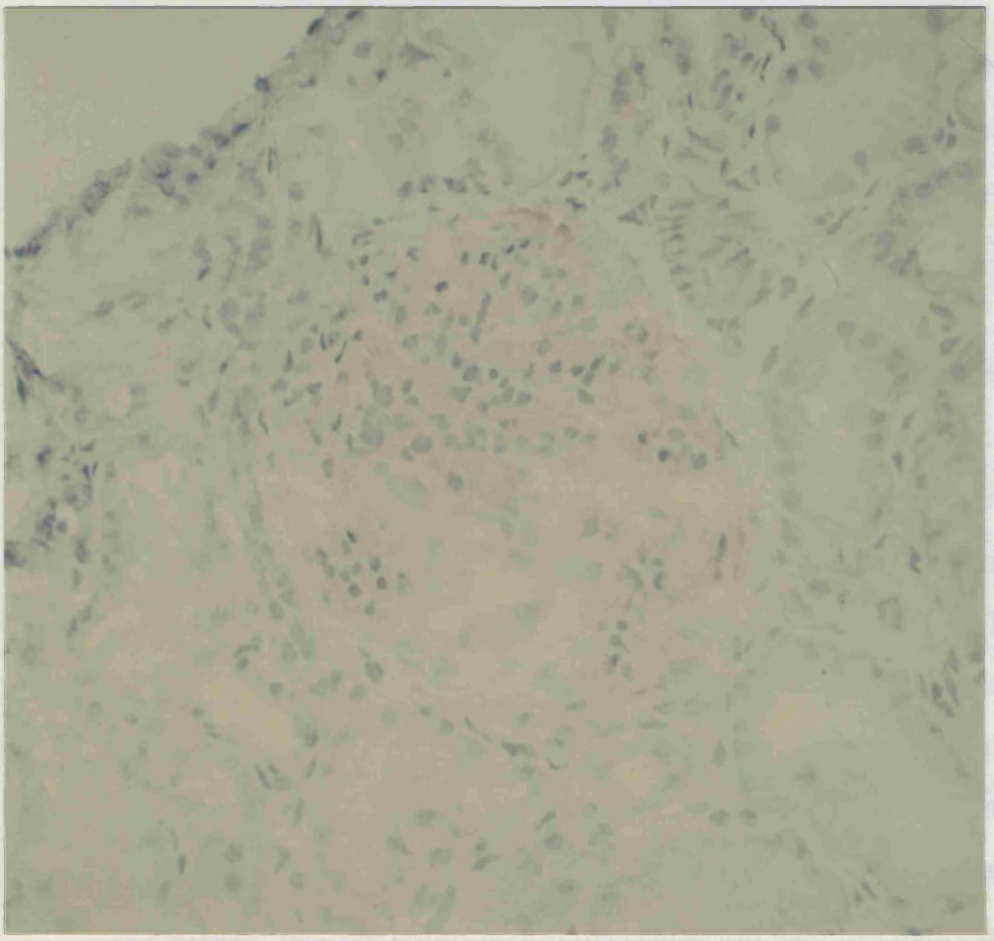


Figure 3.30 (continued)

(b)



3.3 INVESTIGATION OF THE BIOLOGICAL PROPERTIES OF HUMAN MONOCLONAL ANTI-DNA ANTIBODIES

3.3.1 Analysis of the binding properties of monoclonal anti-DNA antibodies *in vitro* and *in vivo*.

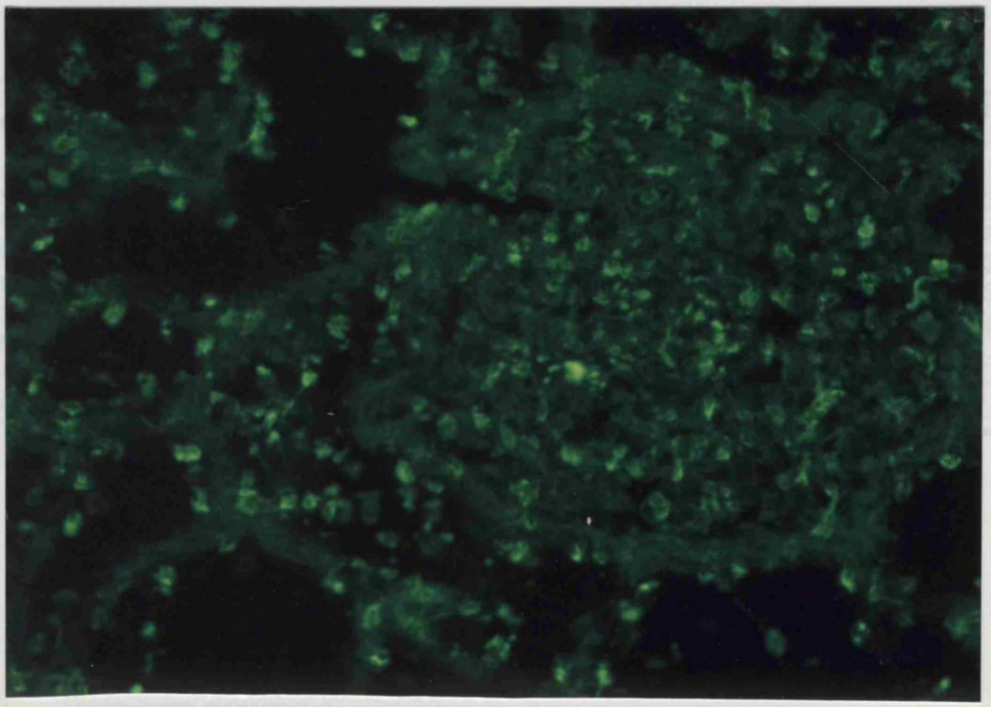
The binding of the two IgG anti-DNA antibodies, B3 and D5, was studied on tissue *in vitro*. Figure 3.31 shows the binding of B3 and D5 to human kidney sections. B3 bound to nuclear components in both the mouse and human kidney. The binding to the two species did not differ in appearance. D5 did not bind to renal tissue from either species.

To study the biological properties of B3 and D5 *in vivo* SCID mice were used. The antibodies were administered both in the form of hybridomas secreting mAb and purified immunoglobulin. Injection of the hybridomas into the peritoneum of the SCID mice produced ascites in all cases. The ascites became clinically apparent between 3-7 weeks after administration. The human immunoglobulin levels in the ascites and serum were similar in all mice indicating that the immunoglobulin transferred freely between the peritoneum and the blood compartments. Table 3.9 shows the time to ascites formation, the serum and ascites immunoglobulin concentration and the degree of proteinuria.

The human IgG concentration in mice injected with the hybridoma secreting D5 was approximately 10 times less than for B3 although the volume of ascites and the weight increase was similar in the two groups. This difference in the concentration between D5 and B3 was mirrored by the concentrations of the two antibodies in culture immediately prior to injection, 0.45μg/ml and 3.5μg/ml respectively. Only the mice that were injected with the hybridomas

Figure 3.31. B3 binds to the nuclei of fixed cells *in vitro*. Immunofluorescence picture of a normal human kidney section (fixed in acetone) incubated *in vitro* with (a) monoclonal antibody B3 showing nuclear staining and (b) D5 showing the absence of staining (x40).

(a)



(b)

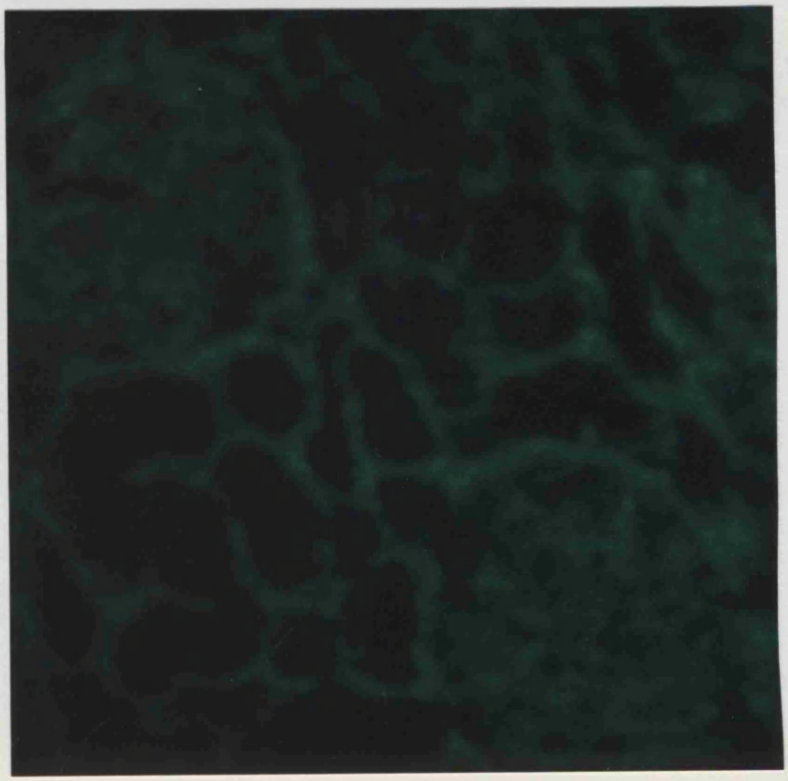


Table 3.9 Data on the SCID mice injected with hybridomas secreting IgG anti-DNA antibodies. Time to development of ascites (increase of weight by 20%), serum and ascites immunoglobulin concentration and proteinuria as recorded by Albustix® (Bayer Diagnostics).

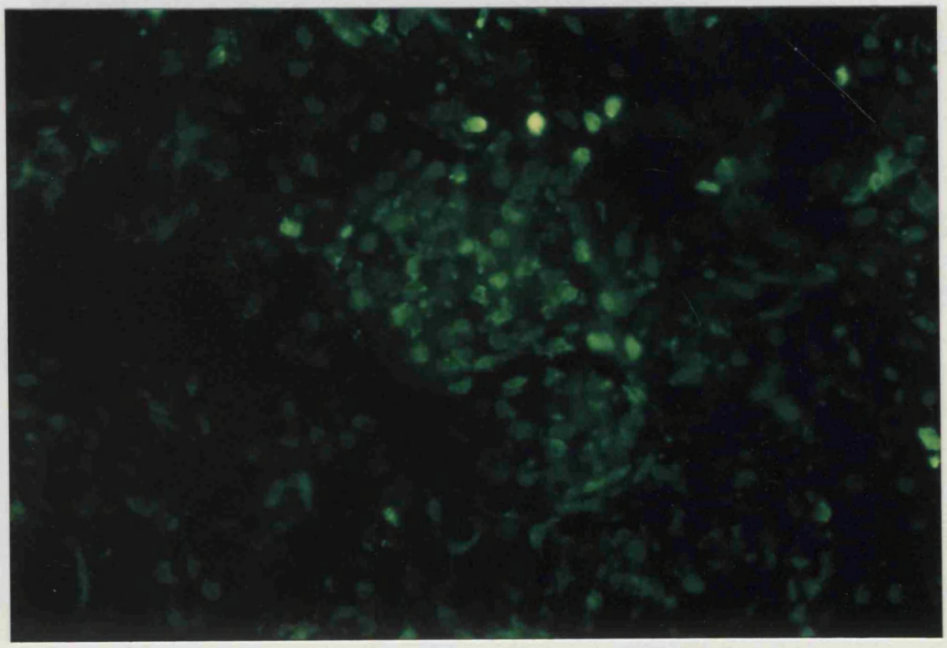
Number of Mice	Hybridoma	Time to ascites (days)	Human Ig ascites mean ± s.d. (µg/ml)	Human Ig serum mean ± s.d. (µg/ml)	Proteinuria (dipstick) mean/range
6	B3	45±10	122±56	124±50	2.4/2-3+
6	D5	37±11	15±6	14±5	0.2/Trace-1+
5	35.21	30±5	556±181	612±174	2.2/2-3+
6	33.C9	41±8	386±121	314±104	2.4/2-3+
5	32.B9	29±7	409±92	422±77	0.8/Trace-2+
5	33.H11	-	0	0	Trace
4	CB-F7	28±8	0	0	Trace
4	-	-	0	0	Trace

producing B3 developed significant proteinuria. Although the mice that had received B3 had significantly elevated levels of proteinuria compared to D5 and the control mice as determined by Albustix®, the creatinine levels were not significantly raised (data provided by the Biochemistry Department, University College London). The mice that received B3, either intravenously or by hybridoma injection, had detectable human Ig deposits in the tissues. B3 was found on cell surfaces in all the organs examined (Figure 3.32 a kidney; b skin). The pattern of staining in the mice that received B3 was the same irrespective of the method of administration. In the skin, cells which stained positive for B3 were in the dermis. Neither pooled human IgG injected intravenously, nor D5 given by either route were deposited in the sections examined (Fig 3.32c). The architecture of the kidneys and other organs appeared normal (Figure 3.33, kidney) (as assessed by Dr. D Katz).

In an additional set of experiments four human monoclonal IgG anti-DNA antibodies were used (kind gift of Dr. Thomas Winkler, Erlangen, Germany). The binding properties of these antibodies are shown in section 2.7.1. The hybridomas secreting these antibodies were injected into the peritoneum of SCID mice (5-6 mice per antibody). Table 3.9 shows the time to ascites formation, the serum and ascites immunoglobulin concentration and the degree of proteinuria. Three out of the four hybridomas caused ascites formation, two of produced detectable antibody deposition in the mice. The antibody designated 33.C9 deposited exclusively in the glomeruli of the kidneys (but not in any other organ) (figure 3.34a). In contrast the antibody 35.21 was found on cell surfaces in the kidney (figure 3.34b) and in the other organs examined. Thus the appearance found with 35.21 was similar to that found with B3. There were no abnormal pathological changes, but proteinuria was detected in the mice that had received 35.21 and 33.C9 (table 3.9). An identical staining pattern was seen in all the mice that had received the same hybridoma.

