An Investigation of the Molecular Basis of Interactions Between Human Monoclonal Antibodies and Antigens that are Clinically Relevant in Systemic Lupus Erythematosus and the Antiphospholipid Syndrome

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ABSTRACT

Autontibodies to a wide variety of antigens are associated with systemic lupus erythematosus (SLE) and the Antiphospholipid Syndrome (APS). Previous studies have demonstrated the importance of somatic mutations and arginine residues in the complementarity determining regions (CDRs) of pathogenic anti-dsDNA and antiphospholipid antibodies. This thesis describes the study of two human monoclonal IgG antibodies, B3 (anti-DNA) and IS4 (antiphospholipid) that were derived from a patient with active SLE and primary APS respectively. I have demonstrated *in-vitro* expression and mutagenesis of B3 and IS4 and used this expression system to investigate the importance of the arginine residues in $B3V_H$ and $IS4V_H$. The mutant heavy chains, as well as the wild-type V_H were expressed with different light chains and the resulting antibodies assessed for binding to nucleosomes, alpha-actinin, cardiolipin (CL), phosphatidylserine (PS), beta-2-glycoprotein I (β_2GPI), and the N-terminal domain of β_2GPI (Domain I) using direct binding assays.

The results obtained have shown that the presence of arginine at position 53 in $B3V_H$ was essential but not sufficient for binding to dsDNA and nucleosomes. Conversely, the presence of this arginine reduced binding to alpha-actinin, β_2GPI and Domain I of β_2GPI. The fact that the arginine to serine substitution at position 53 in $B3V_H$ significantly alters binding of B3 to different clinically relevant antigens, but in opposite directions implies that this arginine residue plays a critical role in the affinity maturation of the antibody B3. Furthermore, of four arginine residues in $IS4V_H$ CDR3 substituted to serine, two at positions 100 and 100g reduced binding to all antigens, while two at positions 96 and 97 reduced binding to β_2GPI but increased or decreased binding to CL and PS. Only one H/L chain combination bound neutral phospholipid and none bound dsDNA; hence, these effects are particularly relevant to antigens important in APS. Therefore, my findings suggest that these four arginine residues have developed as a result of somatic mutations driven by an antigen containing both phospholipid and β_2GPI. These results extend our knowledge of the structure-function relationship of human anti-DNA and anti-phospholipid antibodies and aid in our understanding of how these antibodies lead to pathogenicity and what we need to target in the future for possible therapies.

This thesis is dedicated to my family for their love,
constant support and encouragement

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DECLARATION

The work described in this thesis was carried out by the author unless otherwise stated.

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ABBREVIATIONS

ANA	Anti-nuclear antibodies
aCL	Anticardiolipin antibodies
Anti-dsDNA	Antibodies to double stranded DNA
Anti-ssDNA	Antibodies to single stranded DNA
APC	Antigen presenting cell
aPL	Antiphosholipid antibodies
APS	Antiphospholipid syndrome
BCR	B cell receptor
β_2GPI	β_2-Glycoprotein I
BIC	Bicarbonate buffer
bp	Base pairs (of DNA)
BSA	Bovine serum albumin
BWF1	1st generation (F1) of New Zealand Black x New Zealand White mice
CDR	Complementarity-determining region
C_H	Constant domain of heavy chain
CHO cell	Chinese hamster ovary cell
CL	Cardiolipin
C_L	Constant domain of light chain
CR	Complement receptor
DC	Dendritic cell
DI	Domain I
dhfr	dihydrofolate reductase gene
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EBV	Epstein-Barr virus
EC	Endothelial cell
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen binding fragment of antibody
Fc	Crystallizable fragment of antibody
FR	Framework region
GBM	Glomerular basement membrane
H1	Histone 1 (also H2A, H2B, H3 and H4)
H	Heavy chain

HCLV	Heavy chain loss variant
HCMV	Human cytomegalovirus
HLA	Human leucocyte antigen
HS	Heparan sulphate
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropylthiogalactoside
J	Joining region gene
Kb	Kilobase
kDa	Kilodalton
L	Light chain
LA	Lupus anticoagulant
LB	Luria-Bertani
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MRL/*lpr*	MRL/Mp-*lpr/lpr* (lymphoproliferative gene) mice
MW	Molecular weight
(NZB/W)F_1	First generation (F1) of New Zealand Black and New Zealand White
NF-κB	Nuclear factor κB
NK	Natural killer cell
nRNP	nuclear ribonucleoprotein
OD	Optical density
PAPS	Primary Antiphospholipid Syndrome
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
pG1D1	Heavy chain expression vector (AERES Biomedical, London, UK)
pG1D210	Heavy chain expression vector (AERES Biomedical, London, UK)
PL	Phospholipid
pLN10	Light chain expression vector (AERES Biomedical, London, UK)

PS	Phosphatidylserine
R	Arginine *(see the complete amino acid-single letter code)*
RNA	Ribonucleic acid
SAP	Serum amyloid P
scFv	Single chain Fv fragment of Antibody
SCID	Severe Combined Immunodeficiency
SD	Standard deviation
SEC	Sample/enzyme/conjugate dilution buffer
SLE	Systemic Lupus Erythematosus
Sm	smith antigen (spliceosome)
SNF_1	First generation (F1) of Swiss-Webster and New Zealand Black mice
SnRNP	small nuclear ribonucleoprotein
SPR	Surface plasmon resonance
TAE	Tris Acetate EDTA buffer
TBE	Tris Borate EDTA buffer
TBS	Tris buffered saline
T_C	T cytotoxic cells
TCR	T cell receptor
TE	Tris EDTA buffer
TF	Tissue factor
TGFβ	Transforming growth factor-beta
Th	T helper cell
TNF	Tumour necrosis factor
UV	Ultraviolet
V	Variable
VCAM-1	Vascular cell adhesion molecule-1
V_H	Variable domain of heavy chain
V_κ	Variable domain of kappa light chain
V_λ	Variable domain of lambda light chain
V_L	Variable domain of light chain

AMINO ACIDS

One letter codes and three letter codes

Amino acid	Letter code	Three letter code
Alanine	A	GCT, GCC, GCA, GCG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Asparagine	N	AAT, AAC
Aspartic acid	D	GAT, GAC
Cysteine	C	TGT, TGC
Glutamic acid	E	GAA, GAG
Glutamine	Q	CAA, CAG
Glycine	G	GGT, GGC, GGA, GGG
Histidine	H	CAT, CAC
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Lysine	K	AAA, AAG
Methionine	M	ATG
Phenylalanine	F	TTT, TTC
Proline	P	CCT, CCC, CCA, CCG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Threonine	T	ACT, ACC, ACA, ACG
Tryptophan	W	TGG
Tyrosine	Y	TAT, TAC
Valine	V	GTT, GTC, GTA, GTG

CHAPTER ONE

Introduction

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INTRODUCTION

1.1 The immune system

1.1.1 Components of immunity

Immunity is the body's capability to repel foreign substances and cells. The nonspecific component, innate immunity, is the first line of defense and consists of a set of disease-resistance mechanisms that are not specific to a particular pathogen. Nonspecific responses block the entry and spread of disease-causing agents. In contrast, the highly specific component, adaptive immunity, is the second line of defense and is adapted to an individual threat therefore displaying a high degree of specificity as well as the remarkable properties of "memory". Antibody-mediated and cell-mediated responses are the two types of specific response (Roitt and Delves, 2002). Unfortunately, when the target of the immune response becomes self-antigen, as opposed to foreign antigen, these components of the immune system may be unable to remove the antigen completely, resulting in a sustained immune response, chronic inflammation and self-tissue damage as seen in autoimmunity. Autoimmune diseases are classified as organ-specific - affecting a single organ, or systemic - affecting multiple organ systems.

1.1.2 Cells of the immune system

The immune system is composed of many interdependent cell types with specialised functions that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumor cells. An effective immune response involves two major groups of cells: lymphocytes, which arise in the bone marrow (B cells and T cells) and antigen-presenting cells.

1.1.2.1 B cells

The human body makes millions of different types of B cells each day, and each type has a unique receptor protein [referred to as the B cell receptor or immunoglobulin

(Ig) or antibody] on its membrane that will bind to one particular antigen. A mature B cell leaves the bone marrow expressing membrane-bound Ig of the IgM and IgD class with a single antigenic specificity. These naïve B cells, which have not encountered antigen are circulating in the blood and lymph and are carried to the secondary lymphoid organs, most notably the spleen and lymph nodes. Once the B cell encounters its cognate antigen, the cell proliferates by clonal expansion and it can further differentiate to generate a population of antibody-secreting plasma cells and memory cells. The B cell can either directly become one of these cell types or go through an intermediate differentiation step, the germinal center reaction, in which the B cell will hypermutate the variable (V) region of the antibody and possibly class switch. Only 10% of the potential B cells reach maturity and exit the bone marrow. In the absence of antigen-induced activation, naïve B cells in the periphery die within a few days (Goldsby et al., 2000, Janeway et al., 2001).

Plasma cells secrete antibodies, which effect the destruction of antigens by binding to them and making them easier targets for phagocytes. Most live only for a few days, do not express membrane bound antibody but instead produce large amounts of the antibody in secreted form. Memory B cells are formed specific to the antigen encountered during the primary immune response and are able to live much longer (Dorner and Radbruch, 2005). These cells can respond quickly upon second exposure to the antigen for which they are specific, therefore expressing membrane bound antibody with the same specificity as their parent cell (Goldsby et al., 2000, Janeway et al., 2001).

1.1.2.2 T cells

T lymphocytes are usually divided into two major subsets that are functionally different. Both types of T cells can be found throughout the body. They often depend on the secondary lymphoid organs (the lymph nodes and spleen) as sites where activation occurs, but they are also found in other tissues of the body, most evidently the liver, lung, blood, and intestinal and reproductive tracts.

The two types of T cells are distinguished by the presence on their surface of one or the other of two glycoproteins designated CD4 and CD8. The T helper subset, also called the $CD4^+$ T cell, co-ordinates immune regulation. The main function of the T helper cell is to augment or potentiate immune responses by the secretion of specialised factors that activate other white blood cells to fight off infection. $CD4^+$ T

cells bind an epitope consisting of an antigen fragment lying in the groove of a class II histocompatibility molecule. $CD4^+$ T cells are essential for both the cell-mediated and antibody-mediated branches of the immune system. The T helper (Th) cells may be subdivided according to their cytokine secretion profile. Th1 cells secrete interleukin IL-2, IL-3 and gamma interferon IFN-γ that play a central part in cell mediated immunity and acute allograft rejection. In the cell-mediated immunity, the Th1 cells bind to antigen presented by antigen-presenting cells (APCs) like phagocytic macrophages and dendritic cells. The T cells then release cytokines that attract other cells. The Th2 cells secrete interleukin (IL)-4, IL-5, IL-6, IL-9 and IL-10, which enhance antibody production. The Th2 cells participate in the antibody-mediated immunity where they bind to antigen presented by B cells. The result is the development of clones of plasma cells secreting antibodies against the antigenic material (Roitt and Delves, 2002).

The other important type of T cell is called the T cytotoxic (Tc) subset or $CD8^+$ T cell. These cells are important in directly killing certain tumor cells, viral-infected cells and sometimes parasites. The Tc cells are also important in down-regulation of immune responses. Under the influence of Th-derived cytokines, a Tc cell that recognises an antigen-Major Histocompatibility Complex (MHC) Class I molecule proliferates and differentiates into a cytotoxic T lymphocyte (CTL) which does not secrete many cytokines and instead exhibits cytotoxic activity. The CTL has a crucial function in monitoring the cells of the body and eliminating any virus-infected cells, tumour cells, or cells of a foreign tissue graft (Goldsby et al., 2000, Janeway et al., 2001).

1.1.2.3 Antigen Presenting Cells

Macrophages are important in the regulation of immune responses. They are referred to as antigen-presenting cells (APC) because they identify, pick up and ingest foreign materials and present these antigens to other cells of the immune system notably T cells and B cells. This action is one of the important first steps in the initiation of an immune response. Stimulated macrophages exhibit increased levels of phagocytosis and are also secretory. These cells are part of the innate response. Unlike T and B cells, they do not contain any antigen specific receptors. Macrophages continuously phagocytose self-proteins and cells in their vicinity, during normal tissue repair and aging (e.g. old red blood cells). All of these proteins are degraded and presented with MHC-Class II molecules. These self-proteins however, do not fully activate T cells, because in the absence of infection, macrophages

express low levels of MHC-II, and almost no co-stimulator (B7). B7 molecules are members of the Ig superfamily; they have a single V-like domain and a single constant (C)-like domain. They are constitutively expressed on dendritic cells and induced on activated macrophages and activated B cells. In the case of infection, however, macrophages posses certain types of receptors that recognise differential carbohydrate patterns on foreign cells. They also have receptors for specific bacterial products such as LPS (endotoxin). These receptors, called Toll-like receptors (TLRs), are transmembrane proteins that serve as a key part of the innate immune system and are also able to stimulate activation of the adaptive immune system, linking innate and acquired immune responses. TLRs are considered pattern recognition receptors (PRRs), binding to pathogen-associated molecular patterns (PAMPs) small molecular sequences consistently found on pathogens. Their function is the recognition of pathogens and the activation of immune cell responses directed against those pathogens. Macrophages are therefore stimulated to up-regulate MHC-II and B7, providing these cells with strong antigen presentation properties. They also start to secrete cytokines that aid in their functions (IL-1, 6, 8, 12 and TNFα). It is at this point that antigen presentation by MHC Class II will activate Th cells (Goldsby et al., 2000, Janeway et al., 2001).

Dendritic cells, which also originate in the bone marrow, function as antigen presenting cells. These cells can be classified by their location either in skin and mucous membranes, in the lymphoid organs such as the thymus, lymph nodes and spleen, or in the bloodstream and other organs of the body. They capture antigen or bring it to the lymphoid organs where an immune response is initiated. These cells continuously express high levels of both MHC class II molecules and co-stimulatory B7. For this reason, they are more potent than macrophages and B cells, both of which need to be activated before they can function as APCs. After recognition of foreign particles, dendritic cells migrate into the blood or lymph and circulate to various lymphoid organs, where they present the antigen to T lymphocytes (Peakman and Vergani, 1997).

1.1.3 The role of antibodies in the immune system

The first insights into the mechanism of immunity and the first description of antibodies were provided in 1890 by the experimental work of Emil von Behring and Shibasaburo Kitasato. Antibodies are specialised proteins produced by plasma cells

that circulate in the blood recognising and binding to foreign proteins, microorganisms or toxins in order to neutralise them. They are also known as Igs.

1.1.3.1 Antibody structure and function

The basic antibody structural unit made of amino acids contains four polypeptide chains (Figure 1.1). There are two heavy (H) chains (MW 50,000-70,000) and two light (L) chains (MW 25,000). Disulphide bonds connect each H chain to its partner L chain, and connect the two H chains. When individual Ig molecules of the same type are compared, some portions of the molecule are identical while others are extremely variable. The V parts are the N-terminal domains of both H and L chains and are called V regions (V_H and V_L). The remainder of the H and L chains are identical in all molecules of the same class and are called C regions (C_H1-C_H3 and C_L) (Roitt and Delves, 2002).

V regions form the antigen-binding sites. Antibodies as a group can bind many different antigens because there are many different kinds of V domains. The antigen-binding site is formed from the V domains of one H chain and one L chain. Within an Ig molecule, two H chains are always identical, as are the two L chains, so each Ig unit has two identical binding sites. Antigen and antibody are both sterically and chemically complementary with the interacting surfaces forming multiple bonds. Antigens are often macromolecules and an antibody therefore binds to only a small portion of an antigen's surface, an epitope or antigenic determinant. A large, complex, antigen such as a protein has many epitopes. When the immune system reacts to an antigen, although each individual Ig molecule is epitope-specific, the population of antibodies produced will bind to many different epitopes. Since each distinct type of antibody is produced by a different clone of plasma cells, this response is termed a polyclonal response. Not all epitopes provide equally strong stimuli, and antibodies to a few immunodominant epitopes may constitute most of the antibodies formed. Dominant epitopes are typically present in many copies (Roitt et al., 1996).

Most of the amino acid differences among antibodies fall within areas called complementarity-determining regions (CDRs), and it is these CDRs, on both L and H chains, that constitute the antigen-binding site of the Ig molecule. Each V domain has 3 CDRs (Chothia et al., 1989). The remainder of the V regions is largely composed of β-sheets and comprises the framework regions (FRs), which are more

conserved and perform a structural role, to hold the CDRs in place (Wu and Kabat, 1970). When antibody V region sequences are compared, CDRs are much more different than FRs. Because of this difference the CDRs are also called hypervariable regions. CDRs of both V_H and V_L make up the antigen-binding site and they are held in place by interactions between them and between their associated C_H1 and C_L domains.