Figure 3.32. B3 binds to cell surfaces in vivo. Immunofluorescence photograph of (a) renal tissue, (b) skin derived from a SCID mouse injected with hybridomas secreting B3; (c) (page 175) shows the kidney of a mouse injected with hybridomas secreting D5 as a negative control. (Magnification x40).

(a)



(b)

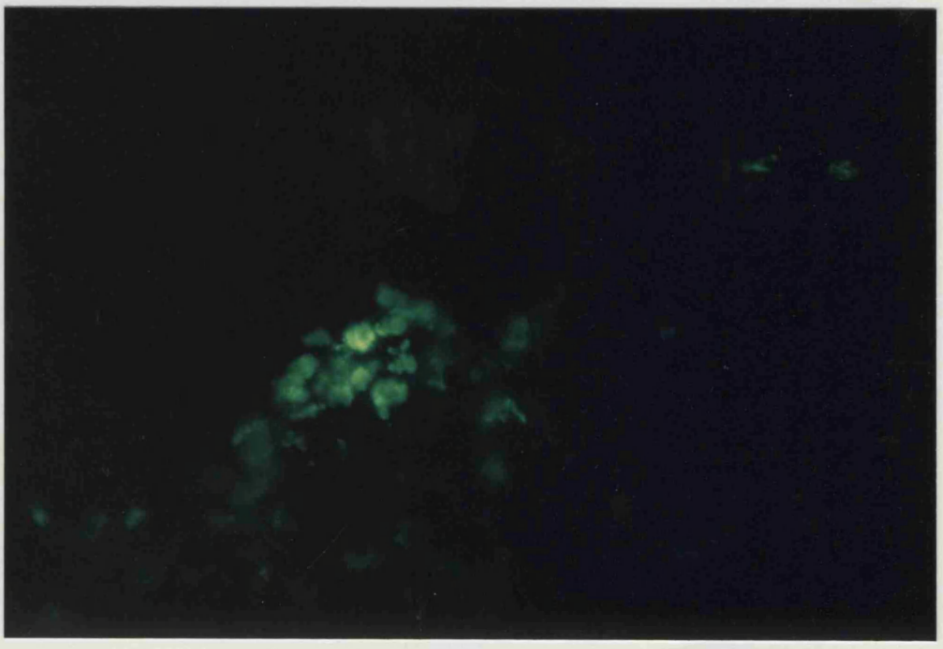


Figure 3.32 (continued)

(c)

Figure 3.33. No pathological changes were observed in the kidneys of SCID mice. Kidney section from a SCID mouse injected with hybridomas secreting B3 stained with haemotoxylin showing a normal gomerulus (Magnification x40).

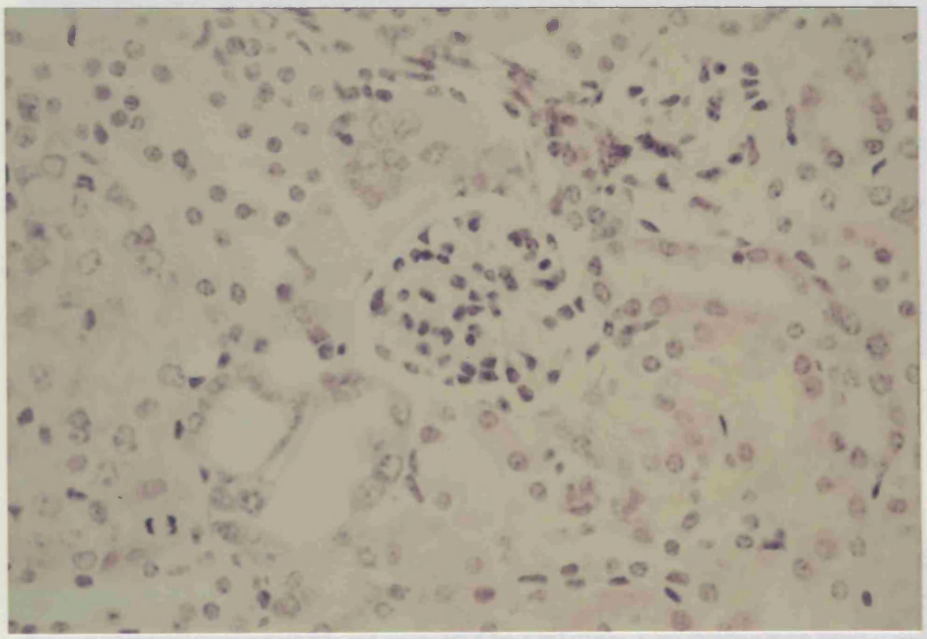
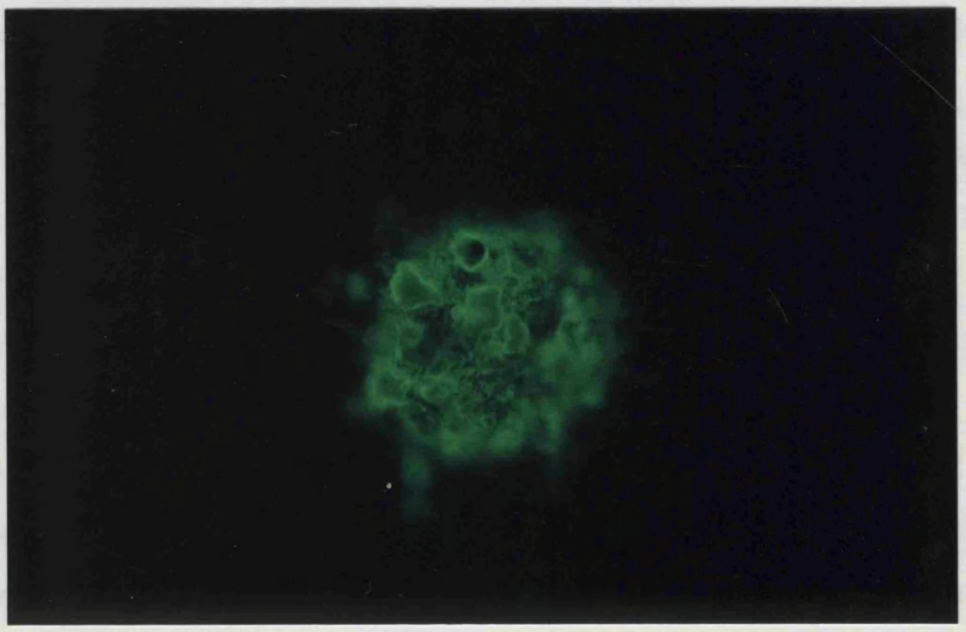
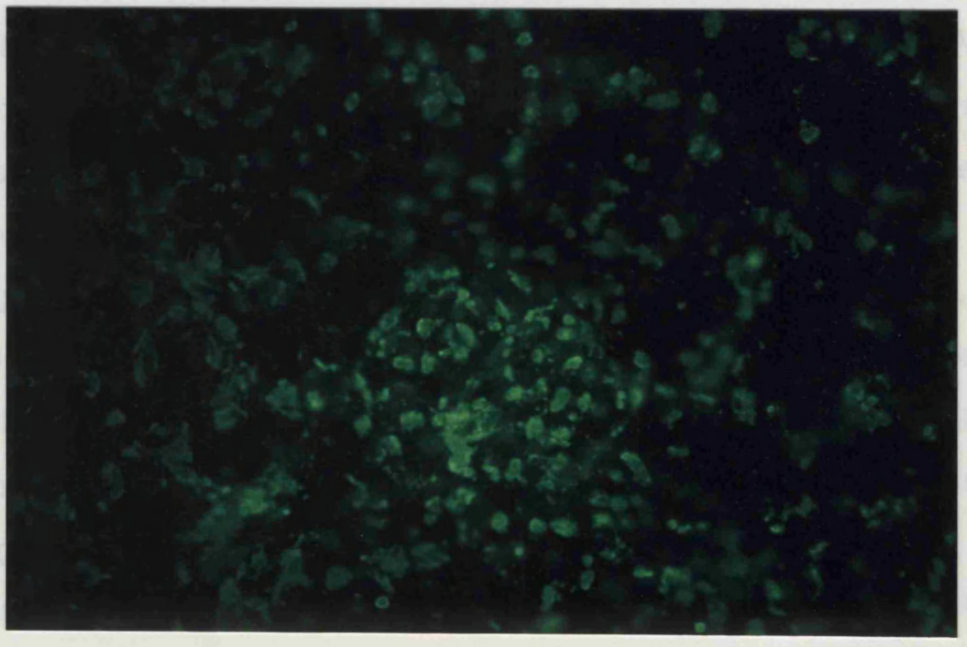


Figure 3.34. The binding pattern of two additional human monoclonal anti-DNA antibodies in vivo. Kidney section from a SCID mouse injected with hybridomas secreting (a) 33.C9 where IgG was found exclusively in the glomerulus and (b) 35.21 where IgG was found on cell surfaces.

(a)



(b)



3.3.2 Immunisation of normal mice with anti-DNA/DNA complexes

These experiments were performed to identify whether a human monoclonal IgG anti-dsDNA mAb could induce disease when used to immunise normal mice. Apart from its binding characteristics (i.e. anti-DNA), the monoclonal antibody B3 was chosen for two reasons. Firstly, because B3 induced anti-DNA antibodies in a normal rabbit when it was immunised with B3 to produce anti-idiotypic antibodies (see figure 3.10) and secondly because its heavy chain utilised VH-26, the heavy chain thought to encode all 16/6 idiotype positive antibodies which have been shown in some, though not all, laboratories to induce a lupus like disease in normal mice (section 1.14). Since it was not practical to use a large number of rabbits for the experiments, BALB/c mice were instead. A final consideration was that B3 (unlike D5) bound to DNA in the supernatant and that the DNA bound to the antibody through purification unless it was specifically removed using 3M NaCl. This contamination of purified anti-DNA antibodies with DNA may lead to the injection of immune complexes rather than simply the antibody alone.

Two series of experiments were performed as described in section 2.7.2 to explore the effects of immunising the mice with B3 complexed to DNA using two different routes. The first experiment used the intraperitoneal route, and in the second the antigens were injected subcutaneously. In addition, different amounts of DNA and B3 were used in the latter experiment.

Intraperitoneal immunisation. Three months after the first immunisation, the levels of anti-DNA antibodies in the mice in the group which had received B3 and DNA had significant levels of anti-dsDNA ($p<0.05$) and ssDNA ($p<0.01$) antibodies compared to controls. The levels in the serum of mice in the other two groups were not significantly increased above those of the controls. By six months the response in the group that had received the combination of B3 and

DNA had started to decrease, in two of the mice the anti-DNA antibody levels were comparable to those in the PBS control group. All of the mice remained well and none developed proteinuria. No clinical manifestations similar to SLE such as arthritis or alopecia were observed. The organs were not examined histopathologically. The experiment was terminated at six months. Figure 3.35 shows the anti-DNA response at the three time intervals tested.

Subcutaneous immunisation. The levels of anti-DNA antibodies were also elevated in the mice that had received the combination of B3 and DNA. The levels of anti-DNA antibodies were generally lower than in the first experiment (intraperitoneal immunisation) compared to a serum sample from an MRL/lpr mouse (Figure 3.36). However, the ODs recorded in the anti-DNA ELISA for some of the mice in the PBS control group of the first experiment were higher than the comparative figures in this experiment. With the increase in the amount of DNA and B3 injected together, there was a concomitant rise in the anti-DNA antibody levels. The level of significance quoted at three months (Figure 3.37) illustrates this point; the level of significance between the higher combination groups of DNA and B3, and the PBS control tended to be greater than for the lower combination groups. As with the first experiment using intraperitoneal immunisation all the mice remained visibly well and did not develop proteinuria. Mice from both experiments were tested for the presence of the B3-RId but none were found to be positive.

In summary, a combination of the mAb B3 complexed to DNA induced anti-DNA antibodies in the serum of normal mice. However, the antibody levels in the serum were at a relatively low titre and the mice remained healthy.

Figure 3.35 Intraperitoneal immunisation of B3 and DNA into BALB/c mice (5 mice per group). Anti-DNA antibodies in the serum of the mice injected with B3 and/or DNA intraperitoneally. For comparison, levels of significance are shown only for serum tested at three months for the mice that had received combinations of B3 and DNA. The other groups that were injected with B3 or DNA alone were not significantly different from the PBS group at three months. The serun from an MRL/lpr mouse is shown for comparison.

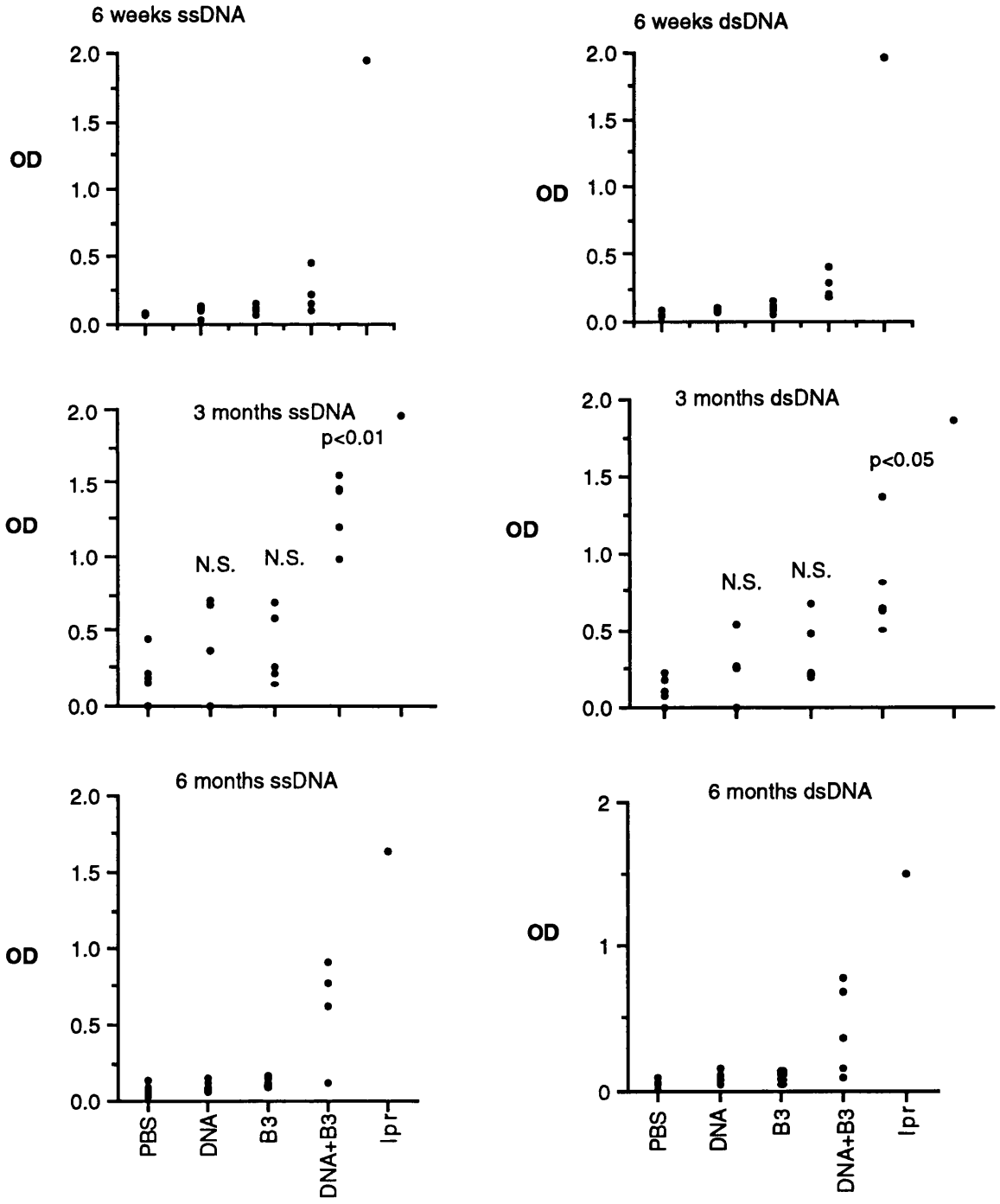
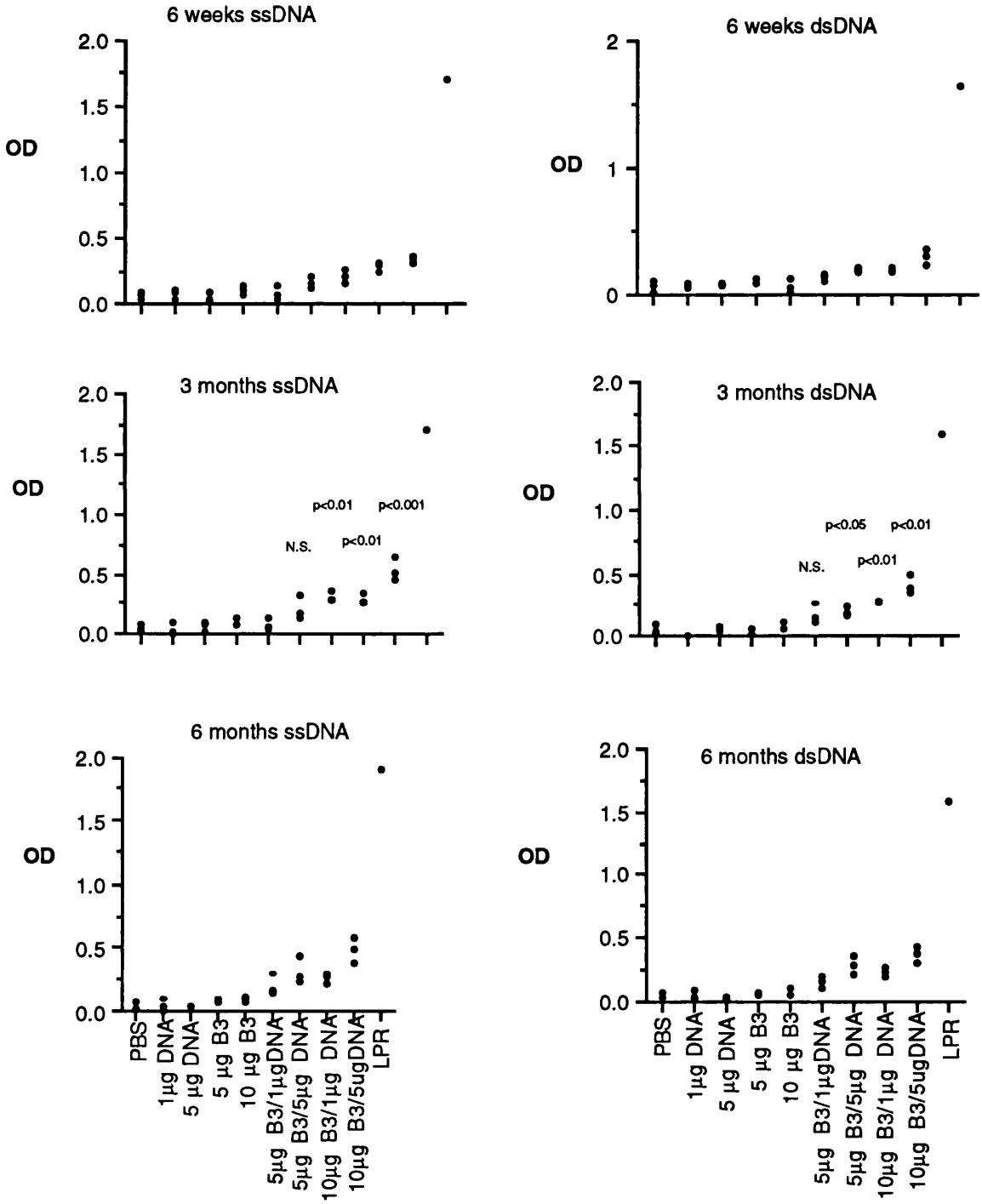


Figure 3.36. Subcutaneous immunisation of B3 and DNA into BALB/c mice (three mice per group). Anti-DNA antibodies in the serum of the mice injected with B3 and/or DNA subcutaneously. Levels of significance are shown only for serum tested at three months for the mice that had received combinations of B3 and DNA. The other groups that were injected with B3 or DNA alone were not significantly different from the PBS group.



*3.4 SEQUENCES OF B3 AND D5 (by kind permission of Ms Celia Longhurst)

The sequences of the heavy and light chain variable regions of B3 and D5 were determined by Ms Celia Longhurst under the supervision of Professor David Latchman (University College London) and in collaboration with Dr. Freda Stevenson [Southampton University] (Stevenson et al, 1993; Ehrenstein et al, 1994). The sequences are shown here and a brief description given. The sequences are discussed in section 4 only in relation to the other data presented in the preceding three sections.

3.4.1 Sequence of B3

The nucleotide sequence of the Vλ region of the IgG anti-dsDNA antibody B3 (Fig 3.37a) indicates that it has 90% homology with the germline gene Vλ 2.1 (Brockly et al, 1989), and 85% homology at the amino acid level (Fig 3.37b). There is a total of 14 replacement mutations, 6 of which are in CDR3. The replacement to silent ratio in CDR3 of the light chain is 7 implying a possible antigen-driven mechanism. The Jλ region is the unmutated Jλ2 sequence. A sequence comparison between B3 and PV11, an SLE-derived, IgM DNA non-binding monoclonal antibody, which carries the 8.12 Id (Paul et al, 1992) revealed a 96% homology at the nucleotide level and 89% homology at the amino acid level. There are four changes to positively charged amino acids (three arginines) in the CDRs of B3 λ chain compared to none in the λ chain of PV11.

The nucleotide sequence of the heavy chain of B3 (Fig 3.38a) has 93.5% homology with the germline gene segment VH26 (Chen et al, 1988) and 89% at

the amino acid level (Figure 3.38b). The D region could be aligned to the D gene segment DN1. The CDR3 region contains seven amino acids including one asparagine residue. There are no charged residues present in the CDR3 region. There is a replacement mutation at position 54 in CDR2 from serine to arginine resulting in a gain in positive charge. Lysine is replaced by glutamine at position 65 where a positive charge is lost. There are nine other replacement mutations within the VH region none of which lead to any change in charge. The framework 4 region consists of a JH4 gene segment with one mutation from serine to tyrosine.

Figure 3.37. Nucleotide (a) and amino acid (b) sequence of the light chain of B3 aligned to its most homologous counterpart: germ-line gene V lambda 2.1 and PV11, a non-DNA binding antibody.

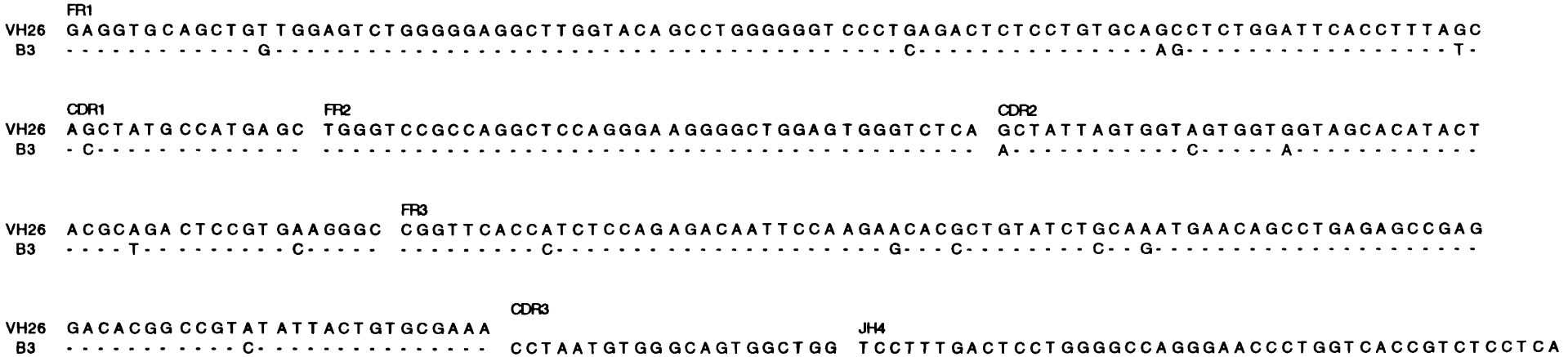
(a)

```
          FR1                                                                                                  CDR1
Vλ2.1  CAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATCTCCTGC ACTGGAACCAGCAGTGATGTT
B3     ------------------------------------------------------------------ ------------A--A--C--C
PV11   ------------------------------------------------------------------ ----------------C---

                                    FR2                                                                        CDR2
Vλ2.1  GGGAGTTATAACCTTGTCTCC TGGTACCAACAGCACCCAGGCAAAGCCCCCAAACTCATGATTTAT GAGGGCAGTAAGCGGCCCTCA
B3     --TG--------T-------- --------G--A------------------------------- ----T----C-T---------
PV11   --TG--------TA------- ----------A-------------------------------- --T-T------T---------

          FR3
Vλ2.1  GGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCT GACAATCTCTGGGCTCCAGGCTGAGGACGAGGCTGATT
B3     ----------C----------C--------------G---------------C-------------------------------C-
PV11   ------------------------------------------------C-------------------------------------

                CDR3
Vλ2.1  ATTACTGC TGCTCATATGCAGGTAGTAGCACTTTA Jλ
B3     -------- A--------AGCA-C-CC-CTCG-... GTGGTATTCGGCG GAGGGACCAAGCTGACCGTCCTAGGT
PV11   -------- A--------A--A-C--C---T--... ----------------------------------------
```

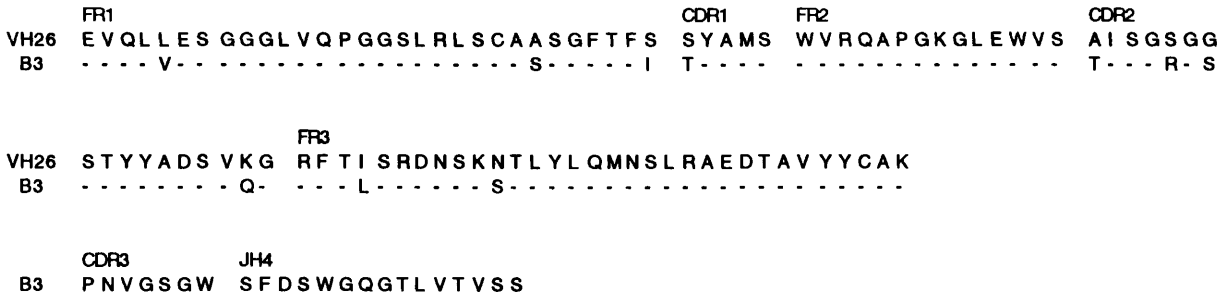
(b)

```
          FR1                          CDR1                FR2                  CDR2
Vλ2.1  QSALTQPASVSGSPGQSITISC TGTSSDVGSYNLVS WYQQHPGKAPKLMIY EGSKRPS
B3     ---------------------- ---RR---G--F-- --------------- -V-H---
PV11   ---------------------- --------G--Y-- --------------- DV-N---

          FR3                                   CDR3          Jλ
Vλ2.1  GVSNRFSGSKSGNTASLTISGLQAEDEADYYC CSYAGSSTL
33     ---T---A----S------------------- S--SSTTR. VVFGGGTKLTVLG
PV11   ------------------------------- S--TS--S. ---------------
```

Figure 3.38. Nucleotide (a) and amino acid (b) sequence of the heavy chain of B3 aligned to its most homologous counterpart: germ-line gene VH-26.