There are five different classes of antibodies, with distinct biological properties. Each class has a H chain with a different amino acid sequence. The five Ig classes are designated IgA, IgG, IgM, IgE and IgD and the five types of H chains are designated by the corresponding lowercase Greek letter, α, γ, μ, ε, and δ. There are two different kinds of L chain designated κ and λ and either type of L chain can associate with any H chain. In humans 60% of the L chains are κ and 40% are λ. IgG has four subclasses (IgG1, IgG2, IgG3, IgG4) and IgA has two subclasses (IgA1, IgA2).

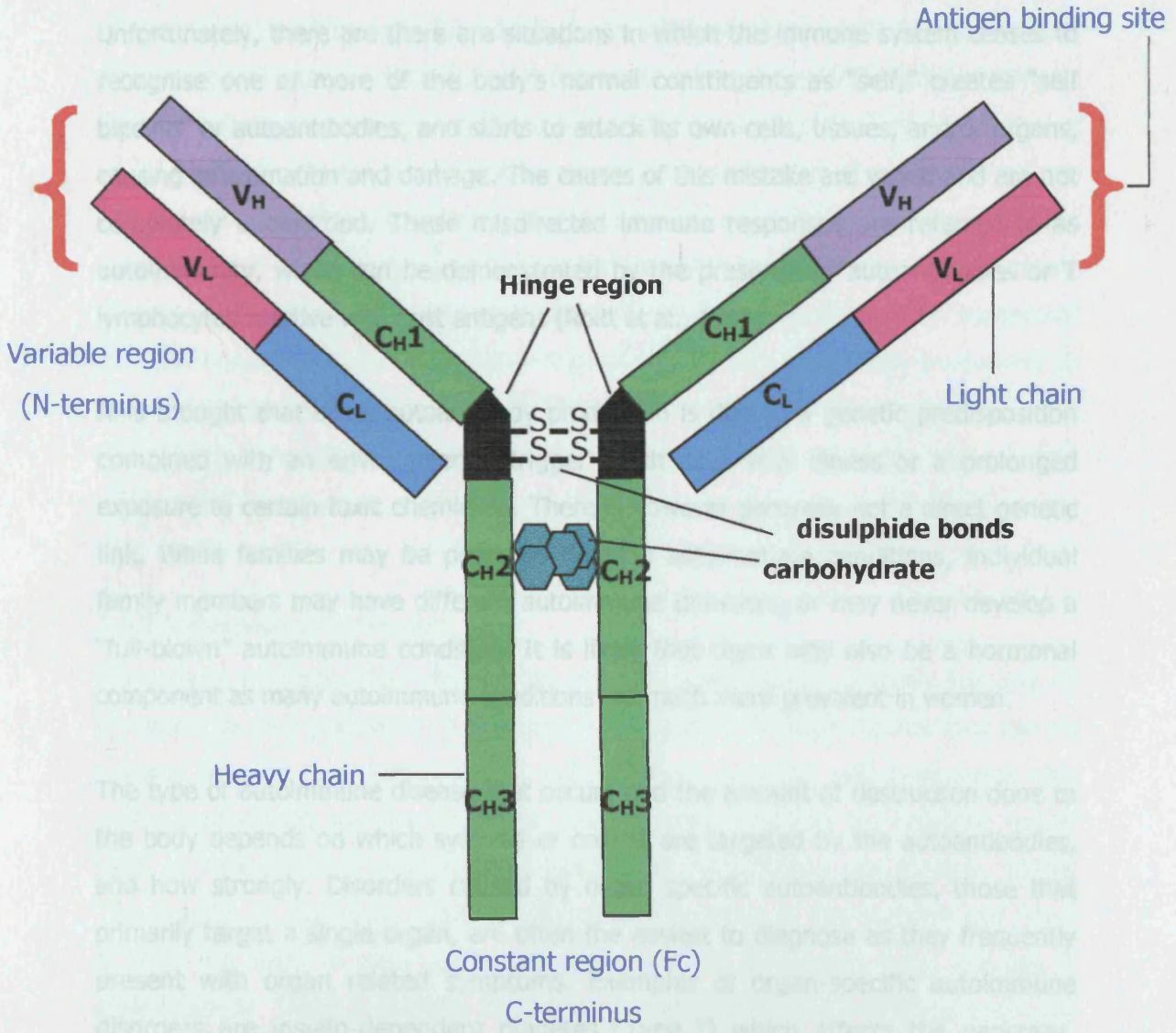


Figure 1.1 Schematic diagram of the structure of an IgG molecule

Immunoglobulin molecules consist of two identical heavy chains (50 KD each) and two identical light chains (25 KD each), which are linked by disulphide bridges. The heavy chains comprise four domains and the light chains each have two domains. Three of the heavy chain domains (C_H1, C_H2 & C_H3) are constant in all antibodies of the same isotype and one (V_H) is highly variable between individual antibody molecules. The light chain has one constant (C_L) and one variable (V_L) domain. The variable domains, and in particular the hypervariable regions (termed complementarity determining regions, CDRs), of the amino terminal domains (N-terminus) of both the heavy and light chains form the antigen binding site of the antibody. The carboxyl terminal domains (C-terminus) of the heavy chains form the Fc (crystallisable fragment) region, this region determines the isotype of the antibody molecule and controls the antibody's effector functions.

1.1.3.3 Autoimmunity and autoantibodies

Unfortunately, there are there are situations in which the immune system ceases to recognise one or more of the body's normal constituents as "self," creates "self binding" or autoantibodies, and starts to attack its own cells, tissues, and/or organs, causing inflammation and damage. The causes of this mistake are varied and are not completely understood. These misdirected immune responses are referred to as autoimmunity, which can be demonstrated by the presence of autoantibodies or T lymphocytes reactive with host antigens (Roitt et al., 1996).

It is thought that some autoantibody production is due to a genetic predisposition combined with an environmental trigger (such as a viral illness or a prolonged exposure to certain toxic chemicals). There is however generally not a direct genetic link. While families may be prone to develop autoimmune conditions, individual family members may have different autoimmune disorders, or may never develop a "full-blown" autoimmune condition. It is likely that there may also be a hormonal component as many autoimmune conditions are much more prevalent in women.

The type of autoimmune disease that occurs and the amount of destruction done to the body depends on which systems or organs are targeted by the autoantibodies, and how strongly. Disorders caused by organ specific autoantibodies, those that primarily target a single organ, are often the easiest to diagnose as they frequently present with organ related symptoms. Examples of organ-specific autoimmune disorders are insulin-dependent diabetes (Type I) which affects the pancreas, Hashimoto's thyroiditis and Graves' disease which affect the thyroid gland, pernicious anemia which affects the absorption of vitamin B12, Addison's disease which affects the adrenal glands, and chronic active hepatitis which affects the liver. Disorders due to systemic autoantibodies can be much more difficult to diagnose. Symptoms may include: joint pain, fatigue, fever, rashes, cold or allergy-type symptoms, weight loss, and muscular weakness. Even if they are due to a particular systemic autoimmune condition, the symptoms will vary from person to person, vary over time, vary with organ involvement, and they may decrease or flare unexpectedly. Examples of non-organ specific autoimmune disorders are rheumatoid arthritis, systemic lupus erythematosus (SLE) and the Antiphospholipid syndrome (APS) (Roitt et al., 1996). SLE and APS will be discussed more extensively later in this chapter.

Autoantibodies found in SLE and APS patients tend to differ from natural autoantibodies found in healthy individuals in that they are generally IgG isotype and have a high affinity for particular autoantigens. In healthy individuals however, low titres of low affinity autoantibodies are part of the normal B cell repertoire (Peeva et al. 2002). These natural autoantibodies resemble the antibodies of a primary immune response in that they are primarily IgM and exhibit polyreactive binding to a wide variety of both autoantigens and foreign antigens. Natural autoantibodies are mainly germline encoded and some germline autoantibody genes are used in the absence of an autoimmune disease. Some autoantibodies might actually be the precursors to pathogenic autoantibodies in SLE. Shlomchik et al., (1987) suggested that pathogenic autoantibodies arise by the somatic mutation of genes that encode protective autoantibodies.

1.1.3.3 Monoclonal Antibodies

Monoclonal antibody (mAb) technology allows the production of large amounts of homogenous pure antibodies. A myeloma is a tumor of the bone marrow that can be adapted to grow permanently in cell culture. When myeloma cells were fused with antibody-producing mammalian spleen cells, it was found that the resulting hybrid cells, or hybridomas, produced large amounts of mAb (Kohler and Milstein, 1975). This product of cell fusion combined the desired qualities of the two different types of cells: the ability to grow continually, and the ability to produce large amounts of pure antibody. These antibodies are called monoclonal because they come from only one type of cell, the hybridoma cell; antibodies produced by conventional methods, on the other hand, are derived from preparations containing many kinds of cells, and hence are called polyclonal. Because selected hybrid cells produce only one specific antibody, they are more pure than the polyclonal antibodies. mAbs are useful because they allow us to study autoimmune rheumatic diseases such as SLE.

1.2 What is Systemic Lupus Erythematosus?

SLE is a complex, heterogeneous autoimmune rheumatic disease characterised by the production of antibodies to components of the cell nucleus in association with a diverse array of clinical manifestations. It is a disabling disease and the primary pathological findings in patients involve widespread inflammation, vasculitis and immune complex deposition. Virtually every organ and/or system of the body may be

involved although the skin, joints, kidneys, lungs and the central nervous system are most commonly affected. The term "lupus erythematosus" was first used in 1852 by Cazenave and Calausit (Amital et al., 1999, Morrow et al., 1999) and its systemic nature has been accepted for the past 100 years (Isenberg et al., 1997).

Lupus is found worldwide. The incidence rate of SLE in the UK is approximately four cases per 10^5 people every year (Johnson et al., 1995). Like many other autoimmune diseases, women are more commonly affected than men and the age of onset is, on average, between 20 and 40 years (Alarcòn-Segovia et al., 1999). It is much more common among black females in the UK, the West Indies, and the United States (Nived and Sturfelt, 1997). Although lupus is widely regarded as a serious and potentially fatal disease, the overall probability of survival in patients with SLE has improved dramatically over the last few decades. Whereas earlier studies in 1955 showed a survival rate of less than 50% in 5 years reports from the last decade revealed that over 90% of patients with SLE survive for 10 years (Abu-Shakra et al., 2004). The exact cause of SLE is not known. However it is clearly a multi-factorial disease influenced by genetic, hormonal and environmental factors.

1.2.1 Aetiology of Systemic Lupus Erythematosus

1.2.1.1 The Genetics of SLE

The exact aetiology of SLE remains elusive. There is however clearly a genetic component to disease susceptibility in SLE. The disease shows a strong familial aggregation, with a much higher frequency among first-degree relatives of patients. Moreover, in extended families, SLE may co-exist with other organ specific autoimmune diseases such as haemolytic anaemia and thyroiditis. The concordance of the disease in monozygotic twins is approximately 25% compared with dizygotic which is about 2% (Pisetsky and Drayton, 1997, Deapen et al., 1992). Therefore, the increased risk of developing the disease in siblings of SLE patients reflects a polygenic inheritance of the disease.

Multiple genes contribute to disease susceptibility and SLE is believed to result from the effects of a number of gene interactions. For instance, patients with homozygous deficiencies of the early components of complement are at risk of developing an SLE-like disease. The genes of the MHC - also known in humans as the Human Leukocyte Antigen System A (HLA)- have been most extensively studied for their contribution in

SLE. An increased frequency of HLA-DR2 and DR3 has been reported in Caucasian SLE patients (Eroglu and Kohler, 2002, Jonsen et al., 2004). However it is possible that some individuals may possess all these genes but still not develop SLE. Further aspects on the genetics of SLE are dicussed in section 1.2.4.1.

1.2.1.2 Hormonal Factors in SLE

SLE is predominantly a female disease, affecting women during the child-bearing years. Females are commonly affected up to ten times more than males. It has been demonstrated that in both sexes, low endogenous oestrogen concentrations are protective and in males, low androgen values increase risk. Oestrogen can act as a potent disease stimulator in lupus-prone mice (Carlsten et al., 1990, Roubinian et al., 1979). In addition, there is evidence from mouse models that androgens may have a protective effect (Lucas et al., 1985, Roubinian et al., 1979). These observations suggest a role for sex hormones in disease predisposition.

1.2.1.3 Environmental Factors in SLE

Various viruses have been implicated in the aetiology of SLE and Epstein-Barr virus (EBV) is the most popular candidate. Infection with EBV results in the production of the viral protein Epstein-Barr virus nuclear antigen-1 (EBNA-1), antibodies against which cross-react with lupus-associated autoantigens, including Ro, Sm B/B', and Sm D1, in lupus patients (Poole et al., 2006).

Photosensitivity is a common presenting symptom of SLE. Ultraviolet (UV) light exposure provokes an abnormal cutaneous reaction and also initiation or activation of systemic flare in susceptible individuals (Millard and Hawk, 2001). UV irradiation induces apoptosis of human keratinocytes and changes the subcellular location of the ribonucleoprotein autoantigens targeted in SLE (Ro, La, small nuclear ribonucleoproteins) (Casciola-Rosen et al., 1994). These antigens stained in a punctate pattern at the cell surface of irradiated cells, a site at which they were never present in unirradiated cells. Little is known about other possible environmental or occupational exposures in relation to SLE disease risk.

1.2.2 Clinical Features of Systemic Lupus Erythematosus

SLE can cause a wide variety of clinical manifestations. The American College of Rheumatology (ACR) has designated a set of criteria (Table 1.1) to aid the diagnosis of SLE (Tan et al., 1982, Hochberg, 1997). To be classified as having SLE, a patient must either have consecutively, or concurrently, satisfied four or more of these criteria. Dermatological problems such as the classic butterfly rash found over the bridge of the nose and malar bones, alopecia, vasculitic skin lesions, buccal and nasal ulceration are very frequent and are present in up to 85% of patients (Isenberg and Horsefall, 1998). Many of these skin lesions are photosensitive thus sun protection forms an important part of management of SLE. Musculoskeletal problems are also a common feature of lupus and these include arthralgia (about 90% of patients) as well as myalgia, muscle weakness and tenderness. In advanced disease, almost any system in the body may be involved, with renal, cerebral and pulmonary manifestations being most dangerous. Significant renal disease will develop in approximately 30% of SLE patients.

Patients with SLE appear to be at high risk for coronary artery disease (Jonsson, 1989, Rahman et al., 1999). The pericardium, myocardium and endocardium may all be affected. Chest pain that increases with respiration and breathlessness are common symptoms in SLE. Infections of the respiratory and urinary systems are frequent and difficult to distinguish from flares of lupus activity (Edworthy, 2001). Normocytic anaemia is present in up to 70% of SLE patients and a Coombs` positive haemolytic anaemia in 10% of patients. Leucopenia and lymphopenia are the most frequent abnormalities of the white blood cell count found in SLE patients (Morrow et al., 1999). Vascular lesions as well as cutaneous vasculitis, ulcers and digital gangrene are also well recognised in SLE patients. Abdominal pain is found in 10 – 20% of the patients, the causes of which range from mild non-specific gastroenteritis to life threatening mesenteric vasculitis. Hepatomegaly and/or persistent liver function test abnormalities as well as splenomegaly are found in up to 10% of patients but pancreatitis is less common (Morrow et al., 1999).

The clinical manifestations of SLE are fundamentally the same in children and adults. SLE can be fatal with the most frequent cases of death divided equally between infections (due to use of immunosuppressive drug treatments), cardiovascular disease and cancer (Cervera et al., 1999). The disease is characterised by cycles of relapse and remission due to the disease activity fluctuating over time between

organs and tissues involved (Morrow et al., 1999). Flares of the disease may require drug treatment with non-steroidal anti-inflammatory drugs, antimalarials, corticosteroids and immunosuppressive agents. Hydrochloroquine sulphate has a place in the management of SLE because of its low record of toxicity and its potential role in lowering cholesterol (Hodis et al., 1993). Severe organ involvement, such as nephritis or vasculitis, requires treatment with a combination of corticosteroids and either azathioprine or cyclophosphamide. Cyclophosphamide, azathioprine and methotrexate, as well as more recent immunosuppressive agents such as mycophenolate (MMF) -see below- may be used to treat more severe forms of SLE (such as renal, cerebral and haematological manifestations). However, they inhibit the inflammatory immune response non-specifically and are therefore associated with a host of unpleasant side effects that may require further therapeutic intervention. For instance, corticosteroids are associated with weight gain, osteoporosis and diabetes and cyclophosphamide is associated with bladder toxicity and infertility.