(b)



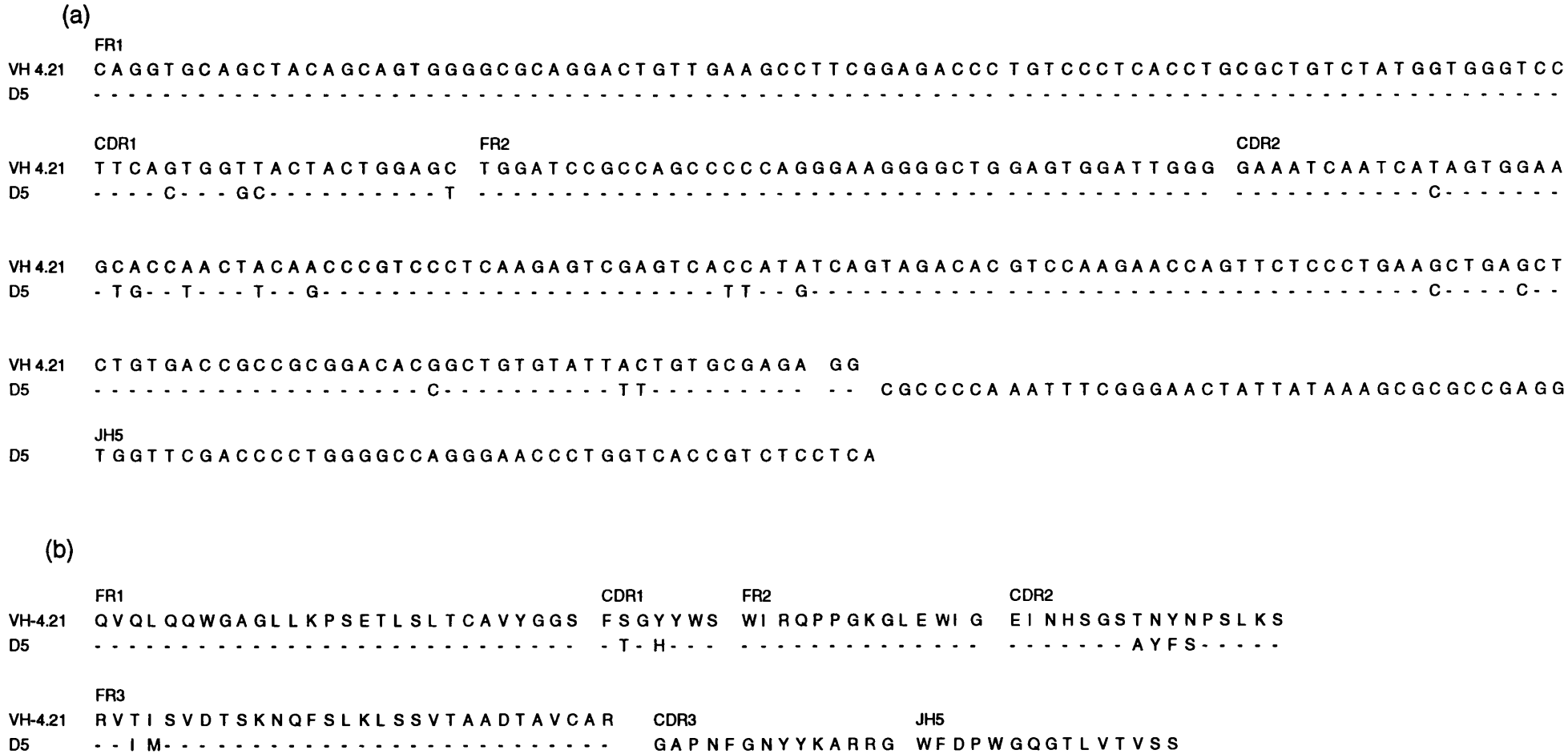
3.4.2 Sequence of D5

The sequence of the light chain of D5 (Figure 3.39) indicates that it belongs to the VkIIIb family and has 95.5% homology to the germ-line gene HumIGKVQ (Chen et al, 1987). Of the 13 mutations in the VL of D5, nine give rise to replacements, but five are in the CDRs and four in FW1 and FW2, and the R:S ratios again do not indicate a selective pressure on the CDRs. The replacement mutations show two changes to asparagines which may be important in the binding of DNA (Diamond et al, 1992).

The VH region of D5 shows 93% homology to the the VH-4.21 germ-line gene at the nucleotide level and 88% homology at the amino acid level (Figure 3.40). D5 is positive for the 9G4 idiotope (unpublished data) which has been mapped to the FR1 region of the VH-4.21 heavy chain (Potter et al, 1993). The mutations are found in the CDRs and FW3 and the R:S ratio are 1.5 and 2.5 respectively, which does not indicate any selection for amino acid changes in the CDRs. The D segment of D5 contains elements derived from the DXP'1 gene with various N region additions. The deduced sequence of 14 amino acid of the CDR3 region is basic with two arginines, one lysine and no acidic residues, and the J region is unmutated JH5. As with the couplet of arginines in the CDR1 region of the light chain of B3, the arginine couplet may also be important in DNA binding.

Figure 3.39. Nucleotide (a) and amino acid (b) sequence of the light chain of D5 aligned to its most homologous counterpart: germ-line gene HumIGKVQ (Chen et al, 1987).

Figure 3.40. Nucleotide (a) and amino acid (b) sequence of the heavy chain of D5 aligned to its most homologous counterpart: germ-line gene VH-4.21.



CHAPTER FOUR DISCUSSION

4.1 GENERATION OF HUMAN MONOCLONAL IgG ANTI-DNA ANTIBODIES

4.1.1 Patients

SLE manifests in numerous ways and probably encompasses several different though linked conditions. Consequently information relating to the patient population used in this thesis is crucial to the data contained herein. The most complete analysis of this patient population was undertaken by Worral et al (1990). This study suggested that the patient population was similar to other reported series except that there was less renal disease compared to some other groups. This observation may have important implications on the generation of monoclonal human IgG anti-DNA antibodies since it is with severe renal disease that IgG anti-DNA antibodies have been most clearly associated. The only other report of the production of human monoclonal IgG anti-DNA antibodies derived from the fusion of peripheral blood lymphocytes with a immortalised cell line indicated that the patients studied had severe renal disease. No IgG anti-DNA antibodies were generated from patients with less active disease, and the investigator took two years to find these patients finally using lymphopharesis (a procedure not commonly used in this country) to obtain sufficient cells (Winkler et al, 1991 and personal communication). Research into SLE relies on the availability of suitable material. The priority for treating lupus patients at the earliest possible opportunity, often with immunosuppressive therapy improves the outlook for the patient but reduces the numbers of suitable patients available for fusion at an appropriate time i.e. when their disease is most active.

As indicated in table 3.1 during the first eight months of this thesis the patients that were available did not all have very active disease. The median BILAG global score was only six indicating mild to moderate disease; there was only one patient with severe active renal disease. Although no clear conclusions can be drawn, the only patient that did yield IgG monoclonal anti-DNA antibodies had the highest BILAG global score i.e. very active disease. Further, the fusion of this patient's peripheral blood lymphocytes when her disease was relatively inactive yielded monoclonal IgM anti-DNA antibodies rather than IgG anti-DNA antibodies. Although suggestive, whether this indicates an isotype switch similar to that observed in the monoclonal antibodies derived from murine models of SLE at different phases of disease (Shlomchik et al, 1990; Tillman et al, 1992) is not proven. Interestingly, the patient had active arthritis when the IgG antibodies were produced indicating that patients with active disease other than renal disease can generate these antibodies. As mentioned in the introduction not all patients with high DNA binding titres have active renal disease. In one study using a purely human fusion partner, successful fusions were more frequently observed when the peripheral blood lymphocytes from active SLE patients were used. It was not clear whether the production of IgM anti-DNA antibodies per se occurred more often from active rather than inactive SLE patients (Watts et al, 1990b). The data in this thesis indicates that disease activity had no effect on the success of the fusion in terms of immunoglobulin production of any specificity.

The source of lymphocytes from patients with SLE for fusion experiments is thought to be important. In the study referred to above (Watts et al, 1990b), the spleen was superior in terms of hybridomas generated compared to peripheral blood lymphocytes. No such comparative data exists for the CB-F7 heteromyeloma cell line. During the period of this thesis only peripheral blood was available as a source of lymphocytes.

4.1.2 Fusion or EBV transformation for the generation of monoclonal IgG antibodies?

To determine the most efficient method of producing monoclonal IgG antibodies, three methods were compared in this thesis using the lymphocytes from three patients with SLE. Direct fusion with the CB-F7 cell line resulted on average in a higher proportion of IgG antibodies to IgM antibodies compared to EBV transformation. EBV transformation followed by fusion yielded many clones, in particular IgM secreting hybridomas. These results suggest that direct fusion with CB-F7 would be the most successful method of generating monoclonal IgG anti-DNA antibodies. As discussed in the introduction, there is much debate in the literature as to whether EBV transformation or fusion is the best technique to produce antibodies most characteristic of the immune (and autoimmune) response. No previous comparison has been made between these two methods in patients with SLE and it is not possible to compare the results here with other reported data. Further there is no data as to whether heteromyeloma cell lines select for a particular population as has been suggested for purely human fusion partners which are thought to fuse preferentially with activated cells (Olsson et al, 1983).

Does the higher ratio of IgM to IgG antibodies obtained with EBV transformation indicate that there are more IgM secreting cells in the peripheral blood of SLE patients, or does EBV select for IgM secreting cells? There is some controversy about the type of cells that are susceptible to EBV transformation (see section 1.16). However, an analysis of studies comparing EBV transformation to other methods of enumerating the precursors of autoantibodies from patients with autoimmune disorders suggests that EBV does not transform a proportion of those cells producing pathogenic (or at least disease associated) autoantibodies (Moynier et al, 1991; Shibata et al, 1992). The data in this thesis suggests that EBV selects for IgM producing B cells from

SLE patients and that fusing the EBV transformed cell lines results in more IgM secreting clones which would hamper the isolation of an IgG producing hybridoma of interest.

4.1.3 The paucity of monoclonal IgG anti-DNA antibodies generated from SLE patients

After establishing that direct fusion with CB-F7 yielded the highest ratio of IgG to IgM secreting cells, direct fusion was performed on a further 16 patients. Despite the fact that IgG antibodies were consistently produced from the fusions, only one fusion produced IgG anti-DNA antibodies. Conversely, IgM anti-DNA antibodies were often generated from the fusions. As mentioned in section 1.8, the ratio of IgM to IgG anti-DNA antibodies present in the peripheral blood of SLE patients would predict a more frequent occurrence of IgG antibodies. Although the data does not suggest that the heteromyeloma cell line has the marked bias for B cells producing IgM antibodies of any specificity as seen with EBV transformation, there is a tendency to fuse with cells secreting IgM compared to cells secreting IgG anti-DNA antibodies. This contrasts somewhat from the purely human fusion partners used previously in our laboratory and elsewhere. Using these lines the number of IgG antibodies of any specificity produced was low and results in the over representation of IgM antibodies as seen with EBV transformation (Ravirajan et al, 1992). A further difference between the purely human fusion partners such as GM 4672 and the heteromyeloma line CB-F7 is the higher fusion frequency of the latter. The fusion frequency of 5.0 per 10^7 lymphocytes fused observed with the GM 4672 line has been calculated by counting the number of wells with dividing cells and dividing this number by the number of lymphocytes used for the fusion. This method assumes that there is only one dividing clone per well which is not the

case when more than a third of the plate's wells has dividing cells as occurred in the majority of fusions performed in this thesis. The fusion frequency observed with the CB-F7 line in other studies was two orders of magnitude higher than the GM 4672 line (Winkler et al, 1991).

Although the heteromyeloma does not have the same bias towards B cells secreting IgM antibodies observed with purely human fusion partners, it did not yield the expected number of monoclonal IgG anti-DNA antibodies when fused with PBL from SLE patients. This situation contrasts with data from murine anti-DNA antibodies where IgG anti-DNA antibodies have been consistently produced from lupus models. In fact IgG anti-DNA antibodies are often the dominant isotype for this specificity. In one study using MRL/lpr mice, 25 out of 31 anti-DNA clones from 5 mice with active disease were of the IgG isotype (Shlomchik et al, 1990). There may be several reasons for this difference in the mouse model including the properties of the mouse fusion partners compared to the human, the fact that B cell function and distribution in murine models of lupus are different to human SLE, or the fact that murine fusions use spleen cells. However, an alternative explanation may be that, as mentioned in the introduction, the disease activity in human SLE, often treated with immunosuppressants, is not comparable to the relentless disease progression seen in murine SLE. Therefore, one might conclude that if the patients studied had severe, untreated disease, IgG anti-DNA antibodies would be generated more frequently. The data in this thesis would support this hypothesis as would the description of the production of IgG anti-DNA antibodies from Winkler and colleagues. Further, when the IgG anti-DNA antibodies were generated from patient HAM when her disease was very active, no IgM anti-DNA antibodies were produced. This mirrors the situation described by Shlomchik et al (1990) in murine models where very few IgM anti-DNA antibodies were generated from the mice with active disease.

The fact that five IgG anti-DNA antibodies were generated from one patient as compared to none from the other patients would also suggest that the fusion frequency or selective bias towards IgM anti-DNA antibodies is not responsible for the usual paucity of IgG anti-DNA antibodies. If these explanations were valid one might expect that only one IgG anti-DNA antibody would be produced from patient HAM when her disease was active, or alternatively the number of IgG anti-DNA antibodies generated from the other patients would vary from 0-5. Rather, the sudden increase from no IgG anti-DNA antibodies in all but one of the patients to five in the other suggests that a discontinuous variable/s govern the production of IgG anti-DNA antibodies. Further, these variables do not operate at all times since no IgG anti-DNA antibodies were generated when the patient's disease was inactive, six months earlier, despite a successful fusion.

4.1.4 Binding specificity of the IgG and IgM anti-DNA antibodies generated from the same patient.

The IgG anti-DNA antibodies differed from the IgM anti-DNA antibodies generated from patient HAM in several ways. Only one out of the five IgM anti-DNA antibody clones bound dsDNA, but three of the IgG antibodies bound dsDNA. Whereas none of the IgM anti-DNA antibodies were positive in the Crithidia assay, two out of the five IgG anti-DNA antibodies were reactive. Thus some of the IgG anti-DNA antibodies were of higher affinity than the IgM anti-DNA antibodies. This reflects the changes observed in the binding of the sera to Crithidia. Only on the second occasion when the patient's disease was active did her serum bind Crithidia (IgG fraction). Moreover, the amount of IgM anti-dsDNA antibodies (as measured by ELISA) present in the serum on the second occasion was reduced by almost fourfold as compared to the first occasion when her disease was inactive. This would be consistent with a "switch"

towards the production of IgG anti-DNA antibodies. However, not all of the monoclonal IgG anti-DNA antibodies were of high affinity to DNA as judged by Crithidia binding supporting the view that isotype switching is not always associated with high affinity antibodies (Casali and Notkins, 1989).

Two of the IgG anti-dsDNA monoclonal antibodies would have not been identified if the supernatant had not been pretreated by DNAase. Change in the reactivity of anti-DNA antibodies by DNAase has been reported by other workers, but not to the extent that without prior DNAase treatment the antibodies were undetectable (Brinkman et al, 1989). It is therefore important when screening for monoclonal antibodies for DNA reactivity by ELISA to include DNAase treatment as a routine, otherwise high affinity antibodies may be missed. This may explain the lack of IgG anti-DNA antibodies identified previously. I did not find that lower affinity antibodies of both isotypes, particularly those that bound predominantly to ssDNA, were affected by DNAase treatment. This may be due to the type of DNA present in the supernatant i.e. it is in a dsDNA form. Bell et al (1990) have demonstrated that cells in culture release DNA containing nucleosomes in vitro and that these nucleosomes stimulate proliferation and immunoglobulin synthesis of normal mouse lymphocytes. Further, hybridomas have also been found to release nucleosomes indicating that they are undergoing apoptosis (Franek and Dolníková, 1991). Of relevance, a recent report suggests that these nucleosomes occur in much higher concentrations in the plasma of SLE patients compared with normal controls (Rumore and Steinman, 1990).

The DNA bound to one of the IgG anti-DNA antibodies selected for further study, i.e. B3, was isolated during the purification procedure and gave a ladder pattern on agarose gel electrophoresis, typical of the appearance seen with nucleosomes. This DNA was derived from the supernatant containing B3 and indicates that the hybridoma generating B3 was undergoing apoptosis. The

properties of B3 that resulted in it being complexed with DNA in the supernatant have not been clearly delineated. If it were simply ascribed to B3 binding to DNA then D5 would also have complexed to this DNA. One difference between B3 and D5 is that the former bound to dsDNA whereas the latter bound weakly. In addition, B3 bound to histones partly through antibody/DNA complexes unlike D5 which did not bind to histones either alone or complexed to DNA. Thus the DNA that allows anti-DNA/DNA complexes to bind to histones must be in a dsDNA form as is found in nucleosomes. This reactivity to histones by a high affinity anti-dsDNA antibody is in keeping with reports suggesting that antibodies to the DNA/ histone complex may be partly responsible for causing tissue pathology (Termaat et al, 1992; Chan et al, 1992).

4.2 ANALYSIS OF IGG ANTI-DNA ANTIBODY IDIOTYPES

The idiotypes described in this thesis are the first to be defined by anti-idiotypes raised by human monoclonal IgG anti-DNA antibodies. Since monoclonal IgM anti-DNA antibodies have been generated from normal individuals, whereas monoclonal IgG anti-DNA antibodies have only been produced from patients with active disease, the idiotypes identified on IgM anti-DNA antibodies may be "natural idiotypes". In contrast, idiotypes identified on IgG anti-DNA antibodies are more likely to identify "pathogenic" idiotypes, or at least those associated more clearly with SLE. Idiotypes defined by anti-idiotypes raised by IgM antibodies have been found on IgG antibodies for example B3 carries an idiotype, WRI-176 Idβ, first defined on a human monoclonal IgM anti-DNA antibody (Dr. J Kalsi, personal communication).

In structural terms one difference between IgM and IgG associated

idiotypes is that the latter are more likely to involve somatic mutations. Nevertheless, anti-idiotypes generated from a human monoclonal IgG anti-DNA antibody may identify germ-line encoded structures. Much of the sequences of B3 and D5 are germ-line providing ample epitopes for the anti-idiotype to bind. However, there is some evidence in this thesis that the anti-idiotypes tended to recognise IgG antibodies, particularly in lupus patients. Since it has been established that the genes used to encode these antibodies are similar, if not identical, to genes associated with IgM anti-DNA antibodies, the explanation that the IgG monoclonal antibodies are encoded by a gene used late in the immune response cannot be correct. The alternative explanation that the anti-idiotypes are recognising epitopes that have been generated by somatic mutation of the nucleotide sequence seems more likely. The hypothesis that these epitopes are part of the idiotype raises a number of issues, since only a minority of amino acids have arisen by somatic mutation. In particular, are these amino acids more antigenic in the context of an immune response? The answer to this point is not known, but the recent re-examination of idiotypes by Jefferis may have some bearing on this (Jefferis, 1993). He proposed that idiotypes that are formed by germ-line sequences are not true idiotypes but isotypes: can these isotypes form part of the idiotype network? If they do not then in terms of the idiotype network, idiotypes which are formed by amino acids arising by somatic mutations would be the only antigens recognised. However, it should be remembered that these idiotypes are not defined through a naturally occurring idiotype network but through a xenogeneic response. Whether the rules governing an autologous response are similar to those pertaining to a xenogeneic immune response, with respect to idiotypes, has not been determined.

4.2.1 The B3-RId

4.2.1.1 B3-RId: Characterisation

The B3-RId was defined by a polyclonal anti-idiotype raised in a rabbit and was found to be specific for B3. The B3-RId was situated at or near the binding site for DNA as determined by inhibition ELISA. The anti-idiotype bound to the light chain of B3 as demonstrated by Western blotting. This does not imply that all the epitopes that the anti-idiotype recognised were present exclusively on the light chain, some may have been conformational i.e. composed of elements of both the heavy and the light chain. However, one important conclusion from this data is that the light chain is involved in the binding to DNA.

To pursue the structural features of the idiotype further a number of techniques may be used, most of which rely on the sequence of B3 being known. Probably the simplest technique that has been used in the past is the investigation of other monoclonal antibodies whose nucleotide sequence has been established. A panel of monoclonal antibodies which have similar features can be used to determine the amino acids that are being recognised by the anti-idiotype. This has been used successfully to further characterise anti-DNA associated idiotypes such as the 3I, 8.12 and the 16/6 idiotypes (Mannheimer-Lory et al, 1991b; Paul et al, 1992; Young et al, 1990). B3 has been sequenced by Ms Celia Longhurst and is shown in section 3.4. The light chain of B3 where the B3-RId is situated showed the highest homology (90% at the nucleotide level and 85% at the amino acid level) to the Vλ 2.1 germline. This germ-line belongs to the VλI I family which encodes another DNA associated idiotype, 8.12 (Paul et al, 1992). This idiotype has been associated with anti-DNA antibodies and has been found in the renal lesions of some SLE patients. The 8.12 idiotype is recognised by a monoclonal antibody raised by

injection of affinity purified anti-DNA antibodies derived from a lupus patient and is not related to the DNA binding site. It therefore differs in a number of ways from the B3-RId. A bank of lambda monoclonal antibodies, some of which were encoded by the V λI I family were tested for the presence of the B3-RId and all found to be negative; whereas B3 was positive for the 8.12 Id (Dr. Betty Diamond, personal communication). A comparison of the light chain variable region sequences of B3 to the other monoclonal antibodies revealed a putative site for the idiotype. In the CDR1 region of the light chain of B3 two adjacent arginines are present (positions 26, 27). These amino acids differ from the two serine residues that are found in the other monoclonal antibodies. Arginines have been shown to be important in conferring specificity to murine anti-DNA antibodies to DNA (Radic et al, 1993). This study revealed that a similar change from serine to arginine in CDR1 of the heavy chain increased the binding for DNA of the original anti-DNA antibody, and that changing two neutral charged amino acids (such as serine) to arginines had an additive effect on the binding to DNA. Since the B3-RId is at or near the binding site for DNA the suggestion that the two arginines from part of the idiotype is attractive. Interestingly the position of two other human light chain anti-DNA antibody associated idiotypes has also been tentatively sited in the CDR1 of the light chain i.e. the 3I and 8.12 idiotypes (Mannheimer-Lory et al, 1991b; Paul et al, 1992). The 8.12 Id is thought to be dependent on the tyrosine at position 32 which is preserved in the B3 antibody.

Despite this analysis of the position of the B3-RId there is one piece of information that does not appear to be consistent. That is the detection of the B3-RId on IgM antibodies in the serum of a proportion of patients with rheumatoid arthritis. There are a number of reasons that may be responsible for this apparent discrepancy. The first relates to the study of idiotypes in general.

B3-RId is a polyclonal preparation and therefore contains a wide variety of antibodies with varying affinities for the idiotype and all binding to varying epitopes. Consequently the epitopes recognised in one situation may be different from another. This may be especially true when analysing serum where there are two sets of polyclonal preparations. However, this is not the case when monoclonal antibodies are tested for the presence of the B3-RId, therefore information from monoclonal antibodies may be regarded as more "precise". Certainly there was much less background binding when a "negative" monoclonal antibody was tested in the idiotype ELISA compared to when a "negative" serum was tested (data not shown). The second point relates to the nature of rheumatoid factors. Although the presence of the idiotype on IgM antibodies did not correlate with the presence of rheumatoid factor, the measurement of rheumatoid factor may not detect all the anti-immunoglobulin present in the sera. Some rheumatoid factors may be directed towards the relatively constant components of variable regions. Although the idiotype ELISA is designed so that any Fc binding to the rabbit anti-Id id is mirrored by binding to the normal rabbit serum, the epitopes may not be identical on the two sides of the ELISA plate perhaps because of the presence of different IgG subclasses.

There are a number of ways of confirming the hypothesis that the B3-RId involves the two adjacent arginines in CDR1. Firstly, the analysis of human monoclonal antibodies should be extended: it is important to identify other B3-RId positive clones and determine whether they also have arginines in the same positions. However, one analogous study failed to delineate precisely the site of an idiotype (16/6) originally identified on an IgM monoclonal anti-DNA antibody using a bank of other monoclonal antibodies (Young et al, 1990). The mapping of the 9G4 Id has been successfully achieved by site directed mutagenesis of the putative site of the idiotope (Potter et al, 1993). Both of these idiotypes are

probably encoded by germ-line genes, consequently the frequency of monoclonal antibodies encoded by the gene would be relatively high compared to the frequency of monoclonal antibodies bearing an idiotype that is derived by somatic mutation. A paucity of B3-RId positive monoclonal antibodies would make the idiotype difficult to map. This may explain why the only other anti-DNA antibody associated with IgG antibodies, F4 (Davidson et al, 1989), and perhaps derived from somatic mutation, has not been mapped. This is despite the fact that the group who described this idiotype mapped two other DNA associated idiotypes (to germ-line sequences) successfully using a bank of monoclonal antibodies (Mannheimer-Lory et al, 1991b; Paul et al, 1992).

The second way of detailing the location of the B3-RId involves epitope mapping which has been used successfully for other idiotypes (e.g. Staines et al, 1993b). This however presupposes that the idiotype involves a continuous stretch of amino acids. Finally probably the most comprehensive system for mapping idiotypes involves X-Ray crystallography (Bentley et al, 1990). However, this technique requires that both the idiotype positive antibody and the anti-idiotype are monoclonal antibodies and is not therefore available for mapping the B3-RId.

Finally it should be noted that there is no definitive proof that the two adjacent arginines present in the CDR1 of the light chain of B3 arose by somatic mutation. This can only be ascertained by sequencing the germ-line of the patient from which these monoclonal antibodies arose. However, the couplet of arginines in CDR1 has not been found in any reported sequence of the VλI I family (Paul et al, 1992; Williams and Winter, 1993).

4.2.1.2 B3-RId: Distribution

The distribution of the B3-RId was examined in the sera of normal individuals and patients with a variety of conditions including SLE and other autoimmune rheumatic diseases. The idiotype when detected appears to be present predominantly on the IgG population in SLE sera, but also intriguingly on the IgM population of sera from some rheumatoid arthritis patients (see above). The increased expression of B3-RId on IgG antibodies in SLE patients did not merely reflect an overall increase in IgG in SLE since there was no correlation between the B3-RId level and total IgG levels. Further there was no correlation between the B3-RId and the two idiotypes associated with D5 which were also elevated in the serum of lupus patients. Therefore, the increase in idiotype levels is unlikely to be simply due to hypergammaglobulinaemia secondary to polyclonal activation. In patients with primary Sjogren's syndrome the B3-RId was not detectable either on IgM or IgG antibodies. In contrast other idiotypes identified on anti-DNA antibodies are frequently found in other autoimmune rheumatic diseases particularly Sjogren's syndrome. In a collaborative study of 19 different idiotypes 9 were present in more than 30% of Sjögren's syndrome and none of the idiotypes were disease specific (Isenberg et al, 1990).