Because of the side effects from the treatments listed above and the limited prevention measures available, there is a continuing need to increase our understanding of the pathogenesis of the disease. A number of new treatments for SLE are now being introduced and these have been developed owing to a better understanding of the immunology of the disease. New immunosuppressive agents include MMF [reviewed in (Isenberg and Rahman, 2006)]. MMF is the prodrug of mycophenolic acid, which inhibits the enzyme inosine-5'-monophosphate dehydrogenase, resulting in inhibition of purine synthesis, lymphocyte proliferation and T-cell-dependent antibody responses. MMF is as effective as intravenous cyclophoshamide for the treatment of lupus nephritis and other major SLE manifestations. It is also associated with fewer side effects than cyclophosphamide and does not seem to cause infertility (Contreras et al., 2004). B-cell depletion is another approach for treating patients with SLE. The treatment incorporates the use of two infusions each of methylprednisolone, cyclophosphamide and rituximab. This approach results in significant clinical improvement and normalises two of the biomarkers (CD20 and CD22) that reflect disease activity. Thus, it can lower anti-dsDNA antibody levels. Although manifestations of SLE in the major organs or systems involved can improve with this treatment, some of the clinical features return with the return of B cells (Leandro et al., 2005, Looney et al., 2004).

Additionally, therapies directed at the T cell-APC interaction are being investigated. The use of the fusion protein CTLAa-Ig, which blocks the binding of the costimulatory B7 molecules expressed on APCs with CD28 on activated T cells, seems promising. Anticytokine therapy is also under investigation. Infliximab, a chimeric mAb, has high affinity for TNF and neutralises its activity. In an open-label study of infliximab in six patients with SLE, Smolen and colleagues have demonstrated significant clinical improvement (Aringer et al., 2004). Another approach that targets cytokines has been the development of B lymphocyte stimulating protein (BLyS) antagonists. Complement therapy is also an attractive approach. Eculizumab is a humanised mAb that exerts its effects by blocking the generation of the terminal complement component C5a and C5b-9. Early human studies indicate that this molecule is safe and well tolerated (Rother et al., 2004). The new therapeutic approaches described above have shown promise in early studies however large clinical trials are awaited.

1.2.3 Immunopathogenesis of Systemic Lupus Erythematosus

1.2.3.1 Autoantibodies in SLE

The central immunological disturbance in patients with SLE is autoantibody production. These antibodies are directed at several self molecules found in the nucleus, cytoplasm, and cell surface, in addition to soluble molecules such as IgG and coagulation factors. It is now almost 50 years since the discovery of anti-nuclear and anti-DNA antibodies in the serum of patients with lupus. Antibodies to DNA were first isolated by four different groups in 1957 (Ceppellini et al., 1957, Robbins et al., 1957, Miescher and Strassle, 1957, Seligmann, 1957). A broad spectrum of autoantibodies was identified in patients with SLE over the next two decades.

Antinuclear antibodies (ANA) are present in more than 95% of patients, making the ANA test the most sensitive test for lupus. However, ANA are not specific, because they can be detected in a variety of autoimmune, rheumatic, and infectious conditions. Moreover, ANA can be detected in normal individuals, especially in the elderly. However, the absence of ANA makes the diagnosis of lupus much less likely, although still possible. ANA are most commonly detected using the indirect immunofluorescent technique on a tissue culture cell substrate.

Anti-double stranded DNA (dsDNA) and anti-Sm antibodies are unique to patients with SLE. In fact, their presence is included in the classification criteria of SLE (Table 1.1). The Sm antigen is designated as a small nuclear ribonucleoprotein (snRNP) and is composed of a unique set of uridine rich RNA molecules bound to a group of core proteins associated with the RNA molecules. Anti-Sm antibodies react with snRNP core proteins. Antibodies to both denatured/single-stranded (ssDNA) and native/ dsDNA are often present in SLE patients, however the latter notably of the IgG isotype are more strongly associated with the renal pathology (Okamura et al., 1993). Other autoantibodies have been associated with SLE and are summarised in Table 1.2. Anti-dsDNA autoantibodies will be discussed in more detail in section 1.2.4. Arbuckle at el., (2003) studied 130 patients with SLE and showed that at least one SLE autoantibody was present in 115 patients before diagnosis. They proposed that there is a progressive accumulation of specific autoantibodies before the onset of SLE, while patients are still asymptomatic (Arbuckle et al., 2003).

1. Malar rash (butterfly rash)	
2. Discoid rash (erythematosus raised patches)	
3. Photosensitivity (skin rash to sunlight)	
4. Oral ulcers	
5. Arthritis (involving two or more peripheral joints)	
6. Serositis	(a) Pleuritis
	(b) Pericarditis
7. Renal disorder	(a) Proteinuria
	(b) Cellular casts
8. Neurological disorder	(a) Seizures
	(b) Psychosis
9. Haematological disorder	(a) Haemolytic anaemia
	(b) Leucopaenia
	(c) Lymphopaenia
	(d) Thrombocytopenia
10. Immunological disorder	(a) Positive for lupus anticoagulant
	(b) Raised anti-native DNA/ Anti-Sm antibody binding
	(c) Abnormal serum level of IgG or IgM anti-cardiolipin antibodies Anti-Sm antibody
	(d) False-positive serological test for syphilis, present for at least six months
11. Anti-nuclear antibody in raised titre	

Table 1.1 The American College of Rheumatology criteria for the diagnosis of SLE
Adapted from Tan et al., (1982) and updated Hochberg, (1997)

	Antibody Specificity	Prevalence (%)
Antibodies to cell nucleus components	Anti-nuclear antibodies	>90
	Anti-dsDNA antibodies	40-90
	Anti-ssDNA antibodies	up tp 70
	Anti-ssRNA antibodies (including anti-poly A, -poly C, -poly I, -poly U)	-
	Anti-ssRNA antibodies (including anti-poly-rU, -poly-rA)	-
	Anti-poly (ADP-ribose) antibodies	-
	Anti-histone antibodies	30-80
	Anti-ribonucleoprotein antibodies	20-35
	Anti-Sm antibodies	5-30
	Anti-SS-A(Ro) antibodies	30-40
	Anti-SS-B(La) antibodies	10-15
Antibodies to cytoplasmic antigens	Heat Shock proteins	5-50
Antibodies to cell membranes	Lymphocytotoxic antibodies	-
	Anti-neurone antibodies	-
	Anti-erythrocyte antibodies	-
	Anti-platelet antibodies	-
	Anti-phospholipid antibodies (cardiolipin)	20-50
Antibodies to serum components	Anticoagulants (lupus anticoagulant)	10-20
	Antiglobulins (rheumatoid factor)	25
	C1q	20-45

Table 1.2 Autoantibodies found in SLE and the approximate prevalence detectable in the serum of patients
Adapted from Morrow et al., (1999).

1.2.3.2 The role of complement in SLE

The complement system consists of a large number of proteins, some of which are soluble plasma constituents and others cell membrane proteins. A main task of complement components is to stop the invasion of the body by microbes and membrane complement proteins act as receptors. The complement molecules coat the microorganism and the cell takes up the whole complement-microorganism complex, a process called opsonization. The complement system is not only involved in killing of invasive pathogens, but also contains important regulators and activators of several humoral and cellular immune functions (Bohana-Kashtan et al., 2004). Regulators of complement activation protect self-tissue from damage (Walport, 2001a, Walport, 2001b). The actions of complement involve activation of one of three pathways, all generating C3 convertase: the classical pathway, the mannose-binding lectin (MBL) pathway and the alternative pathway.

Complement has both beneficial and harmful roles in the pathogenesis of SLE. On the one hand, patients with SLE present with decreased complement levels and with complement deposition in inflamed tissues, suggestive of a damaging role of complement in the effector phase of disease. On the other hand, homozygous deficiency of any of the classical pathway proteins is strongly associated with the development of SLE. The classical pathway is mainly initiated by interaction of C1q with antibodies (IgG and IgM) in immune complexes and is the main effector of antibody-mediated immunity (Sturfelt and Truedsson, 2005).

Complement is strongly activated in patients with SLE. Deposits of C3, C4, and associated complement proteins can be easily detected in biopsies from inflamed tissues from patients with SLE (Manderson et al., 2004). Complement activity and classical pathway protein levels are generally reduced (especially C1, C4 and C2 levels) in relation to disease activity and increase following treatment (Walport, 2002). The initial cause of complement activation in SLE is thought to be the formation of high levels of immune complexes that, in turn, activate complement via the classical pathway. Disease activity however, as well as the presence of autoantibodies directed against complement proteins (anti-C1q antibodies) may influence the degree of reduction of levels of complement components (Manderson et al., 2004).

Inherited complement deficiencies are rare but when they occur these individuals may develop a form of SLE. Complement deficiencies may be partial (heterozygous) or complete (homozygous). In recent years, evidence has emerged that homozygous deficiency of any of the early components of the classical pathway of complement activation (C1q, C1r, C1s, C4, and C2) predisposes to the development of SLE. In fact, these deficiencies are the strongest susceptibility factors for the development of SLE identified up to now in humans (Manderson et al., 2004). There is an association of disease prevalence and severity within the classical pathway, with patients deficient in one of C1-complex proteins exhibiting the strongest prevalence (>80%) and the most severe disease, followed in turn by C4 and C2-deficient patients (Pickering and Walport, 2000).

Several mouse strains have been generated with classical pathway complement deficiencies and have provided important insights into the pathogenesis of SLE. The genes for C1q, C4 and C3 have been successfully targeted in mice, and these mouse strains showed a hierarchy of disease susceptibility similar to that observed in humans. C1q has been shown to bind directly to apoptotic blebs and apoptotic bodies are found in the glomeruli of the C1q deficient mice (Korb and Ahearn, 1997) (Taylor et al., 2000). A recent study performed by Trouw and colleagues in MRL/*lpr* mice has shown that anti-C1q antibodies were present in the sera and deposited in the kidney (Trouw et al., 2004). Gene-targeted C1q-deficient mice have also been shown to develop a syndrome reminiscent of SLE with antinuclear autoantibodies and proliferative glomerulonephritis (Mitchell et al., 2002, Botto et al., 1998). In another study by Paul et al., (2002) using C4-deficient mice, higher levels of anti-dsDNA antibodies developed compared to C4 sufficient littermate controls. At 3 months of age, females with and without C4 have equivalent levels of DNA binding. By 6 months however, anti-dsDNA antibodies of the IgM isotype are elevated in $C4^{-/-}$ females compared to $C4^{+/+}$ controls and by 9 months IgG anti-dsDNA antibodies also increase (Paul et al., 2002). Furthermore, they observed that the kidneys of $C4^{-/-}$ mice had glomerular immune complexes. Hence, these findings demonstrate that C4 normally helps prevent early stages of autoimmune disease and that C4 deficiency predisposes to abnormal regulation of autoreactive B cells.

The levels of complement receptors, CR1 and CR2, are also altered in patients with SLE. CR1 expressed on peripheral B cells, erythrocytes, monocytes and macrophages, binds and clears activated complement components (C3b, iC3b and

C4b) and their associated immune complexes. Walport and Lachmann, (1988) have described decreased expression of CR1 on erythrocytes and peripheral blood leukocytes in patients with SLE and have shown that levels correlated with disease activity. CR2 is expressed on B-cells and follicular dendritic cells. Furthermore, Tolnay and Tsokos, (1998) have shown that MRL/*lpr* mice deficient in CR2 have more severe disease.

1.2.3.3 The role of apoptosis in SLE

Apoptosis, or programmed cell death, is a process that leads to the ordered destruction of cells, avoiding the release of intracellular contents into the extracellular microenvironment, where they have a powerful inflammatory effect. During apoptosis intracellular structures, including the nucleus, are broken down via certain pathways by specific enzymes. Casciola-Rosen et al., (1994) demonstrated that these intracellular components often make up the spectrum of target autoantigens in lupus (for example, nucleosomal DNA, Ro, La and RNP particles) and cluster in blebs on the surface of apoptotic cells enabling them to be presented as antigen. These observations provide a possible connection between apoptosis and SLE.

Under normal circumstances, apoptotic cells are engulfed by macrophages in the early phase of apoptotic cell death without inducing inflammation or the immune response. Recent studies have shown that the clearance of apoptotic cells by macrophages in patients with SLE is impaired (Herrmann et al., 1998). This impairment has generated much interest in recent years, with defective clearance of apoptotic fragments summarised as the 'waste disposal hypothesis' (Walport, 2001a, Walport, 2001b). An early suggestion that defective clearance of apoptotic cells might predispose to SLE came from the observations of those who observed that C1q-null mice develop SLE-like autoimmunity in the presence of defective clearance of apoptotic cells (Botto and Walport, 2002). These mice show glomerulonephritis with deposits of immune complexes and apoptotic cells in the glomeruli (Botto et al., 1998). Interestingly, C1q binds to apoptotic cells, and human serum depleted of C1q does not allow efficient uptake of apoptotic cells by macrophages (Korb and Ahearn, 1997, Taylor et al., 2000).

Cocca et al., (2002) have also illustrated that the clearance of apoptotic cells may be slowed in patients with SLE. They showed that a murine autoantibody can bind

directly to blebs on apoptotic cells, due to the presence of certain ligands, and consequently prevent another molecule, annexin V from recognising ligands on the surface and binding. Their results suggest that if autoantibody binding can prevent annexin V from binding then it is possible that autoantibody binding to blebs may slow apoptotic cell clearance further when other molecules recognising factors on surface of apoptotic cells such as phagocytes and complement are prevented from reaching their receptors and aiding apoptotic cell removal.

A number of studies with animal models support the role of apoptosis in SLE. Fas is a 43-kDa glycoprotein molecule which is involved in inducing apoptosis in both B and T lymphocytes. One of the pathways which leads to apoptosis involves Fas (CD95) and Fas ligand (FasL). MRL/*lpr* mice are characterised by the presence of the lymphoproliferation (*lpr*) gene, which is associated with defective Fas (CD95) receptors on the surface of lymphocytes (Suzuki et al., 1998). In the murine MRL/*lpr* model of SLE, the *lpr* mutation results in defective transcription of the gene that codes for the Fas protein (Singh, 1995). MRL mice which carry the homozygous recessive *lpr* mutation develop a lupus-like disease characterised by high IgG autoantibody levels and immune complex-mediated glomerulonephritis. Takahashi and colleagues reported that Gld/gld mice, characterised by a mutation in the Fas ligand (FasL) gene leading to a non-functional FasL molecule, also developed lymphoproliferation, hypergammaglobulinaemia, and Ig deposits in the kidneys (Takahashi et al., 1994). *Bcl-2* is a proto-oncogene, which exerts a regulatory function during development and maintenance of adult tissue, by preventing apoptosis in specific cell types. Evidence has demonstrated that mice transgenic for overexpression of *bcl-2* develop prolonged B cell survival and other features in common with SLE patients [reviewed in Morrow et al., (1999)]. Therefore, these animal models suggest that these molecules, which play critical roles in apoptosis, may play a role in the pathogenesis of SLE.

The impaired clearance mechanisms for these apoptotic materials seen in patients with SLE, may predispose individuals to the development of antibodies against nucleosomes, which contain materials such as histones and dsDNA. Radic et al., (2004) have shown that autoantibodies specific for the nucleosome core or its molecular components selectively precipitated a complex of core histones and DNA from the cytosol at four hours after induction of apoptosis. Their results indicate a direct role for nucleosomes in the execution of apoptosis, clearance of apoptotic cells

and regulation of anti-nuclear autoantibody production (Radic et al., 2004). Therefore, as the experimental evidence discussed above suggests, the release of nucleosomes from the nucleus and their exposure at the cell surface is important in the pathogenesis of SLE. The role of nucleosomes is further discussed in section 1.2.5.

1.2.3.4 Cellular dysfunction: The role of B cells and T cells in SLE

SLE is characterised by abnormalities of the immune system that involve B cells and T cells. B cell activation is abnormal in patients with SLE. The precise role of B cells in systemic autoimmunity is incompletely understood. Although B cells are necessary for expression of disease, it is unclear whether autoantibody production, antigen presentation, and other B cell functions are required for the complete pathologic phenotype. The number of B cells at all stages of activation is increased in the peripheral blood of patients with active SLE (Klinman et al., 1991). Autoantibody secretion is not the only role of B cells in patients with SLE. B cells are extremely efficient antigen presenting cells for antigens taken up specifically through the B cell receptor (Ig receptor). Although B cells are necessary for expression of disease, it is unclear whether autoantibody production, antigen presentation, and other B cell functions are required for the complete pathologic phenotype. To address this issue, Chan et al. (1999) analysed the individual contributions of circulating antibodies and B cells using MRL/lpr mice that expressed a mutant transgene encoding surface Ig, but which did not permit the secretion of circulating Ig. These mice developed nephritis, characterised by cellular infiltration within the kidney, indicating that B cells themselves, without soluble autoantibody production, exert a pathogenic role. The results indicated that, independent of serum autoantibody, functional B cells expressing surface Ig are essential for disease expression, either by serving as antigen-presenting cells for antigen-specific, autoreactive T cells, or by contributing directly to local inflammation (Chan et al., 1999). New evidence for the strength of the role of B cells in disease development comes from a recent preliminary study of B-cell depletion therapy (involving Rituximab and cyclophosphamide) in 24 patients with SLE resistant to conventional therapies. The results showed a beneficial response in the majority of treated patients (Leandro et al., 2002). There is also evidence that B cells in patients with SLE are more sensitive to the stimulatory effects of cytokines such as IL-6 and it appears that are more prone to polyclonal activation by antigens and cytokines (Linker-Israeli et al., 1991).