The increased expression of this idiotype in SLE patients is largely confined to lupus patients who have active arthritis, though only a proportion of these were positive. Moreover, the level of B3-RId showed a closer correlation to arthritis activity than to overall disease activity in four patients studied serially. Since SLE often involves several organs at the same time though to variable degrees, previous attempts at linking the levels of idiotypes to disease using global indices may have failed to detect underlying disease fluctuations in individual systems. The BILAG index, which was designed to distinguish individual organ/system involvement and incorporates a rate of change

element, allows the fine dissection of disease activity.

A further source of obfuscation may arise from the failure to distinguish which individual immunoglobulin class the idiotypes are present on. Of the separate analysis of IgG and IgM antibodies that have been reported in the literature, the 16/6 idiotype has been found to be expressed on both IgG and IgM antibodies in tissue lesions (Isenberg and Collins, 1985). The PR4-Id derived from a patient with leprosy has been found to be expressed on IgG, IgM and IgA antibodies in lupus patients, but no correlation was found between the IgG, IgA and IgM PR4-Id levels and a variety of parameters in lupus patients including disease activity (Pilcher et al, 1991). The 3I idiotype has been found on both IgG and IgM antibodies; anti-DNA binding was not associated with the IgM class on myeloma proteins but was on SLE derived IgG and IgM monoclonal antibodies derived from EBV transformed lymphocytes (Mannheimer-Lory et al, 1991a). Switching of an idiotype from one immunoglobulin class to another may be linked to the development of more pathogenic antibodies. The B3-RId does not appear to switch from IgM antibodies to IgG antibodies since it is rarely present on IgM antibodies in normal individuals or SLE patients, rather it may be associated with IgG antibodies in the context of active SLE. As mentioned earlier an idiotype derived from the purified serum of an SLE patient known as F4 was also found almost exclusively on IgG antibodies (Davidson et al,1989). This idiotype is located on the heavy chain but is associated with 3I idiotype expression found on the κ chain.

Some of the implications of the presence of the B3-RId on IgM antibodies in the serum of rheumatoid arthritis have been discussed. Other DNA associated idiotypes, such as PR4, have also been found in the serum of rheumatoid patients (Williams et al, 1988). Interestingly, IgM rheumatoid factors

have been found to bind DNA/histone complexes (Agnello et al, 1980). This provides a link between the elevation of the B3-RId and the joint disease seen in SLE and rheumatoid arthritis. This link is potentially strengthened because the monoclonal B3 binds DNA/histone complexes, a feature not common to all DNA binding antibodies (e.g. D5). The somatic mutations of B3 may not only yield anti-DNA antibodies but also specificities or particular idiotypes associated with arthritis. Moreover, the eliciting antigen may not be DNA, and amino acid substitution may occur to provide increased binding to an unidentified antigen. The DNA absorption studies indicate that the idiotype is present on antibodies that do not bind DNA as well as antibodies that do bind DNA.

4.2.2 Comparison of a monoclonal and polyclonal anti-idiotype against a human IgG anti-DNA antibody: D5-RId and D5-MId.

This section analyses the information obtained when a polyclonal and monoclonal anti-idiotype are raised against the same human monoclonal IgG anti-DNA antibody, D5. The definition of idiotypes has been questioned recently (Jefferis, 1993)(see section 1.12). The different methods of generating the anti-idiotypes used previously may be partly responsible for the properties ascribed to the idiotypes studied. However, the results presented in this thesis indicate that the information ascertained regarding the idiotypes associated with the monoclonal D5 using a polyclonal and monoclonal reagent are broadly similar. Both idiotypes are at or near the binding site for DNA and the anti-Ids inhibit each other's binding to D5. Thus the position of the idiotypes probably overlap but are not identical. This overlap is also responsible for the considerable proportion of patients that express both D5-RId and D5-MId in their serum and the fact that the levels of the two idiotypes are correlated in the SLE patients. However, there is a variation in the expression of the idiotype levels in these

patients, and on immunoglobulins deposited in the kidneys of patients with SLE, indicating that the idiotypes occur on differing antibodies i.e. not only antibodies identical to D5. In contrast, there is no significant correlation between the expression of the B3-RId and the two idiotypes related to D5 in the serum of SLE patients.

It is known that monoclonal antibodies derived from different V genes, and even different V gene families, can express the same idiotype. For example, the BEG-2 Id β is expressed by monoclonal antibodies derived from the VH-4 and VH-6 families (Watts et al, 1991). It is possible that some antibodies express D5-MId or D5-RId separately but the regulation of the two idiotypes is linked. By Western blotting the polyclonal, rabbit anti-Id bound to reduced D5 but the mouse monoclonal anti-Id did not. A number of epitopes that the polyclonal anti-Id bound to may also be destroyed by the reducing process though a considerable proportion probably remain. Thus the structural correlate of the overlap between the idiotypes may be a conformational determinant/s.

Although both anti-Ids inhibited each others' binding to D5 the reduction in inhibition of the binding of anti-D5M to D5 by anti-D5R observed with higher concentrations of anti-D5R is not explained fully. The observation that the rabbit anti-Id can bind to the mouse anti-Id is intriguing. One possibility is that a small proportion of antibodies present in the rabbit anti-Id preparation, or antibodies of low affinity, can cross-link the monoclonal D5 to the mouse anti-Id. A less likely explanation is that there is a small proportion of antibodies directed against the rabbit anti-Id in the rabbit polyclonal preparation i.e. an anti-anti-idiotype. These would then bind to the rabbit anti-Id and prevent the latter from binding to D5 at high concentrations of the rabbit polyclonal preparation.

The serological assays used to detect the two idiotypes indicate that the

spread of results is broadly similar with one important difference. The upper limit of normal (as defined by the mean plus two standard deviations of the idiotype levels in the normal population) is approximately 2.5 times lower for the D5-MId assay than for the D5-RId assay. The reasons for this are a matter of conjecture; the possibilities include an artifact of the assay system or that the rabbit anti-idiotype, being a polyclonal reagent, is more likely to identify a variety of structures on the D5 monoclonal antibody which could be shared by a greater number of different antibodies in different individuals. The polyclonal reagent could be recognising one epitope in individual A, another epitope in individual B or even both in individual C. The mouse anti-Id, which is a monoclonal reagent, probably recognises a smaller number of structures than the rabbit anti-Id. Intriguingly, serum studies for the presence of the 32/15 idiotype, identified by a polyclonal reagent, and 32/15 idiotope, identified by a monoclonal antibody, both of which were derived from a human monoclonal IgM anti-DNA antibody, did not show an elevation in SLE patients for the idiotype but did for the idiotope with little overlap between patients (Isenberg et al, 1984b).

The presence of D5-RId and D5-MId were analysed both on the IgG and the IgM population of antibodies. Although the idiotypes in sera were expressed, often at high levels, on IgG antibodies, the ELISA readings for the IgM idiotype assays were low for all sera studied except when rheumatoid factor was present which interfered with the assay and led to high readings both against the anti-Id reagents and the normal mouse and rabbit immunoglobulin. This contrasts with the observations regarding the B3-RId. None of the 6 sera containing an IgM monoclonal paraprotein reacted with the two anti-idiotypes and none of a bank of 34 IgM human monoclonal antibodies, derived from a patient with SLE, reacted with either anti-Id. The same considerations apply to these idiotypes as with the B3-RId. Thus the anti-Ids directed against D5 may identify somatically mutated idiotypes, or perhaps idiotypes which are linked to

the expression of IgG antibodies. The D5 antibody has been sequenced by Ms Celia Longhurst (section 3.4) and the heavy chain is known to arise from the VH4-21 gene. The light chain, where the D5-RId is situated, is derived from the VkIIIb family and has two replacement mutations to asparagines which might be relevant to recognition of DNA (Diamond et al, 1992). Both the light and heavy chain have a number of mutations away from the germ-line both in the CDR and the framework regions but with no clear evidence for antigen selection. Several monoclonal antibodies that utilised the V kIIIb family were negative for the presence of the D5-RId. The differences relating to these monoclonal antibodies and D5 were two the asparagines in the light chain (Dr. Caroline Chapman, personal communication). Epitope mapping is required to further identify the position of the idiotypes and whether they include replacement mutations.

About a third of SLE patients have the D5-RId and/or the D5-MId implying that both idiotypes are public. Raised levels of the idiotypes are observed in a significantly lower proportion of patients with other autoimmune rheumatic diseases and normal controls. Therefore, these idiotypes are associated with IgG antibodies in SLE patients but do not show the association with an organ/system as seen with the B3-RId. In addition, analysis of serial bleeds from four patients that had raised levels of both idiotypes associated with D5 showed no correlation with overall BILAG score or DNA binding. Four (out of 24) healthy relatives expressed raised levels of the idiotypes indicating a possible genetic predisposition to SLE, linked to immunoglobulin expression, which has been observed with other idiotypes (Isenberg et al, 1990). The increased occurrence of idiotypes in SLE relatives provides some evidence that a genetic susceptibility to SLE might be linked to the immunoglobulin locus since the direct analysis of V region genes does not indicate that particular V region polymorphisms account for autoantibody production. However, a

restriction fragment length polymorphism in the VH gene has been associated with a genetic susceptibility to SLE (Olec et al, 1991).

A large proportion of the idiotype present in some of the sera of D5-MId and D5-RId was present on the DNA binding fraction of the sera though the two idiotypes were also present on non-DNA binding antibodies as was the case with the B3-RId. Indeed, in some SLE patients the idiotype was present without significantly elevated titres of DNA binding antibodies. There are several other precedents for this. For example, the 16/6 Id first found on an IgM human monoclonal anti-ssDNA antibody is also present on anti-Klebsiella K-30 antibodies (El Roiey et al, 1987). Similarly, the 3I idiotype first found on anti-DNA antibodies has been found on anti-pneumococcal antibodies (Grayzel et al, 1991). Purification of the DNA binding antibodies present in the serum to ascertain whether an idiotype is present in that fraction does not reveal the amount of DNA antibodies that are positive for the idiotype. In the case of the idiotypes associated with D5 there was no correlation with the DNA binding in the sera of the patients studies serially and the idiotype levels. One explanation could be that although the idiotype was predominantly expressed on the DNA binding antibodies, this was a small component of the total DNA binding titre. This reinforces the concept that only by generating monoclonal antibodies can a precise link between specificity and idiotypy be made. A major weakness of studies of DNA idiotypes in sera reported to date is that they provide no evidence as to the specificity of the idiotype positive immunoglobulins.

The D5-MId was identified on renal biopsies of SLE patients indicating that it may be a pathogenically relevant antibody. The fact that none of the renal biopsies were positive for the D5-RId may be due to a combination of factors. The polyclonal reagent may not be particularly active on tissue sections; its affinity may be lower than the monoclonal reagent for exposed epitopes; or the epitopes are hidden by the renal antigens to which the antibodies are binding.

The B3-RId was also not found in the renal sections which may be for the same reasons. It should be noted that the patient from whom the monoclonal antibodies were derived did not have active renal disease at the time of the monoclonal antibodies were made but six months later a B score was recorded in the renal system. As discussed in the introduction, SLE patients may have renal disease with little if any overt clinical evidence. This fact also has a bearing on the experiments to analyse the effects of the monoclonal antibodies in vivo.

4.3 INVESTIGATION OF THE BIOLOGICAL PROPERTIES OF HUMAN MONOCLONAL IgG ANTI-DNA ANTIBODIES

4.3.1 B3 binds to cells and induces proteinuria in SCID mice

The data presented in section 3.3.1 represents the first attempt to transfer human IgG anti-DNA antibodies *in vivo*. Similar work has been performed using murine monoclonal anti-DNA antibodies which has shown that a proportion of anti-DNA antibodies induce lupus nephritis in normal mice (Vlahakos et al, 1992a). No such equivalent in vivo data exists for human monoclonal anti-DNA antibodies for a number of reasons. The most obvious is the ethical considerations which prevent *in vivo* experiments, but in addition it has only recently become possible to generate IgG human monoclonal anti-DNA antibodies, the isotype found to be most associated with active SLE. Although it is not possible to analyse the effects of these antibodies in normal individuals, the severe combined immunodeficiency (SCID) mouse offers the opportunity to study the in vivo effects of anti-DNA antibodies. This strain of mouse will not reject foreign cells and has been used for ten years to support the growth of monoclonal antibodies from different species providing a high yield of monoclonal antibody in ascites (Ware et al, 1985; McKnight et al, 1991).

Theoretically, by growing antibody secreting hybridomas in SCID mice, high levels of antibody are achieved over a relatively long period. The experiments confirmed that there was a similar concentration of antibody in the ascites and the blood demonstrating that the human immunoglobulin was able to penetrate into the blood.

In the initial experiments the two IgG anti-DNA antibodies, B3 and D5, described in the other sections of this thesis were used. B3 bound to cell surfaces in all the tissues studied, the classical glomerular staining pattern seen with lupus nephritis was not observed. In total nine SCID mice were exposed to B3 as an intravenous bolus or as hybridomas at different times and all nine produced the same pattern of staining. In contrast to the *in vivo* studies, B3 stained the cell nuclei when incubated in vitro on kidney sections not the cell surfaces. Thus the antibody could not penetrate the cells in vivo, a property that has been attributed to some murine monoclonal anti-DNA antibodies (Vlahakos et al, 1992b). The monoclonal antibody D5 did not bind to any tissue component when injected intravenously or as hybridomas. Quantitative differences are unlikely to explain the difference between B3 and D5 since although the serum concentration of D5 was at least half that found for B3, the same quantity of B3 and D5 were injected intravenously. Recently, mouse anti-DNA antibodies have been found to bind to cell surface proteins present on live cells *in vitro* derived from a number of tissues (Raz et al, 1993).