Abnormalities in T cell function are also evident in patients with SLE. T cell abnormalities are crucial in the pathogenesis of the disease because T cells regulate B cell function and the production of most antibodies is T-cell dependent. The total number of peripheral blood T cells is usually reduced and experiments have shown that the early events of T cell activation are defective in patients with SLE compared with controls (Fernandez-Gutierrez et al., 1998). In patients with SLE there is generally an exaggerated activity of $CD4^+$ T helper cells and diminished function of $CD8^+$ T suppressor cells. This effect is accompanied by reduced antibody-dependent cytotoxicity and imbalanced cytokine production. Due to the loss of effective $CD8^+$ T cells, B cell clones produce autoantibodies directed against an array of intra- and extracellular autoantigens (Kammer et al., 2002). T lymphocytopenia is common in patients with SLE, especially of cells bearing the $CD8^+$ phenotype. Studies in lupus-prone mouse models and patients with SLE suggest that T cell dysfunction is primarily caused by multiple biochemical defects in T cell signalling pathways (Kammer et al., 2002).

Nucleosome-specific T helper (Th) cells provide $CD4^+$-class II-restricted help to nephritogenic ANA-producing B cells in lupus. The autoimmune Th cells of lupus were cloned from (SWR X NZB)F_1 (SNF_1) mice with lupus nephritis, as well as from nephritic patients with SLE (Mohan et al., 1993). In the SNF_1 model, representative pathogenic autoantibody-inducing Th clones rapidly induce immune-deposit glomerulonephritis. Approximately 50% of such pathogenic Th clones respond to nucleosomal antigens and nucleosome-specific T cells are detectable in SNF_1 mice. Thus, nucleosomes are primary immunogens that initiate the cognate interaction between the pathogenic Th and B cells of lupus. However, the lupus Th cells do not respond when components of the nucleosome, such as free DNA or histones, are individually presented by APCs (Kaliyaperumal et al., 1996). Thus critical peptide epitopes for the pathogenic Th cells are probably protected during uptake and processing of the native nucleosome particle as a whole. Kaliyaperumal et al. (1996) synthesised peptides spanning all four core histones in the nucleosome and the nucleosome-specific, pathogenic autoantibody-inducing Th clones were tested for response to these synthetic peptides. The authors reported that the peptide autoepitopes triggered the pathogenic Th cells of SNF_1 lupus mice *in vivo* to induce the development of severe lupus nephritis and stimulated the production of Th1-type cytokines, consistent with IgG2a, IgG2b, and IgG3 being the isotypes of nephritogenic autoantibodies induced in the lupus mice. Interestingly, the Th cell

epitopes overlapped with regions in histones that contain B cell epitopes targeted by autoantibodies, as well as the sites where histones contact with DNA in the nucleosome (Kaliyaperumal et al., 1996).

1.2.3.5 The role of cytokines in SLE

Cytokines are low-molecular-weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to a number of stimuli. They play an essential role in moulding the quality of an immune response to foreign or self-antigens. Many cytokines have been implicated in regulating disease activity and in the involvement of different organs in patients with SLE (Kirou and Crow, 1999). There is however a significant body of data only for IL-6, IL-10, interferon-α (INFα) and tumour necrosis factor-α (TNFα). Cytokine production in patients with SLE differs from both healthy controls and patients with other diseases such as rheumatoid arthritis (RA) but also changes with different disease phenotypes. For example, IL-6 seems to be increased in the cerebrospinal fluid of patients with central nervous system involvement in SLE but not in patients with SLE who lack neurological symptoms (Dean et al., 2000).

A recent study performed by Mathian et al., (2005) indicated that IFNα is involved in pathogenesis of SLE. The authors showed that *in vivo* adenovector-mediated delivery of murine IFN-α resulted in preautoimmune (New Zealand Black (NZB) x New Zealand White (NZW))F_1 [(NZB/W)F_1], but not in normal, mice, in a rapid and severe disease with all characteristics of SLE. Anti-dsDNA antibodies appeared as soon as day 10 after initiation of IFNα treatment. Proteinuria and death caused by glomerulonephritis occurred in all treated mice within, respectively, approximately 9 and approximately 18 week, at a time when all untreated (NZB/W)F_1 did not show any sign of disease (Mathian et al., 2005). Serum levels of IFNα are elevated in SLE patients, and gene expression profiling of peripheral blood cells using microarrays showed that most lupus cases demonstrate an upregulation of IFN-responsive genes (Bennett et al., 2003, Baechler et al., 2003). Of interest, these authors found that the IFN gene 'signature' correlates with more severe disease.

Jacob et al., (1988), used an (NZB/W)F_1 hybrid mouse model with a severe autoimmune disease similar to SLE in humans to study the role of TNFα. They presented evidence that the TNFα gene could be involved in the pathogenesis of

lupus nephritis in (NZB/W)F_1 mice and replacement therapy with recombinant TNFα induced a significant delay in the development of the nephritis (Jacob and McDevitt, 1988). IL-10 both inhibits Th1 responses and is a potent cofactor for B cell survival, proliferation, differentiation, and Ig secretion, therefore it may play a potential role in the pathogenesis of SLE (Beebe et al., 2002). SLE patients produce abnormally high levels of IL-10 (Lacki et al., 1997) and results obtained by another group indicate that serum IL-10 values reflect SLE disease activity suggesting that overexpression of IL-10 might play a pathogenic role in SLE (Houssiau et al., 1995). To explore the consequences of IL-10 neutralization in (NZB/W)F_1 mice, animals were injected with anti-IL-10 antibodies from birth until 8-10 months (Ishida et al., 1994). Anti-IL-10 treatment substantially delayed onset of autoimmunity in (NZB/W)F_1 mice as monitored either by overall survival, or by development of proteinuria, glomerulonephritis, or autoantibodies. Survival at 9 months was increased from 10 to 80% in anti-IL-10-treated mice relative to Ig isotype-treated controls (Ishida et al., 1994). Furthermore, another group reported IL-10 antagonist administration to humans and showed that the use of IL-10 antagonists may be beneficial in the management of SLE (Llorente et al., 2000).

Anti-TNFα therapy is slightly more complicated because of TNFα's dual physiological roles; it is a proinflammatory cytokine but has also been proposed to be an immunoregulatory molecule that can alter the balance of T regulatory cells. Anti-TNFα therapy is effective for the majority of autoimmune patients with RA with end organ destruction due to inflammation, but can worsen or induce autoimmunity for a significant minority of these patients (Kodama et al., 2005). This finding is consistent with numerous animal and human studies showing that reducing TNFα activity aggravates or initiates certain forms of autoimmunity. Anti-TNFα in humans can promote production of anti-DNA antibodies (Ehrenstein et al., 1993b). Kodama et al., (2005) presented a mechanistically based hypothesis that TNFα holds potential therapeutic value because of its demonstrated capacity in animal models of autoimmunity to selectively kill, by apoptosis, autoreactive (pathogenic) T cells but not normal cells. To investigate the safety of therapeutic TNFα blockade, 6 patients with active SLE were given four 300-mg doses of infliximab, a chimeric anti-TNFα antibody (Aringer et al., 2004). The authors proposed that anti-TNF therapy could suppress the local tissue destruction in SLE. Infliximab did not lead to adverse events related to an increase in SLE activity, although autoantibodies to dsDNA and cardiolipin (CL) increased. However, this study was small and was limited to patients

with nephritis or arthritis and low-to-moderate overall disease activity. Thus, to sufficiently address the potential value of TNF-blocking therapy in SLE, larger and controlled clinical trials are necessary.

1.2.4 The role of anti-dsDNA autoantibodies

1.2.4.1 Origin of pathogenic anti-dsDNA antibodies

Autoantibodies that bind dsDNA have generated the most interest since they are believed to be most closely involved with the pathogenesis of SLE (Isenberg, 1997). Even though they are among the most specific autoantibodies in lupus, they are not particularly sensitive because they may be transiently present and found in 60-80% of lupus patients at some point in their disease course (Hahn, 1998). Anti-dsDNA antibodies are very rare in other conditions but anti-ssDNA antibodies can be found in some cases of autoimmune hepatitis and infections including syphilis, parasitic infections, and bacterial endocarditis (Sawalha and Harley, 2004).

Anti-DNA antibodies differ in their properties, including isotype, ability to fix complement, and capacity to bind to the glomeruli causing pathogenicity. Only certain types of anti-DNA antibodies are pathogenic (Hahn, 1998). The most remarkable feature of high-affinity anti-dsDNA IgG antibodies is that they are frequently linked to glomerulonephritis and widely suspected to be responsible for kidney damage in many lupus patients (Putterman et al., 1997). Complement fixation may be essential for tissue damage; thus IgG1 and IgG3 subclasses, which fix complement, are enriched in pathogenic antibodies (Hahn, 1998).

Okamura et al., (1993) studied the correlation between renal histology and class specific (IgG and IgM) antibodies to dsDNA and ssDNA by ELISA in 40 untreated patients with SLE. The levels of IgG antibodies to dsDNA were significantly higher in patients with World Health Organisation class IV nephritis (Hochberg et al., 1992), indicating more severe renal disease, than in those with class I, class II, or class III nephritis. IgG antibodies to ssDNA were higher in patients with class IV than in those with class II nephritis. IgG antibodies to dsDNA showed a close correlation with the renal histological activity score whereas IgG antibodies to ssDNA showed only a weak correlation. IgM antibodies to dsDNA and to ssDNA were not correlated with renal histological features (Okamura et al., 1993).

Cationic charge gives an antibody to dsDNA a pathogenic advantage. Extensive studies in murine models of lupus nephritis have shown that cationic anti-DNA autoantibodies have nephritogenic potential. Suzuki et al., (1993) investigated whether cationic anti-DNA antibodies of IgG class are also produced in vivo in patients with active lupus nephritis. They have shown that the highly cationic anti-dsDNA antibodies of IgG class were prominent in the patients with active lupus nephritis. The cationic anti-DNA antibodies bound to heparan sulphate (HS), a major glycosaminoglycan in the glomerular basement membrane (GBM), much better than neutral anti-DNA antibodies. The results suggest that the cationic anti-DNA autoantibodies may play a certain role in the development of lupus nephritis (Suzuki et al., 1993).

A variety of methods for detecting anti-dsDNA antibodies have been described. Immunofluorescence of the haemoflagellate of *Crithidia luciliae* is generally considered to be the cheapest and most specific test (Hahn, 1998). The high specificity of the test relates to the pure circular dsDNA in the kinetoplast (with no no histones or single stranded DNA in its structure) (Isenberg, 2004). The enzyme-linked immunosorbent assay (ELISA) for antibodies to DNA is widely available, relatively easy to perform and therefore, very popular among many laboratories. In this assay, plastic wells in a microtiter plate are coated with dsDNA and test serum is added. IgG antibodies bound to the dsDNA are detected by adding enzyme-labeled anti-human IgG followed by substrate, which changes colour when acted on by the enzyme. In the Farr assay, radiolabeled DNA is incubated with serum, and the DNA–anti-DNA complexes are precipitated with ammonium sulfate or polyethylene glycol (Hahn, 1998). This is a very accurate method of assessing dsDNA antibody levels and most likely to predict the occurrence of disease flares, particularly flares of glomerulonephritis (ter Borg et al., 1990).

Several mechanisms can lead to the production of antibodies to dsDNA. These antibodies are part of the normal repertoire of natural autoantibodies, most of which are low-affinity IgM antibodies that react weakly with several self-antigens (Putterman et al., 1997). However, natural antibodies can undergo an isotype switch (from IgM to IgG)- see section 1.4.4- and therefore increase their potential to be pathogenic. High-affinity IgG antibodies to dsDNA, can also be produced by somatic mutations in the encoding Ig genes (discussed in more detail in section 1.4).

Antibodies to dsDNA can be induced in mice by stimulation with antigens, such as bacterial DNA or cell-wall phospholipids (PLs); or by DNA and proteins complexes. These induced antibodies have been found to be deposited in the glomeruli, where damage may result. Bacterial DNA has been shown to be much more antigenic than mammalian DNA. Bacterial DNA contains unmethylated CpG motifs, which are immunostimulatory and more common in bacterial DNA than in mammalian DNA and can elicit cytokine production and polyclonal B cell activation (Pisetsky, 1996). Immunisation of BALB/c mice with ssDNA from *Escherichia coli* (*E.coli*) induced a significant anti-DNA response that included antibodies resembling lupus anti-DNA antibodies in their binding properties, although lacking specificity for mammalian dsDNA (Gilkeson et al., 1993). DNA may also be presented to T cells in association with foreign proteins or peptides. Anti-DNA antibodies have been induced in normal, nonautoimmune mice by immunisation with complexes formed with a DNA-binding peptide, Fus1, and native, B form, mammalian DNA (Krishnan and Marion, 1993). Fus1 is a 27-amino acid peptide from the internal domain of a ubiquitin fusion protein from *Trypanosoma cruzi*. From a single BALB/c mouse immunised with Fus1-DNA, six monoclonal anti-DNA antibodies were produced that shared specificity and V region structural similarity with monoclonal anti-DNA antibodies derived from autoimmune (NZB/W)F_1 mice (Krishnan and Marion, 1993). Polyomavirus transcription factor T antigen can induce anti-DNA antibodies in mice and antibodies recognizing dsDNA were confined to patients with frequent polyomavirus reactivations (Rekvig et al., 1997). In these cases, it is believed that immunogenic immune complexes are formed between the host chromatin (packages of nucleosomes connected by DNA linkers) and the DNA-binding large T antigen of polyomavirus.

The ability to make antibodies to dsDNA depends in part on genetic susceptibility. In the NZB/W-related NZM2410 lupus-prone strain, Wakeland and colleagues have identified the genomic positions of three recessive loci (*Sle1*, *Sle2*, and *Sle3* on chromosomes one, four, and seven, respectively) that are strongly associated with SLE-susceptibility (glomerulonephritis and anti-dsDNA autoantibody production) (Morel et al., 1994). Although linkage analyses have provided information about the number and locations of susceptibility genes in lupus-prone strains, they have supplied little information about the nature of the component phenotypes each locus contributes and how they interact to produce the immunopathology characteristic of the susceptible parental strains. To address this issue, Wakeland and colleagues

adopted a congenic dissection approach (Wakeland et al., 1997). The basic principle of this approach is to convert a polygenic system into a series of monogenic systems in individual congenic strains, each carrying a single susceptibility interval on a resistant genetic background (mouse strain C57BL/6 [B6]). Two strains are congenic if they are genetically identical and differ only at a single genetic locus. Any phenotypic differences that can be detected between congenic strains are related to the genetic locus that distinguishes the strains. The authors have produced three congenic strains B6.NZM*Sle1*, B6.NZM*Sle2*, B6.NZM*Sle3*. *Sle1* mediates the loss of tolerance to nuclear antigens; *Sle2* lowers the activation threshold of B cells; and *Sle3* mediates a dysregulation of $CD4^+$ T cells. However, these *Sle* loci are not sufficient individually to mediate fatal lupus nephritis and disease liability increased with the number of susceptibility loci, suggesting that significant penetrance of disease would require two or more loci (Morel et al., 2000). Morel and colleagues reported that *Sle1* in combination with either *Sle2* or *Sle3* as a B6-bicongenic strain develop severe humoral autoimmunity with moderately penetrant fatal glomerulonephritis leading to kidney failure whilst other locus combinations without *Sle1* failed to mediate fatal disease (Morel et al., 2000). The tricongenic strain resulted in fully penetrant, fatal glomerulonephritis. Therefore these loci are sufficient to reconstitute a fully penetrant SLE phenotype in these mice. These results are believed to be relevant to human SLE since these susceptibility loci are syntenic with regions found in human chromosomes. Moreover, several groups have completed linkage analyses in data sets from families containing multiple members affected with SLE. Results have identified chromosomal regions exhibiting evidence for significant linkage to SLE and have been confirmed using independent cohorts (1q23, 1q25-31, 1q41-42, 2q35-37, 4p16-15.2, 6p11-21, 12q24, and 16q12), suggesting the high likelihood of the presence of one or multiple SLE susceptibility genes at each locus [reviewed in (Shen and Tsao, 2004)].

1.2.4.2 The role of antibodies to DNA in the pathogenesis of disease

Anti-DNA antibodies can be isolated from glomerular eluates of patients with active lupus nephritis and can induce nephritis in normal and severe combined immunodeficient mice (Vlahakos et al., 1992, Ehrenstein et al., 1995). However, the correlation between anti-DNA antibodies and lupus nephritis is not absolute because some patients with active nephritis are negative for anti-DNA antibodies, whereas some patients with persistent high titres of anti-DNA may not show renal involvement (Mok and Lau, 2003).

Different mechanisms for how anti-DNA antibodies induce nephritis have been proposed. These follow two main, non-mutually exclusive directions. The first theory of pathogenesis is that circulating antibody-antigen complexes are postulated to deposit in the GBM, forming a site for subsequent inflammatory responses. Anti-dsDNA–nucleosomal DNA complexes get passively trapped via interactions between the positively charged cationic N-terminus of the core histones and the negatively charged HS in the glomeruli (Fournie, 1988, Rekvig et al., 2004, Lefkowith and Gilkeson, 1996, Kramers et al., 1994). Apoptotic blebs are important in antigen presentation, and are believed to be the source of nucleosomal material involved in the formation of these immune complexes. On the other hand, there is also new support for the alternative theory of pathogenesis that a subset of anti-dsDNA antibodies cross-reacts with a non-nucleosomal antigen in the kidney, thereby leading to tissue damage. This will be discussed futher in section 1.2.4.3.

Anti-dsDNA antibodies penetrate living cells, even to the nucleus, and bind various intracellular constituents. Alarcon-Segovia and colleagues reported that anti-DNA antibodies could enter living cells and interact with their intracellular target (Alarcon-Segovia and Llorente, 1983). Subsequent work from other investigators has confirmed these initial observations, and a mechanism of intracellular penetration of anti-dsDNA antibodies into living cells was suggested for the pathogenicity of anti-dsDNA autoantibodies (Yanase et al., 1997). Cellular entry was found to be initiated by the binding of the nuclear localising anti-DNA antibodies to myosin 1, a 110-kDa cell surface receptor. Myosin 1 is expressed on the surfaces of many cell types. Recent evidence suggests that within the cytoplasm, myosin 1 forms a complex with *DNase I* (Yanase et al., 1997). Therefore internalization of anti-DNA antibodies by myosin 1 places them in proximity to *DNase I*, with the potential to interfere with the activity of the enzyme within the cell (such as apoptosis). Other researchers suggested that antibody penetration depends on binding to an extracellular matrix protein in the presence of DNA or a membrane determinant precisely resembling DNA (Zack et al., 1996). It thus appears that a subset of cross-reactive anti-DNA antibodies might exert their pathogenic effect by cellular penetration and nuclear localization (Madaio and Yanase, 1998).

Interestingly, anti-dsDNA antibodies have various effects on a number of cellular cytokines, providing another feature that can explain their potential pathogenic effect in lupus patients. Cellular interactions with anti-dsDNA antibodies have been shown

to be associated with upregulated expression of IL-1, IL-6, IL-8, and transforming growth factor-β (TGF-β) in supernatants of lupus Ig treated cells. IL-1, IL-6, IL-8 and TGF-β were determined in cultured human umbilical vein endothelial cells (HUVECs), incubated with control IgG, anti-dsDNA, or IgG from the same lupus patients depleted of anti-dsDNA by affinity chromatography (Neng Lai et al., 1996, Lai et al., 1997). Compared with control IgG, there was a significant increase of IL-1 in endothelial cells (ECs) incubated with anti-dsDNA. IL-1 and IL-6 were significantly elevated in ECs incubated with anti-dsDNA, compared with those incubated with anti-dsDNA-depleted-IgG (Neng Lai et al., 1996). Furthermore, compared with either control IgG or anti-dsDNA-depleted-IgG, ECs incubated with anti-dsDNA-containing-IgG expressed higher levels of IL-8 and TGF-β (Lai et al., 1997).

In many patients with SLE, increased renal disease activity is associated with rising titres of anti-DNA antibodies, while prophylactic treatment of serologically active patients with corticosteroids significantly reduces the number of subsequent disease flares, contributing to the suspicion that anti-dsDNA is a specificity that is closely allied with clinical disease. Bootsma and colleagues investigated whether giving prednisone when a rise in anti-dsDNA occurs can prevent these relapses (Bootsma et al., 1995). They showed that treatment with prednisone as soon as a significant rise in anti-dsDNA occurs prevents relapse in most cases. However, doubling of the level of anti-dsDNA was taken as sufficient to trigger an increase in steroid dose. This doubling could occur within the normal range or only just outside of it. In addition, the potential serious complications of this study cannot be ignored. The patients were on high doses of steroid so much of the time that they had many side effects such as weight gain and hypertension and made this treatment unattractive for many patients. Furthermore, Alarcon-Segovia and colleagues have recently reported that LJP 394, an immunomodulating agent designed to specifically downregulate anti-dsDNA producing B cells, prolonged the time to renal flare and decreased the number of renal flares in a subgroup of lupus patients (Alarcon-Segovia et al., 2003). However, LJP 394 was not significantly better than placebo when the whole group of patients in the trial is considered.

1.2.4.3 Cross-reactivity of anti-DNA antibodies with renal antigens

Nephritogenic anti-DNA antibodies may be polyreactive. Phosphodiester groups occur in both polynucleotides and PLs, which can account for the ability of some monoclonal and serum derived lupus autoantibodies to bind to both species of

molecules. Therefore, the cross-reactivity of anti-DNA antibodies with CL and other negatively charged PLs suggested that anti-DNA antibodies might react with renal tissue antigens that share epitopes with DNA (Shoenfeld et al., 1983). Furthermore, proteins that form a major structural component of glomerular podocytes and mesangial cells, such as laminin and α-actinin are known to play a prominent role in the pathogenesis of several experimental glomerulopathies.

Laminin is a major non-collagenous protein constituent of the GBM. Sabbaga and colleagues reported that the interaction of a murine monoclonal anti-DNA antibody with extracellular glomerular antigens was found to be due to the binding of this antibody to laminin (Sabbaga et al., 1989). In addition, this interaction was specific, since it was inhibited by laminin, dsDNA and ssDNA in solution. Therefore, the authors concluded that the polyreactivity of the anti-DNA antibody that confers to it the capacity to bind laminin, may account for its ability to form immune deposits by binding directly to non-DNA glomerular antigens. In addition, Amital and colleagues have analyzed the fine specificity of the interaction of pathogenic murine lupus autoantibodies with laminin and found that pathogenic murine lupus autoantibodies react with a 21-mer peptide located in the globular part of the α-chain of laminin. Furthermore, they have shown that immunisation of young lupus-prone mice with this peptide accelerated renal disease (Amital et al., 2005).

Mostoslavsky and colleagues investigated a panel of anti-DNA, anti-histone and anti-nucleosome mAb derived from lupus-prone NZB/W or MRL/*lpr* mice for their *in vivo* pathogenicity, using the RAG-1-deficient mouse model (Mostoslavsky et al., 2001). They have shown in this model a group of anti-DNA, but not anti-histone or antinucleosome mAb were strongly pathogenic and could be distinguished from the nonpathogenic mAb by their cross-reaction with a ~100-kDa glomerular protein, identified by mass spectrometry and Edman degradation as α-actinin. α-actinin is a cross-reactive target for pathogenic anti-dsDNA antibodies in renal tissue, and antigen expression and accessibility may contribute to the susceptibility of particular strains of mice to anti-dsDNA antibody-induced nephritis.

Deocharan and colleagues studied R4A, a pathogenic anti-DNA antibody and found that this antibody bound to and immunoprecipitated a 100-kDa protein identified as α-actinin from mesangial cell lysates (Deocharan et al., 2002). This finding was not the case for a nonpathogenic mutant of R4A. The authors observed that binding was

more apparent using MRL/*lpr* than nonautoimmune BALB/c mesangial cell lysates, suggesting that antigen expression in the kidney plays a role in anti-DNA antibody binding to renal tissue. Furthermore, sera and kidney eluates from lupus mice with active nephritis displayed high titers of anti-α-actinin antibodies. The nature of the anti-DNA-reactive epitope in α-actinin is not known. α-actinin is a dimer composed of two 100-kDa monomers that are arranged in an antiparallel manner to form a rod-shaped molecule, in which there is a high density of acidic residues with negative electrostatic potential. This structure could potentially constitute a structural mimic to the sugar-phosphate backbone of the DNA double helix. Two groups have investigated the differences between a pathogenic and non-pathogenic subset of anti-DNA antibodies and whether a particular antigen was involved in SLE pathogenesis.

What I have discussed so far are the findings of studies carried out using murine anti-DNA mAbs, which suggest that pathogenic anti-dsDNA antibodies cross-react with α-actinin. To further elucidate the nature of DNA/α-actinin cross-reactivity in human lupus and to directly study the pathogenic potential of these antibodies, our group recently studied binding to α-actinin in humans (Mason et al., 2004). Two human mAbs (RH14 and B3), which cause proteinuria in severe combined immunodeficiency mice (SCID) mice bound strongly to α-actinin in ELISA, while nonpathogenic DIL-6 bound very weakly. Serum samples from patients with SLE were also tested. α-actinin binding was found to be significantly higher in patients than in healthy controls. A greater proportion of anti-dsDNA IgG-binding antibodies purified from patients with renal disease bound to α-actinin than did those purified from the sera of patients without renal disease. The results published by our group support the findings of previous studies using murine anti-DNA mAbs, which suggest that some pathogenic anti-dsDNA antibodies cross-react with α-actinin.

The question regarding which glomerular structures (α-actinin, nucleosomes, or others) are recognised by nephritogenic anti-dsDNA antibodies is still controversial. In a recent study, Kalaaji et al., (2006) determined which glomerular structures are recognised by monoclonal and *in vivo* bound nephritogenic antibodies. They analyzed sera from patients with lupus nephritis, from random ANA-positive patients, and paired antibodies from sera and kidney eluates from nephritic (NZB/W)F_1 mice for activity against proteins identified by monoclonal nephritogenic antibodies, and against α-actinin, dsDNA, nucleosomes, histone H1, HS, *DNase I*, and type IV

collagen. Anti-α-actinin antibodies were observed in human and murine lupus nephritis sera and in sera from patients without SLE and were not detected in kidney eluates from nephritic mice. Antibodies to dsDNA and histone H1 were detected in all eluates. Western blot analyses revealed that nephritogenic anti-dsDNA antibodies recognised histone H1. Competitive ELISA demonstrated that nephritogenic mAbs, and dominant antibodies eluted from nephritic kidneys, cross-reacted with dsDNA and H1. Immunoelectron microscopic analysis of nephritic (NZB/W)F_1 mouse kidneys revealed that antibodies eluted from kidneys, but not anti-α-actinin antibodies, bound to distinct nephritis-associated electron-dense structures linked to GBMs (Kalaaji et al., 2006). From their experiments it seems that cross-reactive anti-dsDNA/anti-histone H1 antibodies are central among those deposited in nephritic glomeruli.

Zhao and colleagues analyzed the reactivity and pathogenicity of monoclonal autoantibodies derived from lupus patients with and without renal disease (Zhao et al., 2005). They have extensively characterised a panel of 10 IgM anti-dsDNA/anti-α-actinin antibodies that was generated by Epstein-Barr virus transformation of lymphocytes from patients with SLE and have shown that all anti-dsDNA antibodies isolated also bound α-actinin and cross-reactive antibodies bound to mesangial cells and to isolated glomeruli. In addition the authors reported that binding to glomeruli was not inhibited by *DNase* treatment, but could be inhibited by α-actinin.

1.2.5 The role of nucleosomes in SLE

1.2.5.1 The nucleosome stucture

There is strong evidence to suggest that nucleosomes are important in both the development of pathogenic antibodies in SLE and the interaction of these antibodies with tissues. The nucleosome is the fundamental unit of chromatin and consists of a core particle composed of an octamer of two copies each of histones H2A, H2B, H3, and H4, around which is wrapped round approximately 146 basepairs (bp) of helical DNA (Figure 1.2). Histones in the core associate among themselves primarily through their globular domains, while their basic amino-terminal segments are external and interact with DNA on the surface of the nucleosome. Histone H1 associates through its globular domain with the nucleosome core particle at the entry and exit points of the DNA strands to form a full nucleosome. The molecular weight of a nucleosome particle is 262-kDa with a molar ratio protein/DNA = 1. This highly conserved

structure occurs every 200±40 bp throughout all eukaryotic genomes. Interestingly, chromatin breakdown and DNA fragmentation are common events in apoptotic cell death. Linker DNA, which is accessible, is degraded by nuclear endonucleases upon activation in order to produce mono- and oligo-nucleosomes. Apoptotic cells are then an important source of nucleosomes. In physiological conditions, these apoptotic cells are recognised and engulfed by phagocytes to prevent any release of cell constituents in the extracellular environment. Experimental evidence suggests that SLE patients and lupus mice have been shown to undergo increased rate of apoptosis, leading to an increased nucleosome release (apoptosis is very controversial since reduced apoptosis also mediates pathogenesis of SLE-discussed in more detail in section 1.2.3.3).

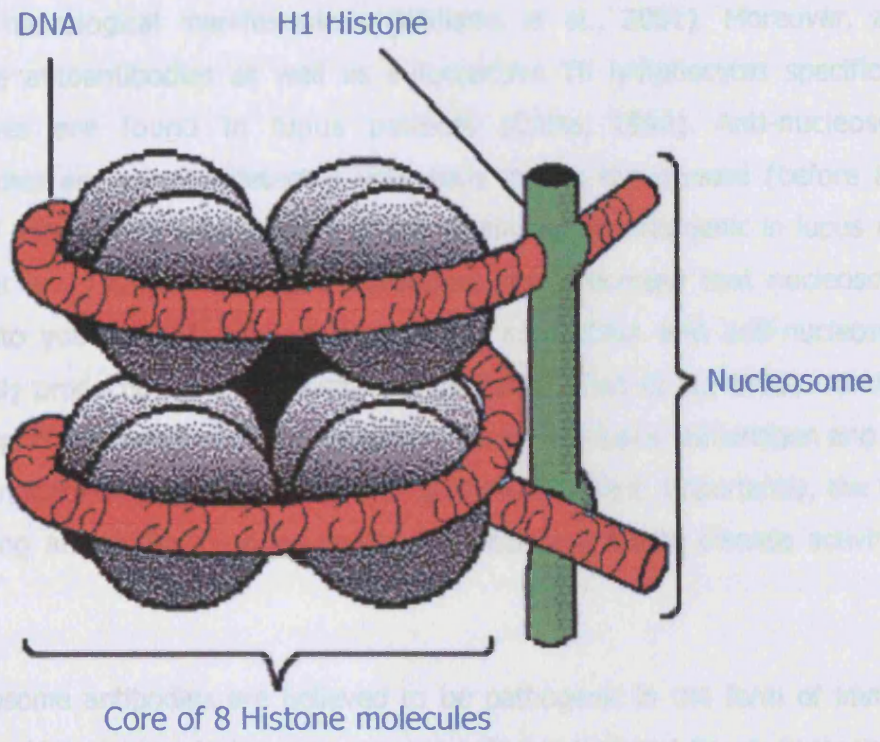


Figure 1.2 Diagramatic representation of the structure of a nucleosome
A nucleosome consists of an octameric histone core, containing two molecules each of H2A, H2B, H3 and H4, surrounded by two turns of the DNA helix. H1 binds to the nucleosome where DNA enters and leaves the nucleosome.

1.2.5.2 The role of anti-nucleosome reactivity in the pathogenesis of SLE

Free nucleosomes have been found in the sera of lupus patients and lupus mice (Rumore and Steinman, 1990). Interestingly, a positive correlation was found between the presence of circulating nucleosomes in patients and the development of renal and neurological manifestations (Williams et al., 2001). Moreover, anti-nucleosome autoantibodies as well as autoreactive Th lymphocytes specific for nucleosomes are found in lupus patients (Datta, 1998). Anti-nucleosome autoantibodies are actually detected very early during the disease (before anti-dsDNA and anti-histone antibodies) and are potentially nephritogenic in lupus mice (Amoura et al., 1994). Mohan and colleagues have reported that nucleosomes injected into young SNF1 lupus mice increase anti-dsDNA and anti-nucleosome autoantibody production and accelerate the disease (Mohan et al., 1993). All these reports support the hypothesis that nucleosome is a main lupus autoantigen and that circulating nucleosomes play a key role in lupus development. Importantly, the level of circulating anti-nucleosome autoantibody is correlated with disease activity in humans.

Anti-nucleosome antibodies are believed to be pathogenic in the form of immune complexes which deposit in kidneys, causing inflammation and tissue damage and also lead to activation of the complement system. Several mechanisms might explain the binding of anti-nucleosome antibodies to kidney cells, either directly or indirectly if nucleosomes are "planted" in the glomerular tissue due to its affinity for extracellular components where nucleosomes work as bridges.