One important consideration is the possibility that this hybridoma cell line has invasive properties which would result in wide dissemination in the tissues. Human immunoglobulin positive cells would appear in the organs studied. In order to eliminate this possibility, the monoclonal antibodies were injected intravenously and a similar pattern of staining was found when B3 was injected intravenously to that observed with intraperitoneal injection. Further, if the hybridomas had a propensity to invade, the mice injected with the cells

secreting D5 would also invade the same tissues and produce a similar pattern of staining. The binding of the immunoglobulin to tissue components could also be mediated by Fc receptors, however this seems unlikely since both D5 and B3 had the same Fc region i.e. IgG1.

One unexpected finding was that the mice receiving hybridomas secreting B3 developed significant proteinuria. It is unlikely that this was due to tumour load as both D5 and B3 resulted in similar weight gain though D5 appeared to divide more rapidly. Since there was no overt nephritis it is unclear whether the proteinuria was glomerular in origin. There was no significant increase in the creatinine levels of the mice although this may not be expected as the elevation of anti-DNA antibodies would have occurred only for a few weeks. One possible explanation for the proteinuria in those mice that received the hybridomas secreting B3 is a direct toxic effect on the kidneys by free light chains. Some immunoglobulin components, particularly light chains, are known to be directly nephrotoxic. Lambda light chains have a propensity to form amyloid deposits in the kidney, whereas kappa light chains cause tubular dysfunction and non-amyloid deposition disease (Feiner, 1988). Bence Jones protein containing light chains can form amyloid deposits in the kidney when injected intravenously into normal mice (Solomon et al, 1991). Although approximately 30% of amyloid deposits do not react with anti-serum directed at intact light chains, in the experiments described by Solomon et al (1991) in which Bence Jones protein was injected, the light chains were easily detectable by antibodies directed at the light chains.

There have been no reports in the literature of inducing amyloid deposits by the injection of whole immunoglobulin and it was therefore important to confirm the pattern of staining by intravenous injection of the antibodies. However, some plasmacytoma cells are known to secrete excess light chain and it is possible that the hybridomas may have similar properties. In the

experiments reported by Vlahakos et al (1992a) these factors were not taken into consideration, and may account for the proteinuria observed. Interestingly, in this report two antibodies produced immune deposits in the kidney when injected in the form of secreting hybridomas but not when injected intravenously in to the mice. This discrepancy may be explained by the occurrence of amyloid or non-amyloid deposition disease of free light chains released by the hybridoma. Separate from these disorders there are a number of nephropathies where whole antibody is deposited in the kidney such as SLE. In one study the light chain component of the antibody found in the kidney was characterised. SLE was one of two disorders where lambda chains were the predominant light chain (Orfila et al, 1988). One case report has described the association of anti-DNA antibodies, microlamellar structures, glomerulonephritis and a monoclonal IgG lambda paraproteinaemia (Joh et al, 1990). Unfortunately this report did not document whether the lambda monoclonal had DNA binding properties or whether the anti-DNA antibodies utilised the same light chain. Lambda light chains are less frequently observed in mice than in humans but a preliminary report has suggested that differences in the kappa chains of anti-DNA antibodies derived from lpr mice may account for nephritogenic potential of the antibodies (Gilkeson et al, 1993c).

4.3.2 One human monoclonal IgG anti-DNA antibody bound exclusively to glomeruli

In an additional experiment four human IgG anti-DNA antibodies (Winkler et al, 1991) were tested for their pathogenicity in vivo. The hybridomas secreting these antibodies were injected into SCID mice in the same way as B3 and D5. Only three of these hybridomas produced ascites. Two of the antibodies bound to the tissues studied, one in a similar manner to B3, but another deposited exclusively in glomeruli (33.C9). This demonstrates that

monoclonal human IgG anti-DNA antibodies are capable of binding to glomeruli *in vivo*. There were no unique features of 33.C9 that distinguished it from the other anti-DNA antibodies. In terms of its fine specificity it did not bind to a variety of other antigens and its high affinity to dsDNA was comparable to the other anti-DNA antibodies (Winkler et al, 1991). In addition, when examining its amino acid sequence there were no changes to arginines which have been implicated in the binding to DNA (Winkler et al, 1992). Moreover, 33.C9 does not show any evidence for positive selection by antigen when the nucleotide sequence is compared to its respective germ-line.

In contrast to the murine monoclonal anti-DNA antibodies that induced nephritis in normal mice (Vlahakos et al, 1992), none of the human antibodies used in this study did so. There are a number of possible explanations for this failure to induce glomerulonephritis. Firstly, in the human disease there may be a range of anti-DNA antibodies deposited in the kidney and only a combination of antibody specificities produces the patterns of immunofluorescent present in lupus kidneys. Secondly, the antibodies may need to be present for a longer period than that achieved in these experiments for pathological changes to be observed. However, the data from Vlahakos and coworkers (1992) in which pathological lesions were induced by individual mouse monoclonal anti-DNA antibodies indicates that these two explanations may not be correct. A third possible explanation is that human monoclonal antibodies may not be able to interact with other components of the immune system, in particular complement. This may explain the lack of immune complexes seen in this study as observed with electron microscopy. It is known that human antibodies do not interact with mouse complement (Winkelhake et al, 1992). This may be more relevant than the fact that functional B and T cells are not present since, in a recent report, murine monoclonal anti-DNA antibodies that induced nephritis in normal mice also produced an identical picture in SCID mice (Ito et al, 1993). Moreover, in

the experiments by Raz and coworkers (1989) using the isolated perfused rat kidney, only when complement was added to the human polyclonal anti-DNA antibodies was there any significant change in renal function or proteinuria.

4.3.3 Immunisation of normal mice with anti-DNA/DNA complexes.

As discussed in the results section these experiments were performed to identify whether B3 encoded by the VH-26 germ-line gene (and potentially 16/6 Id positive) could induce a lupus like syndrome in normal mice as has been reported by some but not other groups. No agreed explanation has arisen to explain the conflicting data (see section 1.14). The presence of DNA in the preparations that have been injected might lead to discrepant results. Thus instead of analysing the immunological consequences of injecting an antibody with a "pathogenic" idiotype, the experiment becomes the analysis of immunising normal mice with immune complexes. As the data in this thesis has shown one out of the two anti-DNA antibodies was complexed with DNA in the supernatant. It is therefore important to examine whether DNA is co-purified with the anti-DNA antibody which is used to immunise mice. The previous reports studying the induction of SLE with a 16/6 positive antibody have failed to clarify whether their preparation of the antibody was DNA free.

The mice that were immunised with B3 and DNA derived from the supernatant consistently showed the presence of anti-DNA antibodies in their serum. The levels of anti-DNA antibodies were lower than that for an lpr mouse and the mice remained healthy throughout the study period. In contrast no mouse that was injected with B3 or DNA alone developed anti-DNA antibodies. The immune response to antibody/antigen complexes rather than to antigen alone is likely to be greater. The majority of reports have found that mammalian dsDNA is not immunogenic in normal animals (section 1.9) but if the DNA is coupled to an immunogenic DNA binding protein anti-dsDNA antibodies are

produced (Desai et al, 1993): B3 could be considered to be acting as a DNA binding protein. It remains to be determined whether other anti-DNA antibodies coupled to DNA would have the same effects.

Two immunisation routes were chosen for the injection to identify whether a particular route is more "pathogenic" than another. The mice that were injected via the intraperitoneal route developed higher titres of anti-DNA antibodies in all four groups. Even when PBS and IFA were immunised some anti-DNA antibodies were detectable, though at low levels. This may be because of the presence of accessory cells in the peritoneum , as well as nucleosomes (Dr. S Muller, personal communication).

None of the sera tested contained the presence of the B3-RId as determined by ELISA. Unfortunately we were not able to test for the presence of the 16/6 idiotype as the relevant reagent was not available. The reasons why a lupus like disease was not induced by these immunisations is not clear assuming that the experiments of Mendlovic et al (1988) are valid. There are a number of differences in the protocols used including the use of IFA rather than CFA, and different antibodies were injected. However, other studies have failed to reproduce Mendlovic and colleagues findings using very similar protocols (Williams and Isenberg, 1994). Serum anti-DNA antibodies have not been found by these groups, but it is not known whether DNA was present in the preparations used.

4.4 CONCLUSIONS

The first part of this thesis described the production of IgG human monoclonal anti-DNA antibodies after first determining the best method to achieve this. The use of the CB-F7 cell line as a fusion partner yielded approximately equal numbers of IgG and IgM antibodies of any specificity from

SLE patients but from only one individual were IgG anti-DNA antibodies derived. Interestingly, this patient had the highest disease activity index out of all the patients. In addition, when this patient's disease was relatively inactive a fusion of the lymphocytes and CB-F7 yielded only IgM anti-DNA antibodies. The isotype and specificity of the monoclonal antibodies generated on the two occasions from this patient reflected to some extent the antibodies present in the sera.

Anti-idiotypic reagents were generated from two of the IgG monoclonal anti-DNA antibodies, B3 and D5. The idiotype associated with B3 was found on the light chain of the monoclonal and was involved in DNA binding. In a comparison of other monoclonal antibodies which utilised the same light chain gene as B3, a putative site for the idiotype was proposed involving two arginines in the CDR1 region of the light chain. The expression of B3-RId in sera was associated with arthritis in patients with SLE. A comparison of one polyclonal and one monoclonal anti-idiotype derived from D5 revealed that there was a correlation between the expression of these two idiotypes in the sera of SLE patients.

A number of human monoclonal IgG anti-DNA antibodies, including B3 and D5, were transferred into SCID mice to assess the biological effects of the antibodies *in vivo*. One antibody deposited exclusively in the glomeruli but did not lead to nephritis. In separate experiments, BALB/c mice were used to determine whether B3 could induce a lupus like disease through the idiotype network when immunised into the mice. Only when the antibody was complexed with DNA were anti-DNA antibodies induced in these normal mice. The mice remained healthy throughout these experiments.

CHAPTER FIVE APPENDICES

5.1TISSUE CULTURE

All the tissue culture work described in this dissertation was carried out in a Gelaire Class II hood which was regularly cleaned using hypochorite and exposed to formalin every six months. Before and after use, the main surface of the hood was cleaned with 70% alcohol. The cells were grown in a Heraeus incubator which was maintained at 37°C with 5% corcon dioxide in a water saturated atmosphere. The incubator was cleaned regularly and copper sulphate was placed in the water at the base of the incubator to reduce the risk of fungal infection. Cell lines were screened every six months for mycoplasma infection (courtesy of the Imperial Cancer Research Fund, UCL).

Tissue culture media

Tissue culture media was made in batches of 500 ml as required. Each batch was sterility tested. Two ml of fresh media was placed in a Universal Container in the incubator and examined for infection after 48hr.

GM Medium

RPMI 1640 (Gibco, Paisley, Scotland)	83.8%
L-glutamine 200mM (100 x) (Gibco)	1%
Hepes 1M (Gibco)	1%
Sodium pyruvate 100mM (Gibco)	1%
MEM non-essential amino acids (100 x) (Gibco)	2%
Penicillin (10000IU/ml) and Streptomycin (10000µg/ml) solution (Gibco)	1%
Gentamicin (10mg/ml) (Gibco)	0.2%
Fetal calf serum (Gibco)	10%

HAT/HT Medium

RPMI 1640	60.8%
L-glutamine 200mM (100 x)	1%
Hepes 1M	1%
Sodium pyruvate 100mM	1%
MEM non-essential amino acids (100 x)	2%
Penicillin (10000IU/ml) and Streptomycin (10000µg/ml) solution	1%
Gentamicin (10mg/ml)	.2%
Fetal calf serum (Gibco)	20%
NCTC-135 with L-glutamine (Gibco)	10%
Insulin solution	1%
HAT or HT x 50	2%

Preparation of HAT/HT solution

One vial of HAT/HT (Sigma) was diluted in 10 ml RPMI to give the following concentrations: HAT, Hypoxanthine 5 x 10^{-3} M; Aminopterin 2 x 10^{-5}M, Thymidine 8 x 10^{-4}M; HT, Hypoxanthine 5 x 10^{-3}M; Thymidine 8 x 10^{-4}M.

Preparation of insulin solution

To prepare a solution containing insulin 0.2IU/ml and oxaloacetic acid 0.1 M. 100mg bovine insulin (Sigma) was diluted in 65.5 ml double distilled water at 37°C, to which was added 1.729g of oxaloacetic acid and 65.5 ml sodium pyruvate (Gibco). The solution was stirred until clear and then filtered using a 0.2µm pore size filter (Gelman acrodisc), aliquoted into 5 ml aliquots and stored at -20°C until use.

Post fusion medium is identical to the HAT medium but without the HAT.

Serum Free Medium

SF-1 (Northumbria Biologicals)	97.8%
L-glutamine	1%
Penicillin and Streptomicin	1%
Gentamicin	.2%

Storage of cell lines

The cells were washed using RPMI medium, counted and spun down. The cells were then resuspended in 50% fetal calf medium, 30% RPMI, and 20% sterile dimethylsulfoxide (Analar, BDH, Poole, UK) at a density of 1-2 x 10^7/ml. The cell suspension was aliquoted into previously labelled freezing tubes (Nunc cryotubes 1.8 ml) and placed in a Nalgene™ Cryo 1°C Freezing Container in a -80°C freezer for a minimum of four hours in order to achieve a -1°C/min rate of cooling using Propan-2-ol (BDH). The tubes were then placed in liquid nitrogen tank for long term storage.