In the direct-binding mechanism of anti-nucleosome antibodies to kidney cells, these antibodies might cross-react with non-nucleosomal kidney antigens, as it was demonstrated for anti-dsDNA antibodies and cross-reaction with α-actinin, which I have discussed above (Mostoslavsky et al., 2001). In the case of the indirect binding via a "planted antigen", nucleosome/anti-nucleosome or nucleosome/anti-DNA immune complexes have been shown to deposit in kidneys, onto the GBM (van Bruggen et al., 1997). The sugar-phosphate backbone of DNA is negatively charged therefore DNA itself is unlikely to be deposited in the GBM due to repulsion by similarly charged molecules. The GBM also carries a strong negative charge due to the presence of glycosaminoglycan side chains, particularly HS. Kramers and colleagues demonstrated that for both monoclonal murine antibodies and polyclonal human antibodies, both DNA binding and ability to cause nephritis could be reduced

by purification under conditions in which antibody-nucleosome complexes dissociate (Kramers et al., 1994). They suggested that the cause of renal damage was not the antibody itself but an antibody-nucleosome complex. The authors investigated this using a rat kidney model. Following renal perfusion of antibody, no binding to the GBM was seen whereas infusion of antibody complexed to nucleosome resulted in binding. Therefore the hypothesis is that the positively charged histones bind to the negatively charged HS in the GBM whilst the DNA part of the nucleosomes is bound by anti-DNA antibodies. Thus nucleosomes may act as a bridge between anti-DNA antibodies and the negatively charged HS in the GBM.

About 30% of SLE patients have no anti-dsDNA antibodies. Among these patients, about 65% are positive for anti-nucleosome antibodies (Amoura et al., 2000b). In this study, the authors have shown that IgG anti-nucleosome antibodies are significantly increased in SLE. Importantly, IgG anti-nucleosome antibodies were detected in both active and inactive SLE in contrast to IgG anti-dsDNA antibodies, which were nearly exclusively detected in active patients. This finding shows that anti-nucleosome maybe more important in some people than others. Moreover, active disease was marked by a unique increase in IgG3 anti-nucleosome antibody levels during SLE flares (Amoura et al., 2000a). This increase was also associated with active nephritis. In my opinion, clinical studies analysing only anti-DNA antibodies ignore anti-nucleosome antibodies, which can be important in disease diagnosis and activity assessment in patients negative for anti-DNA antibodies.

1.3 What is The Antiphospholipid Syndrome?

In 1983, it was recognised that in patients with SLE the presence of circulating antiphospholipid antibodies (aPL), notably lupus anticoagulant (LA) and anti-CL antibodies (aCL), was associated with thrombosis, pregnancy complications and thrombocytopenia (Hughes, 1983). This association was later termed APS (Harris, 1987). During the last 23 years, there has been enormous progress in the knowledge of different aspects of APS, but still a substantial number of questions, mainly about pathogenic mechanisms and treatment, remain unanswered.

1.3.1 Criteria for classification and diagnosis of the APS

By definition, a diagnosis of APS requires persistent presence of medium to high levels of aCL (IgG or IgM isotype), presence of LA or both. A recent consensus

provides simplified criteria for the diagnosis of APS (Table 1.3) (Wilson et al., 1999, Miyakis et al., 2006). A patient with APS must meet at least one of two clinical criteria and at least one of two laboratory criteria. APS is divided into several categories. "Primary" APS occurs in patients without clinical evidence of another autoimmune disease, whereas "secondary" APS occurs in association with another autoimmune disease. SLE is by far the most common disease with which the APS occurs. A minority of patients with APS present with multiple simultaneous vascular occlusions throughout the body, often resulting in death. This acute and devastating syndrome, termed "catastrophic" APS, is defined by the clinical involvement of at least three different organ systems over a period of days or weeks with histopathological evidence of multiple occlusions of large or particularly small vessels (Asherson, 1998). The classification criteria have helped to focus research but have their limitations since they do not include some clinical manifestations associated with APS such as cardiac valve disease and cerebral ischemia. aPL are found among young, apparently healthy control subjects at a prevalence of 1.5% to 5% for both aCL and LA . The prevalence of aPL increases with age and is much higher among patients with SLE, with figures ranging from 12-30% for aCL and 15-34% for the LA (Cervera et al., 1993). APS has been shown to develop in up to 50-70% of patients with SLE who are aPL positive (Petri, 2000). However, many healthy individuals have laboratory evidence of aPL without clinical consequences.

1.3.2 Clinical Features of the APS

Thrombosis within the venous or arterial circulation is a major and most common clinical manifestation of this syndrome (Table 1.4). Any size of vessel of any organ may be affected. In the venous circulation, deep or superficial vein thrombosis of the legs is particularly common occurring in 31 to 55% of patients with the syndrome, half of whom also have pulmonary emboli (Asherson et al., 1989, Cervera et al., 2002). Arterial thromboses are less common than venous thromboses and involve the brain in up to 50% of cases, presenting as transient ischemic attacks or strokes (7 to 13%) (Cervera et al., 2002). Other anatomic sites for arterial thrombosis are the heart (25%), causing coronary occlusion, and the eye, kidney and peripheral arteries (25%). Thrombocytopenia, present in up to half of the patients with APS, is a prominent manifestation of the condition and occasionally patients can also develop a form of haemolytic anaemia (6 to 20% patients). A skin rash known as livedo reticularis, whose severity varies enormously from a confined area of skin to large parts of the arms or legs, is a recognised prominent complication of the

syndrome (occurs in 11 to 22% of patients) (Cervera et al., 2002). These lesions are characterised by thrombi in the intradermal vessels.

A particularly striking feature of PAPS is the development of recurrent, spontaneous fetal loss. The risk of pregnancy loss in women with aPL is greatest from the 10th week of gestation onward (fetal period) (Wilson, 2001). This finding is in contrast to pregnancy loss in the general population, which is most frequent during the first 9 weeks of gestation (Morrow et al., 1999). There is also evidence that women with aPL have an increased risk of giving birth to a premature infant because of pregnancy-associated hypertension and uteroplacental insufficiency (Lima et al., 1996). It has also been proposed that aPL may also impair trophoblast invasion and hormone production (Di Simone et al., 2000). The prevalence of pregnancy loss in patients with aPL has not yet been established, however, in aPL-positive patients, the risk of pregnancy loss is directly related to the levels of aPL, mainly aCL IgG isotype. According to a cohort study of 1000 patients, the most common obstetric complications in the mother were preeclampsia (9.5% of pregnant women), eclampsia (4.4%), and placental abruption (2.0%) (Cervera et al., 2002).

Treatment decisions for the APS fall into four main categories: prophylaxis, prevention of further thromboses of large vessels, treatment of acute thrombotic microangiopathy and management of recurrent fetal loss. Many clinicians use low dose aspirin in patients with no history of thrombosis but persistently high IgG aPL and/or positive LA. The immediate management of arterial or venous thrombotic events follows standard methods of anticoagulation using heparin initially, followed by warfarin. Anticoagulation should be balanced against the risks, particularly of major haemorrhage. Options for the management of pregnancy in aPL positive patients include low dose aspirin or anticoagulation with subcutaneous low molecular weight heparin. In patients known to have aPL, it is generally wise to avoid exposure to other procoagulant factors such as oral contraceptives. Improvement in treatment awaits the further elucidation of pathogenic mechanisms (Ruiz-Irastorza and Khamashta, 2005).

Vascular thrombosis

a) One or more clinical episodes of arterial, venous or small-vessel thrombosis in any tissue or organ AND

b) Thrombosis confirmed by imaging or Doppler studies or histopathology, with the exception of superficial venous thrombosis AND

c) For histopathological confirmation, thrombosis present without significant evidence of inflammation in the vessel wall.

Pregnancy morbidity

a) One or more unexplained deaths of a morphologically normal foetus at or beyond the 10th week of gestation, with normal foetal morphology documented by ultrasound or by direct examination of the foetus OR

b) One or more premature births of a morphologically normal neonate at or before the 34th week of gestation because of severe placental insufficiency OR

c) Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal, anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

Laboratory criteria

a) Anti-cardiolipin antibody of IgG and/or IgM isotype in blood, present in medium or high titre on at least two occasions at least 12 weeks apart, measured by standard ELISA for β_2-Glycoprotein I dependent anti-cardiolipin antibodies OR

b) Lupus anticoagulant present in plasma on two or more occasions at least six weeks apart, detected according to the guidelines of the international Society on Thrombosis and Haemostasis OR

c) Anti-β_2 glycoprotein-I antibody of IgG and/or IgM isotype in serum or plasma, present on two or more occasions, at least 12 weeks apart, measured by a standardised ELISA, according to recommended procedures

Table 1.3 Preliminary classification criteria for the Antiphospholipid Syndrome (Wilson et al., 1999, Miyakis et al., 2006)

ORGAN SYSTEM	PRIMARY PATHOGENIC PROCESS
Arterial	Thrombosis of the aorta or axillary, carotid, hepatic, mesenteric, pancreatic, splenic artery
Cardiac	Angina, myocardial infarction, valvular abnormalities, atherosclerosis
Cutaneous	Superficial thrombophlebitis, splinter hemorrhages, leg ulcers, Livedo reticularis, purpura
Endocrine	Adrenal infarction, necrosis of the pituitary gland
Gastrointestinal	Hepatic infarction, intestinal infarction, splenic infarction, ascites
Haematologic	Thrombocytopenia, haemolytic anaemia
Miscellaneous	Perforation of the nasal septum or avascular necrosis of bone
Neurologic	Transient ischaemic attack, stroke, seizures, multi-infarct dementia, encephalopathy, migraines, cerebral venous thrombosis
Obstetrical	Pregnancy loss, intrauterine growth retardation, uteroplacental insufficiency
Opthalmologic	Thrombosis of the retinal artery, thrombosis of the retinal vein
Pulmonary	Pulmonary emboli, pulmonary hypertension, pulmonary arterial thrombosis, alveolar hemorrhage
Renal	Thrombosis of the renal vein, thrombosis of the renal artery, renal infarction, hypertension, acute renal failure, chronic renal failure, proteinuria, haematuria
Venous	Deep venous thrombosis of the legs or thrombosis of the adrenal, hepatic, mesenteric, or splenic vein

Table 1.4 Clinical manifestations of the Antiphospholipid Syndrome (Levine et al., 2002)

1.3.3 Antiphospholipid Antibodies

1.3.3.1 Detection of clinically relevant aPL

aPL are Igs of IgG, IgM, and IgA isotypes that target PLs and/or PL-binding plasma proteins. There are two broad categories of PLs. The neutrally charged PLs include phosphatidyl ethanolamine, phosphatidyl choline (PC) and platelet-activating factor. Negatively charged PL include phosphatidyl glycerol, phosphatidyl serine (PS), phosphatidic acid, phosphatidyl inositol, and CL (Morrow et al., 1999).

aPL, notably the LA antibodies, aCL antibodies, and anti-β_2-Glycoprotein I (β_2GPI) are the serological hallmarks of APS and the most-useful determinations for screening patients (Levine et al., 2002). Division into these subgroups is broadly based on the method of detection. aPL are detected by coagulation assays that indicate the inhibition of PL-dependent coagulation reactions and by immunoassays that use solid-phase PLs and protein cofactors as antigenic targets. In general, LA antibodies are more specific for APS, whereas aCL antibodies are more sensitive (Levine et al., 2002). Therefore, the most sensitive test for aPL detection is a solid-phase immunoassay for aCL which was initially introduced in 1983 (Harris et al., 1983). IgG and IgM are the isotypes usually measured and they are expressed in GPL and MPL international units (IU), respectively. However, there is no definitive association between specific clinical manifestations and particular subgroups of aPL. Therefore, multiple tests for aPL should be performed, since patients may be negative according to one tests yet positive according to another. There are 30% of patients with only LA or aCL, therefore to identify all patients with aPL both LA and aCL have to be measured (Cuadrado and Lopez-Pedrera, 2003). The anti-β_2GPI assay shows higher specificity than aCL for APS diagnosis. In 3–10% of APS patients, anti-β_2GPI may be the only test positive (Miyakis et al., 2006).

aPL can occur in patients with a variety of infectious or neoplastic conditions or patients exposed to certain medications. However, these antibodies usually are not related to the clinical manifestations of the APS and are different from those generated in patients with the syndrome. They are not associated with an increased risk of thrombosis and bind to both neutral and anionic PL (Hunt et al., 1992). Several groups have reported that the predominant reactivity is against a complex formed by CL and serum PL-binding proteins ("cofactors") rather than reactivity against CL per se (Ordi et al., 1993, Galli et al., 1990, McNeil et al., 1990). aPL have

been shown to be directed against a variety of other PL binding serum proteins which include protein C, protein S and prothrombin (PT) (Bevers et al., 1991, Oosting et al., 1993). The most commonly studied however is β_2GPI, which associates with negatively charged PLs through charge interactions. In contrast to antibodies generated in patients with APS, which recognise β_2GPI on PL-binding proteins and thus are referred to as being cofactor-dependent, the aPL that occur in patients with infections generally recognise PL epitopes and bind directly to negatively charged PLs themselves. This phenomenon distinguishes "non-pathogenic" and "pathogenic" aPL.

McNeil and colleagues purified aPL that exhibit binding in a CL ELISA. However, these highly purified aPL did not bind to the CL when assayed by a modified CL ELISA in which the blocking agent did not contain bovine serum, nor did they bind to PL affinity columns (McNeil et al., 1990). Binding to the PL antigen only occured when normal human plasma, human serum, or bovine serum was present, suggesting that the binding of aPL to CL requires the presence of a serum cofactor. Using sequential PL affinity, gel-filtration, and ion-exchange chromatography, McNeil and colleagues purified this cofactor to homogeneity and have shown that the binding of aPL to CL requires the presence of this cofactor in a dose-dependent manner. Using N-terminal region sequence analysis of the molecule they identified the cofactor as β_2GPI (McNeil et al., 1990). Their findings indicate that the presence of β_2GPI is required for some antibody-PL interaction, suggesting that bound β_2GPI forms the antigen to which aPL are directed.

Ordi and colleagues used a similar approach, a standard ELISA method and a modified ELISA to study four groups of patients (Group 1- patients with aCL and autoimmune disease [SLE and PAPS]; Group 2- patients with aCL, no symptoms and no underlying infection or autoimmune disease; Group 3- patients with aCL and infectious diseases [syphilis, leprosy, HIV infection and Q fever]; Group 4- control group). They observed that 19 of 20 samples from patients in Group 1 had cofactor dependence in aCL activity and 17 of 19 samples from patients in Group 3 had aCL activity, which was independent of the presence of the cofactor. Three of four patients in Group 2 had cofactor independent aCL and one had cofactor dependent aCL activity. None of the samples from the control group had aCL. In addition, the authors have shown that aCL related clinical manifestations (thrombosis) depend on the presence of cofactor dependent aCL and not on cofactor independent aCL (Ordi et al., 1993). Absence of cofactor dependency however was shown with the IgM

APS-derived antibodies, HVA2 and HLC9. These antibodies presented polyreactivity to several PLs- CL, PS, phosphatidyl ethanolamine, phospatidyl inositol and PC- but not to β_2GPI (Buschmann et al., 2005).

Forastiero et al., (2005) assessed the contribution of antibodies to β_2GPI and PT to the thrombotic risk in a cohort of 194 patients with persistent LA and/or aCL. IgG anti-β_2GPI and anti-PT antibodies were associated with an increased risk of thrombosis. Patients testing positive for anti-β_2GPI had a higher rate of thrombosis than did antiphospholipid patients without anti-β_2GPI. Similarly, a higher rate of thrombosis was found in patients with positive anti-PT compared with patients without anti-PT (Forastiero et al., 2005).

1.3.3.2 The role of aPL in the APS

Unlike anti-dsDNA antibodies, aPL do not form immune deposits in the affected tissues and rarely produce significant amounts of inflammation. aPL exhibit multiple effects because of the many biological processes that involve PLs and PL membranes hence it is difficult to determine whether these are clinically relevant. However, the major features of APS arise from thrombosis. No single mechanism is accepted as the main cause of thrombosis in APS. The fact that thrombosis in APS is episodic and localized suggests that the presence of high levels of aPL alone are not enough to trigger it. Any proposed aPL-mediated effects that are based on *in vitro* studies must be assessed *in vivo* in animal models and clinical studies to evaluate significance. Evidence accumulated from animal models of the APS, where different groups induce the APS in normal mice by both the passive transfer of aPL and active immunisation with aPL or β_2GPI, indicate that aPL can play a role in the development of thrombosis and pregnancy loss [reviewed in (Radway-Bright et al., 1999)].

There are few models of the thrombosis found in patients with APS. Pierangeli and colleagues (Pierangeli et al., 1994, Pierangeli et al., 1996) have developed a novel mouse model of induced venous thrombosis to study the role of autoantibodies in thrombus formation. To determine whether Igs from patients with the APS play a role in thrombosis, the authors passively immunised groups mice, intraperitoneally, either with Igs (IgG, IgM and IgA) purified from patients with the APS or with Igs of the same isotype from healthy controls. Each animal was anesthetised and the femoral vein minimally mobilised and subjected to a standardised "pinch" injury to

induce thrombosis. The vessel was transilluminated and clot formation and dissolution were visualised. The authors noted that in eight out of nine Ig preparations from patients with APS there was a significant increase in mean thrombus area and a significant delay in mean thrombus disappearance time compared to mice that received Ig from healthy people (Pierangeli et al., 1994). In addition, Pierangeli and colleagues compared the effects on thrombus formation produced by separate groups of mice injected with affinity-purified IgG or IgM aCL or with normal Igs of the same isotype to determine whether aCL are involved (Pierangeli et al., 1996). They have observed that mean thrombus area and mean disappearance times were again significantly increased in all four groups injected with affinity-purified aCL antibodies. This study was the first to show that aCL of IgG, IgM and IgA isotypes may play a role in thrombosis *in vivo*. The drawback of this work however, is that not all aPL can mediate this effect in the model and the pinch injury used in this model provides a second stimulus for the cause of thrombosis.

In a later study, the same authors (Pierangeli et al., 1999) tested human affinity-purified aPL from patients with the APS, in the mouse model of microcirculation that allows direct microscopic examination of thrombus formation and adhesion of white blood cells to ECs in the cremaster muscle as an indication of EC activation *in vivo*. Adhesion molecule expression on HUVECs after aPL exposure was also performed to confirm EC activation *in vitro*. Pierangeli and colleagues demonstrated that all 6 aPL significantly increased the expression of VCAM-1, with one of the antibodies also increasing the expression of E-selectin on HUVECs *in vitro*. In the *in vivo* experiments, they showed that each aPL antibody, except from one preparation, increased white blood cell sticking compared with control, which correlated with enhanced thrombus adhesion (Pierangeli et al., 1999). Therefore, activation of ECs by aPL *in vivo* may create a prothrombotic state on ECs, which may be the first pathophysiological event of thrombosis in APS.

Jankowski and colleagues adapted a hamster model of arterial thrombosis (Jankowski et al., 2003). This animal model complies with the concept of thrombosis as a "double-hit" phenomenon. Very mild thrombosis is provoked by limited photochemically induced injury to the vessel wall ("first hit"). Three mAbs raised against human β_2GPI were selected on the basis of their crossreactivity with hamster β_2GPI. Two of these, one with LA activity, 5H2, and one with only aCL properties, 11E8, were infused prior to photochemically induced vessel damage. 5H2 promoted

thrombus formation dose dependently. The LA mAb 11E8 reactive with human β_2GPI exclusively, did not significantly promote thrombus formation. mAb 27A8, non–cross-reacting with hamster β_2GPI and chosen as a negative control, had no clear effect.

In another animal model, Fischetti et al., (2005) used intravital microscopy to monitor the vascular changes that occur in rats following the intraarterial injection of aPL. Their results showed that human polyclonal IgG with anti-β_2GPI activity triggers clotting in the microcirculation of rat mesentery in the presence of a priming proinflammatory stimulus. In this case this group did not induce a thrombogenic injury by a mechanical or photochemical damage of the vessel wall. Their approach was to mimic more closely the *in vivo* situation using a cofactor commonly occurring in patients with APS and unable to trigger clotting per se, such as LPS. They showed that the endothelial cells seem to play a critical role in the initiation of aPL antibody-mediated thrombosis in this model. aPL injected into LPS-treated rats targeted to the endothelium of the mesenteric microvessels. The binding appears to be selective for the anti-β_2GPI antibodies because deposits of IgG were not observed in rats receiving aPL-positive IgG depleted of anti-β_2GPI antibodies. Weak deposits of IgG not associated with the formation of thrombi were seen in rats treated with aPL-positive IgG in the absence of LPS. To explain the increased antibody deposition observed after injection of LPS and aPL the authors suggested that LPS up-regulates the expression of the ligand for β_2GPI on endothelial cells or synergizes with the antibodies in clustering the ligand capable of stimulating the endothelial cells (Fischetti et al., 2005).

A study performed by Blank and colleagues provides more direct evidence of a pathogenic role of aPL in obstetric manifestations of APS. Blank et al., (1991) infused murine monoclonal aPL and human polyclonal IgG aPL from a patient with PAPS into the tail vein of pregnant mice to evaluate the effect on fecundity, fetal loss (fetal resorption), and the weight of embryos and placentae. They demonstrated lower fecundity rate, increased resorption index of embryos (equivalent to recurrent fetal loss), lower number of embryos per pregnancy, and lower mean weights of embryos and placentae in comparison to mice infused with appropriate control Igs (murine or human control Ig lacking aPL binding) (Blank et al., 1991). Therefore, their results showed that aPL have direct effects on fecundity and on the outcome of pregnancy in this murine model.

In another study, a significantly higher rate of fetal resorptions with a significant reduction in fetal and placental weight was found in pregnant BALB/c mice injected with human monoclonal IgG aPL from a PAPS patient compared to mice injected with a control human monoclonal IgG antibody lacking aPL activity (Ikematsu et al., 1998).

Other models of APS exist, mainly in mice that are prone to spontaneously develop autoimmune disease (systemic lupus-like illness). Hashimoto and colleagues found that (NZWxBXSB)F_1 mice produce high titres of aCL and develop abnormally high incidence of degenerative coronary vascular disease with myocardial infarction and thrombocytopenia (Hashimoto et al., 1992). Futhermore, MRL/*lpr* mice spontaneously develop some features similar to human APS such as high levels of IgG aCL, thrombocytopenia and poor pregnancy outcome (Gharavi et al., 1989). Another group reported histologic evidence of central nervous system thromboses in MRL/*lpr* mice (Smith et al., 1990). However, there are differences between the models compared to human SLE and APS. The marked lymphadenopathy found in MRL/*lpr* mice is not a common feature of SLE. The autoimmune mice with murine lupus almost invariably die of severe glomerulonephritis, whereas less that 50% of patients with SLE develop renal involvement.

1.3.4 Mechanisms of pathogenesis in the APS

Several hypotheses have been proposed to explain the cellular and molecular mechanisms by which aPL promote thrombosis. The present hypotheses for pathogenic mechanisms in the APS are summarised in Figure 1.3. However, the major mechanisms of hypercoagulability in APS involve up-regulation of the tissue factor (TF) pathway, inhibition of the protein C pathway and the interaction of aPL with the endothelium.

It is known that aPL are able to recognise, injure, and/or activate cultured vascular ECs. Binding of aPL induces EC activation, as evaluated by up-regulation of the expression of adhesion molecules (intercellular adhesion molecule 1 (ICAM-1), VCAM-1 and E-selectin) and up-regulation of cytokine secretion (George et al., 1998a).

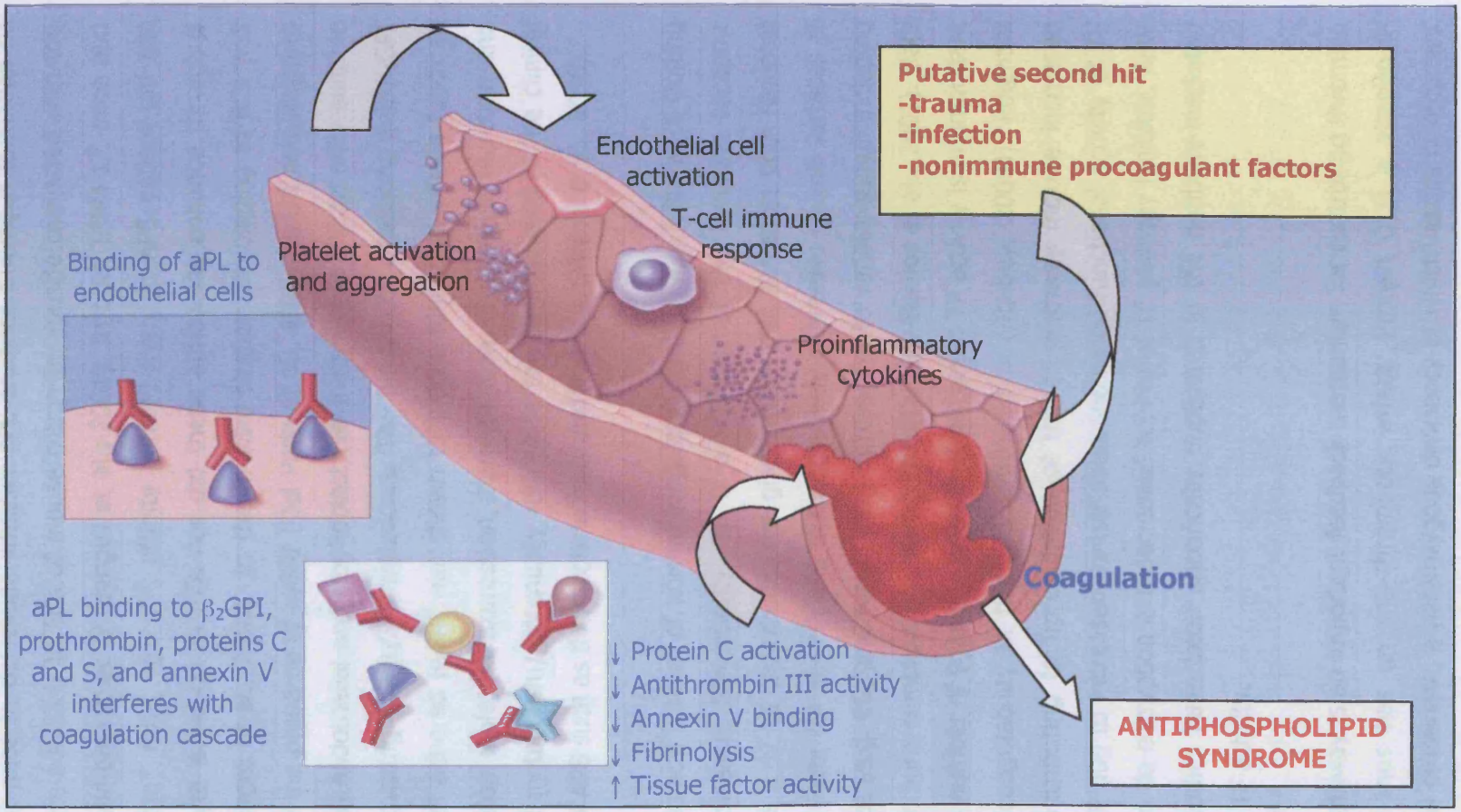


Figure 1.3. Pathogenic Mechanisms in The Antiphospholipid Syndrome
Adapted from Hanly, 2003

Comparable findings have also been reproduced in murine *in vivo* experimental models of microcirculation as discussed in the section above (Pierangeli et al., 1999, Pierangeli et al., 2000). The same authors used ICAM-1–deficient and ICAM-1–/P selectin–deficient mice to determine whether aPL enhance thrombus formation or increase leukocyte adhesion to ECs of mouse cremaster muscle compared with the corresponding wild-type mice (Pierangeli et al., 2001). In both ICAM-$1^{-/-}$ mice and ICAM-$1^{-/-}$/P-$selectin^{-/-}$ mice, IgG aPL affinity purified from an APS patient did not increase leucocyte adhesion to ECs nor did these antibodies enhance thrombus formation (Pierangeli et al., 2001). In the *in vitro* studies aPL binding to ECs was dependent upon the presence of β_2GPI (Del Papa et al., 1995). β_2GPI was found to adhere to human endothelial and trophoblast cell membranes and to offer suitable epitopes for circulating anti-β_2GPI antibodies. The binding of circulating anti-β_2GPI antibodies to cell adhered β_2GPI was shown to induce functional effects (such as increased adhesion molecule expression, up-regulation of cytokine secretion and arachidonic acid metabolism) potentially related to the pathogenesis of the clinical manifestations of APS such as thrombotic events and fetal loss (Meroni et al., 2000).

TF is the physiological initiator of normal coagulation and a major initiator of clotting in thrombotic disease. Structurally, TF is a member of the cytokine receptor superfamily; it is a 47-kd transmembrane glycoprotein that serves as both receptor and enzyme activator for plasma and is constitutively expressed on the surfaces of various cell types (e.g. ECs and monocytes) in response to various stimuli including bacterial LPS, immune-complexes, IL-1 and TNF (Dobado-Berrios et al., 2001). Data obtained from a number of studies suggest that increased TF activity is an important cause of hypercoagulability in the APS [reviewed in (Roubey, 2000)]. Potential mechanisms contributing to upregulation of the TF pathway include increased expression of TF due to increased transcription, increased functional activity of TF molecules due to de-encryption and decreased activity of TF pathway inhibitor. Anti-β_2GPI autoantibodies have been specifically implicated in the antibody-mediated enhancement of TF activity.

The protein C pathway is an important feedback mechanism for controlling thrombin formation, and thus has an anti-thrombotic effect. Protein C is a vitamin-K-dependent serine proteinase, a heterozygous deficiency of which results in recurrent thrombotic disease (Mackworth-Young, 2004). Activated protein C combines with another cofactor, protein S, in the presence of PL to catalyse the degradation of

factors Va and VIIIa of the coagulation pathway. For this to occur, protein C is first converted to its active form by thrombin in the presence of thrombomodulin, an EC-derived cofactor. Protein C is a potential target for antibodies in APS. It has been shown that aPL can interfere with the protein C system by different mechanisms, which include the inhibition of thrombin formation, decrease of protein C activation by the thrombomodulin thrombin complex, inhibition of protein C complex assembly, inhibition of protein C activity directly or via its cofactor protein S, and binding to factors Va and VIIa in a manner that protects them from proteolysis by activated protein C (de Groot et al., 1996). It has been described that aPL isolated from patients can inhibit protein C activity. To investigate if aPL are directed against a combination of PLs and protein C or protein S, Oosting and colleagues studied IgG fractions from the 30 patients, of whom 26 had aPL and 23 had thrombosis (Oosting et al., 1993). The authors found that the antibodies responsible for inhibiting Va degradation were directed against PL-bound protein C or protein S. Other groups have also demonstrated that aPL derived from patients' serum impairs the degradation of factor V by protein C *in vitro* (Marciniak and Romond, 1989, Borrell et al., 1992).

The pathogenic mechanisms contributing to obstetric complications are not fully understood either. Originally aPL were thought to cause pregnancy morbidity by placental thrombosis but recently there has been more compelling evidence of a direct effect upon maternal and fetal cells. For instance, it has been shown that purified aPL can bind specifically to placental antigens, providing a potential mechanism for nonthrombotic placental damage and impaired foetal blood supply. Donohoe and colleagues developed an immunofluorescent technique to detect human aPL binding to human placenta and demonstrated binding to the placenta by affinity purified aPL from five of six patients with the APS (Donohoe et al., 1999). They have also performed preliminary sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting studies that demonstrated that aPL bind a number of placental proteins (Donohoe et al., 1999). aPL and antibodies to β_2GPI have also been found to modify trophoblast proliferation and differentiation (Chamley et al., 1998).

Defective endovascular trophoblast invasion, rather than intervillous thrombosis, is the most frequent hostological abnormality in PAPS-positive associated early pregnancy loss (Sebire et al., 2002). Sebire and colleagues examined placental

histopathology in patients with a history of recurrent miscarriage, both with and without PAPS. They confirmed that the major pathophysiologic mechanism is uteroplacental vasculopathy, such as placental infarction and preeclampsia. In addition, in this study only one case of fetoplacental vascular thrombosis was identified; this occurred in a clinically uncomplicated PAPS–positive pregnancy delivered at 38 weeks. Their findings therefore do not support the hypothesis that pregnancy complications in PAPS are related to intraplacental thromboses, although this could occur in failed pregnancies as opposed to those successfully treated and resulting in live birth (Sebire et al., 2003).

While antigenic epitopes recognised by aPL are important in the pathogenesis of the APS, there is emerging evidence that the complement pathway may mediate fetal damage (Salmon et al., 2002). Holers et al., (2002) used a murine model of APS in which pregnant mice are injected with human IgG containing aPL to investigate whether these antibodies activate complement in the placenta, generating split products that mediate placental injury and lead to fetal loss and growth retardation. They found that inhibition of the complement cascade *in vivo* blocks fetal loss and growth retardation and mice deficient in complement C3 were resistant to fetal injury induced by aPL (Holers et al., 2002). Similarly, (Girardi et al., 2003) also tested complement activation in pregnant mice that received human IgG containing aPL. They identified complement component C5 (and particularly its cleavage product C5a) and neutrophils as key mediators of fetal injury and showed that antibodies that block C5a–C5a receptor interactions prevent pregnancy complications.

Despite the multiple ways by which aPL could cause pathology, many individuals with high IgG aPL levels do not develop features of APS. This may be due to the particular pattern of antibody specificities in their serum. However, it appears that for many people other factors may be needed for the expression of the APS, i.e. a 'second hit' is required. aPL would be responsible for a hypercoagulable state and a second stimulus, such as other thrombotic risk factors, infections, or drugs, would lead to the development of thrombosis.