5.2 PREPARATION AND USE OF SEPHAROSE 4B AFFINITY COLUMNS

The protein to be used, such as immunoglobulin, was coupled to cyanogen bromide activated Sepharose 4B following the method recommended by the manufacturers. Cyanogen bromide activated sepharose 4B was obtained as a freeze dried powder (Pharmacia, UK) and stored at 4°C until use. Freeze dried powder was swollen for 15 min in 1mM HCL (200 ml/g powder) and washed using a sintered glass filter (porosity G3). HCL was added in 5-6 aliquots, the supernatant being sucked off between successive additions. One gram of freezed dried powder swells to give a gel volume of aproximately 3.5 ml.

The gel was then washed with coupling buffer [NaHCO3 buffer (0.1M), pH 8.3 containing NaCl (0.5M)] and mixed with a solution of protein dissolved in coupling buffer (5-10 mg protein/ml gel). The protein-gel mixture was continuosly mixed using a roller mixer at room temperature for 2 hours. The gel

was then transferred to 0.2M glycine in coupling buffer to block any active groups. The mixture was then mixed continously overnight at 4°C.

To remove uncoupled protein, the gel was washed alternately at high pH (coupling buffer) and at low pH (acetate buffer, 0.1M pH 4) for 6 cycles. The gel was then stored at 4-8°C in PBS containing 0.1% azide until use.

The gel was placed in a pharmacia column and washed in PBS. The plunger was inserted ensuring that that no air bubbles were trapped and the column stored at 4°C until use. For use the column was connected to a peristaltic pump (Micoperpex 2132 peristaltic pump, LKB instruments Ltd, Croyden, UK).

5.3 BUFFERS

5.3.1 ELISA

Phosphate buffered saline (PBS)

To prepare 10 litres of 10xPBS:

KCL	20g
KH_2PO_4	20g
Na_2HPO_4	114.8g
NaCl	800g

Adjust to pH 7.4 with 5MNaOH. Dilute tenfold with distilled water and recheck pH before use.

PBS-T

Prepare PBS as above and add 0.05% Tween 20 (Sigma)

Bicarbonate buffer

Sodium carbonate 0.8g

Sodium bicarbonate 1.55g

Make up to 1 Litre. Adjust pH if necessary to 9.5.

5.3.2 Agarose gel electrophoresis

TBE electrophoresis buffer
10x stock solution
108 g Tris base
55g boric acid
40 ml 0.5M EDTA, pH 8.0 (2 mM)

10x loading buffer
20% Ficoll 400
0.1M Na_2EDTA, pH 8
1% SDS
0.25% bromophenol blue
0.25% xylene cyanol.

5.3.3 Western blotting

Running buffer

14.4 g glycine
3 g Tris
10% SDS
in 1 litre of water, pH 8.3

Sample buffer x3:
10% SDS
60% sucrose
100mM EDTA
1 M Tris HCL
Few grains of bromophenol blue
For use add 31 mgs of DTT per ml of sample buffer.

Cathode buffer
25mM Tris
40mM 6 amino-n-hexanoic acid
20% Methanol
80% Distilled water

Anode buffer 1
0.3M Tris
20% Methanol
80% Distilled water

Anode buffer 2
25mM Tris
20% Methanol
80% Distilled water

Ponceau S
3% Trichloroacetic acid (TCA)
0.2% Ponceau
97% Distilled water.

Colour development solution
100mM TrisHcl
100mM NaCl
5mM MgCl2
pH to 9.5

Colour stop solution
20mM Tris HCl
1 mM EDTA
pH 2.9

5.3.4 Affinity chromatography

Coupling buffer

Prepare 0.1M Boric acid (6.18g/l); solution A

0.1 M Sodium tetraborate 10 H20 38.14g/l; solution B

Add solution B to solution A until pH 8.6. Add NaCl to achieve a 0.5M solution.

Acetate buffer

Prepare 0.1M acetic acid (5.8 ml in 1 litre distilled water) containing NaCl 0.5M (29.2 g/l); solution A

0.1M sodium acetate (8.2g/l) containing NaCl 0.5M; solution B. Add solution B to solution A until pH 4.0.

Glycine HCL

Glycine 0.1M 3.75g/500ml distilled water containing 0.15M NaCl 4.4g/500 ml distilled water. Adjust pH to 2.3 with concentrated HCL.

5.3.5 Immunostaining

Tris buffered saline

Stock solution. 60.6 g Tris HCL plus 13.9 g Tris base made to 1 litre with H20. pH to 7.6 with HCL. Prepare stock solution NaCl 8.76 g/l (0.7M)

For use add 9 parts NaCl solution to 1 part Tris stock.

5.4 PREPARATION OF DNA FOR THE DNA ELISA

A stock solution of calf thymus DNA (Sigma) of 1 mg/ml was dissolved overnight in distilled water and sonicated for 4x15 seconds bursts on ice. Single stranded DNA was prepared by diluting this solution to 500µg/ml and boiling for 10 min before cooing on ice for 15 minutes. Double stranded DNA was prepared by adding an equal volume of DNA stock solution to S1 nuclease solution (1U/ml) immediately prior to use for 1 hour at 37°C.

5.5 BILAG SCORING SYSTEM

The following is a copy of the guidance notes for completing a BILAG assessment.

Assumption

It is implicit in the scoring system that all features scored are thought to be due to active lupus.

The questionnaire asks whether features are improving, the same, worse or new during the last month, or since the last assessment if this was performed less than one month ago. If a new feature has developed in the last month (or since the last assessment if less than a month ago) it should be scored as new (ie 4), even if it has subsequently improved or resolved. For the first assessment any response will register the feature as a criterion. For subsequent assessments, features will only contribute to the score if they are the same, worse or new. These different grades have been used so that BILAG can identify all patients who have developed a particular feature for the first time and also to document the response of particular features to treatment.

In the renal and haematological assessments (which include laboratory tests) the assessor will be asked for confirmation that abnormal results are due to active lupus (rather than drug side effects for example).

When going through the individual screens for the first time you may find that you have no data to enter for a particular screen. If this happens you will be asked to assign the patient to either a category E or D for that particular system. You should enter D if the patient has ever had any involvement of that system and E if there has never been previous involvement. Once a patient has scored an A, B, C or D in a particular system she will always score at least a D in the future. The score E implies no involvement of the system ever.

1. GENERAL NON-SPECIFIC MANIFESTATIONS (Gen)

1. Pyrexia
2. Weight loss
3. Lymphadenopathy

4. Fatigue/malaise/weakness
5. Anorexia/nausea/vomiting

Category A
Pyrexia
plus two other

Category B
Pyrexia
or two others

Category C
Any other one criterion

Category D
Previous involvement

Category E
No involvement

Mucocutaneous Disease (Muc)

Category A- any one of
1. Severe maculopapular, discoid or bullous eruption; ie active facial and/or extensive (>2/9), scarring or causing disability.
2. Angio-oedema
3. Extensive mucosal eruption

Category B - any one of
1. Malar erythema
2. Mild maculopapular eruption
3. Panniculitis
4. Localised active discoid lesions incl. lupus profundus
5. Severe active alopecia
6. Subcutaneous nodules
7. Perniotic skin lesions

Category C- any one of
1. Periungal erythema
2. Swollen fingers
3. Sclerodactyly
4. Calcinosis
5. Telangiectasia
6. Mild alopecia
7. Small mucosal ulceration

Category D
Previous involvement

Category E
No involvement

CNS disease attributable to lupus (CNS)
First assessment

Category A
Acute, progressive or recurring:
Any one of
1. Impaired level of consciousness
2. Psychosis or delirium or confusional state
3. Grand Mal Seizure
4. Stroke or stroke syndrome
5. Aseptic meningitis
6. Mononeuritis multiplex
7. Ascending or transverse myelitis
8. Peripheral or cranial neuropathy
9. Chorea
10. Cerebellar ataxia

Category B
Any one of
1. Headache (severe unremitting)
2. Organic depressive illness
3. Chronic brain syndrome including pseudotumor cerebri
4. Disc swelling or cytoid bodies

Category C
Episodic migrainous headaches

Category D
Previous CNS disease

Category E
No previous CNS disease

CNS disease
Subsequent assessments

Category A

Acute, progressive or recurring (scored "worse" or "new").

Any one of

1. Impaired level of consciousness
2. Psychosis or delirium or confusional state
3. Grand Mal Seizure
4. Stroke or stroke syndrome
5. Aseptic meningitis
6. Mononeuritis multiplex
7. Ascending or transverse myelitis
8. Peripheral or cranial neuropathy
9. Chorea
10. Cerebellar ataxia

Category B

Any one of the following "new" or "worse" in the last month

1. Headache (severe unremitting)
2. Organic depressive illness
3. Chronic brain syndrome including pseudotumor cerebri
4. Disc swelling or cytoid bodies

or

Any one of the following "same" or "improving" in the last month on

5. Impaired level of consciousness
6. Psychosis, delirium or confusional state
7. Grand Mal seizure

Category C

1. Episodic migrainous headaches

or

"A" 4-10 or "B" 1-4 "same" or "improving" over the last month.

Category D
Previous CNS disease

Category E
No previous CNS disease

4. MUSCULOSKELETAL DISEASE (M/S)

Category A
One or more of
1. Definite myositis (Bohan and Peter)
2. Severe polyarthritis with loss of function (not responsive to steroids < 10mg/day, antimalarials, NSAIDS)

Category B
One or more of
1. Arthritis
2. Tendinitis

Category C
1. Arthralgia
2. Myalgia
3. Tendon contractures and fixed deformity
4. Aseptic necrosis
5. Mild chronic myositis

Category D
Previous involvement

Category E
No previous involvement

5. CARDIOVASCULAR/ RESPIRATORY DISEASE (CVS/Resp)

Category A
Cardiac failure or symptomatic effusion plus two other criteria
or four from:
1.Pleuropericardial pain
2. Dyspnoea
3. Friction rub
4. Progressive CXR changes - lung fields
5. Progressive CXR changes - heart size

Category B
Any two criteria listed under "A"

ECG evidence of pericarditis or myocarditis
7. Cardiac arrhythmias including tachycardia
> 100 in absence of fever
8. Deteriorating lung function: > 20% of
expected or > 20% fall
9. Cytohistological evidence of
inflammatory lung disease

Category C
Mild intermittent chest pain
or one other criterion

Category D
Previous involvement

Category E
No previous involvement

VASCULITIS
Any one of the following

Category A
1. Major cutaneous vasculitis (including ulcers) accompanied by infarction occurring in the previous month
2. Major abdominal crises due to vasculitis
3. Recurrent thromboembolism (excluding strokes)

Category B
1. Minor cutaneous vasculitis (nail fold vasculitis, digital vasculitis, purpura, urticaria)
2. Superficial phlebitis
3. Thromboembolism (excluding strokes)- first episode

Category C
1. Raynaud's phenomenon
2. Livedo reticularis

Category D

Previous involvement

Category E

No involvement

7. RENAL DISEASE

First assessment

Category A

Two or more of the following provided that 1, 4 or 5 are included

1. Proteinuria
2. Accelerated hypertension
3. Creatinine clearance < 50 ml/min
4. Active urinary sediment (on an uncentrifuged specimen): pyuria (>5wc/hpf); haematuria (>5rbc/hpf) or red cell casts in the absence of infection
5. Histological evidence of active nephritis within the last three months (or since the previous assessment if seen less than 3 months ago)

Category B

One of the following

1. One of the category A criteria
2. Urinary dipstick 2+ or more
3. 24hr urinary protein >0.5g but < 1g

Category C

One of the following

1. Urinary dipstick +
2. Blood pressure >140/90 (5th phase)
3. Creatinine > 130 mmol/l

Category D

Previous renal involvement

Category E

No previous renal involvement

RENAL DISEASE

Subsequent assessments

Category A

Two or more of the following provided that 1, 4 or 5 are included

1. Proteinuria (defined as)

(a) urinary dipstick increased by 2 or more levels or

(b) 24 hour urinary protein rising from >0.20 g to > 1 g or

(c) 24 hour urinary protein rising from >1g by 100% or more or

(d) newly documented proteinuria of > 1 g

2. Accelerated hypertension

3. Deteriorating renal function (defined as)

(a) plasma creatinine >130 μM/L or 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 (as defined above)

5. Histological evidence of active nephritis (as defined above)

Category B

One of the following

1. One of the category "A" criteria

2. Moderate proteinuria (defined as)

(a) urinary dipstick of 2+ or more or

(b) 24 hr urinary protein rising from 1g by > 50% but <100%

3. Moderate decline in renal function (defined as)

(a) plasma creatinine > 130μM/L or having risen to 115% of previous value

Category C

One of the following

1. 24hr urinary protein >0.25g

2. Urinary dipstick 1+ or more

3. Rising blood pressure (defined as)

(a) systolic rise of > = 30mm Hg

(b) diastolic rise of > = 15 mmHg

(provided the recorded values are 140/90)

Category D
Previous renal disease

Category E
No previous renal disease

8. HAEMATOLOGICAL DISEASE (Hae)

Category A
One of the following
1. wcc < 1000
2. platelet count < 25
3. Haemoglobin < 8

and

Category B
One of the following
1. wcc < 2500
2. platelet count < 100
3. haemoglobin < 11
4. Evidence of active haemolysis (raised bilirubin +/- retic count

positive Coomb's test

Category C
One of the following
1. wcc < 4000
2. lymphocyte count < 1500
3. platelet count < 150
4. Coomb's test positive but no evidence of active haemolysis
5. Evidence of circulating lupus anticoagulant detected by functional assays.

Category D
Previous involvement

Category E
No previous involvement

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