1.3.5 The role of T cells and cytokines in the APS

Cytokine abnormalities have been implicated in the pathogenesis of fetal loss, one of the major manifestations of the APS. Interleukin-3 (IL-3) is an important factor that aids in embryo implantation and placental development. IL-3 was found to be

decreased both in pregnant women with APS compared with a control group, and in animal models of APS (Shoenfeld et al., 1998). In another study, where primary APS was induced following immunisation of BALB/c mice with a human monoclonal aPL antibody (H-3), analysis of the cytokine profile of the mice with the APS indicated low production of IL-2, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fishman et al., 1993). The authors concluded that administration of murine recombinant IL-3 led to a dramatic reduction in the fetal resorptions rate and thrombocytopenia associated with the experimental APS was also corrected following IL-3 administration (Fishman et al., 1993).

To examine antigen recognition profiles and Th cell activity in β_2GPI reactive T cells. Arai et al., (2001) generated 14 CD4+ T cell clones specific to β_2GPI from three patients with APS. At least four distinct T cell epitopes were identified, but the majority of the β_2GPI specific T cell clones responded to a peptide encompassing amino acid residues 276 to 290 of β_2GPI containing the major PL binding site. Ten of 12 β_2GPI specific T cell clones stimulated peripheral blood B cells that produced anti-β_2GPI antibody in the presence of recombinant β_2GPI. Th cell activity was found exclusively in T cell clones capable of producing IL-6. In vitro anti-β_2GPI antibody production induced by T cell clones was inhibited by either anti-IL-6 or by the anti-CD40 ligand mAb. In addition, exogenous IL-6 augmented anti-β_2GPI antibody production in cultures of B cells with T cell clones lacking IL-6 expression. The results of this study indicate that β_2GPI specific CD4+ T cells in APS patients preferentially recognise the antigenic peptide containing the major PL binding site and have the capability to stimulate B cells to produce anti-β_2GPI antibody via expression of IL-6 (Arai et al., 2001).

T cells appear to play a major part in the pathogenesis of APS since autoantibody production by B cells generally requires T cell help. Experimental primary APS was induced in mice following active immunisation with aPL mAb and T cells from the APS mice were infused into BALB/c recipients (Blank et al., 1995). The authors observed high serum titers of aPL and clinical features of primary APS, including thrombocytopenia and a high frequency of fetal resorptions. T cell-depleted bone marrow cells did not transfer the disease (Blank et al., 1995). In addition, another group treated BALB/c mice with anti-CD4 mAb either before or after induction of experimental APS and SLE (Tomer et al., 1994). The treated mice did not develop

proteinuria, had fewer episodes of fetal resorption and significantly lower levels of antibodies to dsDNA, histones, CL, and PS.

1.3.6 The importance of β_2-Glycoprotein I in APS

β_2GPI, an abundant glycoprotein in human plasma found at a concentration of approximately 200μg/ml, is synthesized mainly in the liver but also by other cells such as trophoblasts (Li and Krilis, 2003). β_2GPI is a protein of approximately 50-kDa; it is a highly glycosylated, single-chain polypeptide of 326 amino acids containing a high proportion of proline and cysteine residues, with 20% w/w carbohydrate attached. The protein is a member of the complement control protein or short consensus repeat (SCR) superfamily, characterised by repeating stretches of 60 amino acid residues, each with a set of 16 conserved residues and 2 fully conserved disulfide bonds and has 5 of the repeating SCR domains (also known as "sushi domains"). β_2GPI is highly conserved among different mammalian species including humans, bovines, canines and mice (Miyakis et al., 2004).

aPL can recognise β_2GPI directly (in the absence of PL) if the protein antigen is present on microtiter plates at a sufficient density. The crystal structure of the protein has been solved both in solid and fluid phase and bears relevance for the positioning of the domains of β_2GPI involved in the binding of the antibodies (Figure 1.4) (Schwarzenbacher et al., 1999); its structure suggests that the protein binds to PL membranes via the cationic portion of its fifth SCR domain. Domains III and IV are heavily glycosylated and therefore partially shielded from protein-protein interactions and may therefore be regarded as linking domains for the N-terminal domains that are exposed most in the blood (Bouma et al., 1999).

Controversial results have been reported regarding the aPL-specific epitopes of β_2GPI that are crucial in the development of APS. Although, short peptides containing sequences from domain V of β_2GPI inhibited binding to human mAbs to β_2GPI, polyclonal antibodies from human patients did not bind these peptides (Wang et al., 1995). Results from other studies using truncated β_2GPI with various domain deletions indicated that the binding epitopes were not on domain V in most cases but on domain IV (George et al., 1998b).

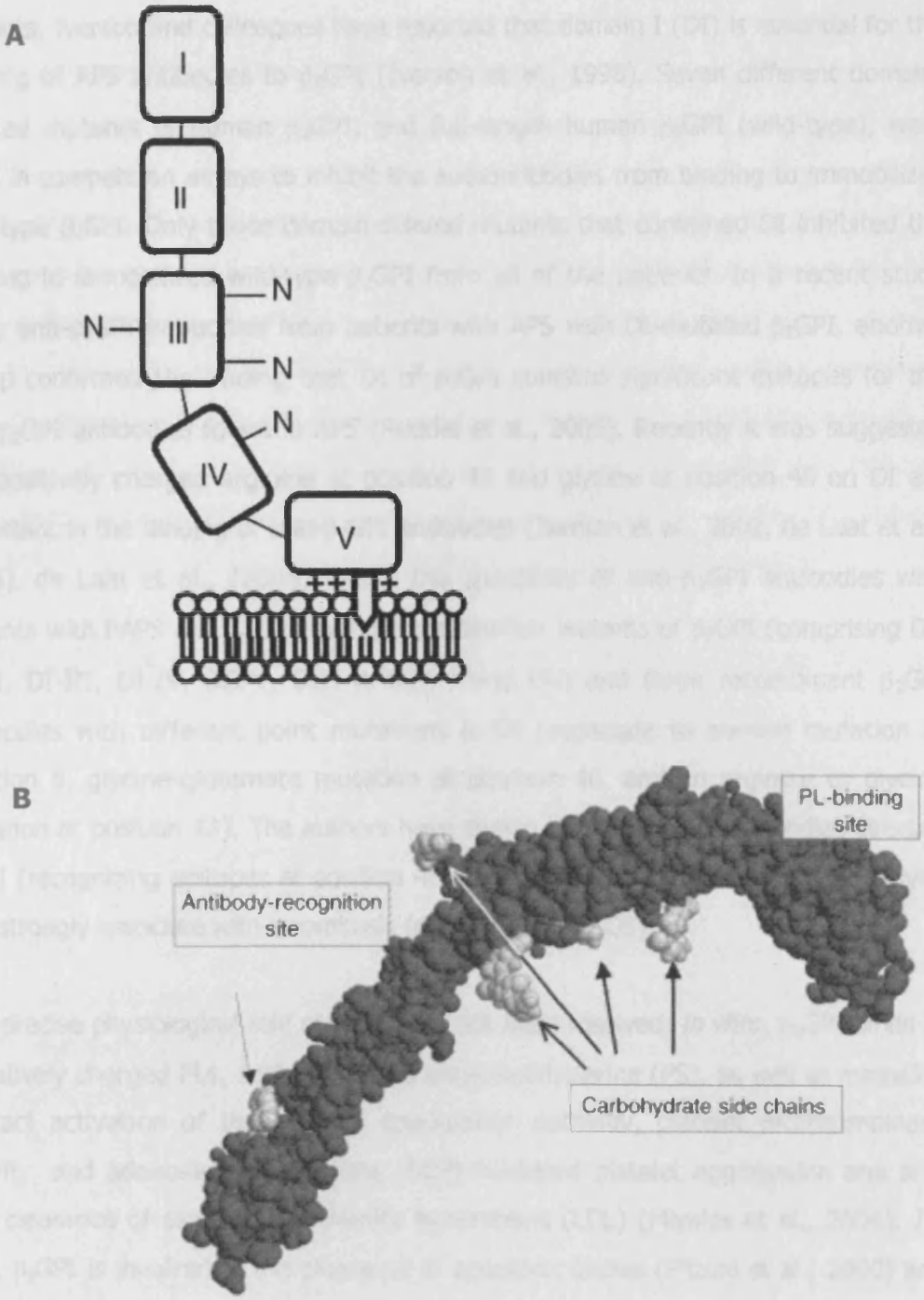


Figure 1.4 Structure of β_2-glycoprotein I

(**A**) Schematic drawing of the domain structure of β_2-glycoprotein I. Domain V is involved in the binding to anionic phospholipids while domain I contains the binding epitope for anti-β_2GPI antibodies. (**B**) Space-filling model of the crystal structure of β_2-glycoprotein I. The antibody recognition site in domain I and the phospholipid-binding site in domain V are indicated. Adapted from (de Groot and Derksen, 2005)

Using anti-β_2GPI antibody from patients with APS and different β_2GPI domain-deleted mutants, Iverson and colleagues have reported that domain I (DI) is essential for the binding of APS antibodies to β_2GPI (Iverson et al., 1998). Seven different domain-deleted mutants of human β_2GPI, and full-length human β_2GPI (wild-type), were used in competition assays to inhibit the autoantibodies from binding to immobilized wild-type β_2GPI. Only those domain-deleted mutants that contained DI inhibited the binding to immobilized wild-type β_2GPI from all of the patients. In a recent study using anti-β_2GPI antibodies from patients with APS with DI-mutated β_2GPI, another group confirmed the finding that DI of β_2GPI contains significant epitopes for the anti-β_2GPI antibodies found in APS (Reddel et al., 2000). Recently it was suggested the positively charged arginine at position 43 and glycine at position 40 on DI are important in the binding of anti-β_2GPI antibodies (Iverson et al., 2002, de Laat et al., 2006). de Laat et al., (2005) tested the specificity of anti-β_2GPI antibodies with patients with PAPS and SLE by using eight deletion mutants of β_2GPI (comprising DI, DI-II, DI-III, DI-IV, DII-V, DIII-V, DIV-V and DV) and three recombinant β_2GPI molecules with different point mutations in DI (aspartate to alanine mutation at position 8, glycine-glutamate mutation at position 40, and an arginine to glycine mutation at position 43). The authors have shown that anti-β_2GPI antibodies directed at DI (recognizing epitopes at position 40 and 43) cause lupus anticoagulant activity and strongly associate with thrombosis (de Laat et al., 2005).

The precise physiological role of β_2GPI has not been resolved; *in vitro*, β_2GPI binds to negatively charged PLs, such as CL and phosphatidylserine (PS), as well as inhibiting contact activation of the intrinsic coagulation pathway, platelet prothrombinase activity, and adenosine diphosphate (ADP)-mediated platelet aggregation and also aids clearance of oxidized low-density lipoproteins (LDL) (Miyakis et al., 2004). *In-vivo*, β_2GPI is involved in the clearance of apoptotic bodies (Pittoni et al., 2000) and liposomes as well as interfering with the protein C pathway (Roubey, 1996). β_2GPI adheres to resting human ECs. β_2GPI, dimerised after binding of circulating aPL, has an increased affinity for cells, such as platelets, ECs and monocytes leading to their activation (Del Papa et al., 1997). In addition, β_2GPI appears to promote clearance of certain products of oxidation and prevent thrombus formation (review in (Inanc et al., 1997)). Therefore, binding of aPL to epitopes on β_2GPI may interfere with these functions, and therefore promote thrombosis.

1.4 The generation of diversity in immunoglobulins

1.4.1 Organisation of immunoglobulin genes

The structure of antibodies has been discussed in section 1.1.3.1. It has been estimated that the mammalian immune system can generate more than 10^{11} different antibodies and is capable of specifically recognising and responding to a limitless array of foreign antigens (Berek and Milstein, 1988). For several decades, immunologists sought to visualise a genetic mechanism that might generate such diversity. One of the theories that emerged is the germline theory, which maintained that the genome contributed by the germ cells contains a large repertoire of Ig genes. In contrast, the somatic-mutation theory maintained that the genome contains a small number of Ig genes, from which a large number of antibody specificities are generated in the somatic cells by mutation or recombination.

Dreyer and Bennett suggested another model which combined elements of the two theories; that two separate genes encode a single Ig H or L chain, one gene for the V region and one for the C region (Dreyer and Bennett, 1965). Moreover, they proposed that a large number of V region genes were carried in the germline, whereas only single copies of C region class and subclass genes exist. Although Dreyer and Bennett provided a good theoretical model, further validation of their hypothesis has been achieved with major technological advances in molecular biology with the use of restriction enzyme analysis and Southern blotting. The first direct evidence was provided by the work of Hozumi and Tonegawa in 1976. Chromosomal DNA from embryonic cells and adult myeloma cells was first cut with a restriction enzyme, and the DNA fragments containing particular V and C region sequences were identified by hybridization with radiolabeled DNA probes specific for the relevant DNA sequences (Hozumi and Tonegawa, 1976). What their experiments showed is that in germline DNA (in embryonic cells), the V and C region sequences identified by the probes are on separate DNA fragments. However, in DNA from an antibody-producing B cell these V and C region sequences are on the same DNA fragment, showing that a rearrangement of the DNA has occurred (Hozumi and Tonegawa, 1976). These results are important since they show that the Ig genes are rearranged in cells of the B-lymphocyte lineage, but not in other cells.

Subsequently, cloning and sequencing of the L and H chain DNA was accomplished and showed that the κ and λ L chains and the H chains are encoded by separate

multigene families each containing several gene segments. Tonegawa et al., (1978) cloned the germline DNA for the mouse V_λ and determined its complete nucleotide sequence. The λ V region is encoded by two fused gene segments in the order V-J (V–joining) (Tonegawa et al., 1978). Furthermore, Sakano and colleagues determined the entire nucleotide sequence for mouse Ig κ chain gene and as in the λ multigene family, the V_κ and J_κ gene segments encode the V region of the κ L chain (Sakano et al., 1979).

The organisation of the Ig H chain genes is similar but more complex. Schilling et al., (1980) determined the complete V region sequences from ten antibodies and two myeloma proteins binding alpha-1,3 dextran and found that the V_H gene segment encodes amino acids 1 to 94 and the J_H gene segment was found to encode amino acids 99 to 107. The most striking feature of their data is the extensive sequence variability of a region that corresponded to amino acids 95 to 97 of the H chain, which they denoted the D (diversity) segment (Schilling et al., 1980). From these results, the authors proposed that a third germline gene segment is located at the junction between the V and J segments in the centre of the third hypervariable region and must join the V_H and J_H gene segments to encode the entire V region of the H chain. The existence of D_H gene segments was later confirmed by Kurosawa and Tonegawa (1982). They located the D gene segments within mouse germline DNA with a cDNA D-region probe, which hybridized with a stretch of DNA lying between the V_H and J_H gene segments (Kurosawa and Tonegawa, 1982).

DNA sequencing studies revealed the presence of conserved sequences flanking each germline V, D, and J gene segment. The conserved heptamer/nonamer sequences have been termed recombination signal sequences (RSSs) and function as signals for the recombination process that rearranges the genes. These conserved palindromic heptamers (CACTGTG) and conserved nonamer sequences (GGTTTTTGT) are located 3′ to each V gene segment, 5′ to each J gene segment, and on both sides of each D gene segment and are separated by an intervening sequence of 12 or 23 bp. Early and colleagues observed that these intervening 12 and 23 bp sequences correspond, respectively, to one and two turns of the DNA helix (one-turn recombination signal sequences and two-turn signal sequences) (Early et al., 1980). Signal sequences having a one-turn spacer can only join with sequences having a two-turn spacer (the one-turn/two-turn joining rule). This ensures that a V_H, D_H, J_H segments join in the

proper order and that segments of the same type do not join together (for example a V_H segment does not join to another V_H segment).

This work was all carried out in mice. Matthyssens and Rabbits cloned the first human V_H gene, which has been isolated from human fetal liver DNA by using a cDNA plasmid probe containing a mouse V_H sequence (Matthyssens and Rabbitts, 1980). The human J_H and the first D_H genes were subsequently cloned and sequenced (Ravetch et al., 1981, Siebenlist et al., 1981). When the V_κ (Bentley and Rabbits, 1980), V_λ (Brockly et al., 1989), J_κ (Hieter et al., 1980) and J_λ (Vasicek and Leder, 1990) genes were also cloned, it was found that the same principle applied to human gene segments as well as mice, confirming the presence of RSSs and the one-turn/two-turn joining rule.

1.4.2 The mechanism of rearrangement of immunoglobulin genes

The recombination of V region gene segments, is a site-specific recombination process that occurs only in developing lymphocytes and only in between Ig and TCR gene segments flanked by RSSs (Bassing et al., 2002). The complex of enzymes that act synergistically to mediate V(D)J recombination is termed the V(D)J recombinase. The products of the two genes RAG-1 and RAG-2 (recombination-activating genes) comprise the lymphoid-specific components of the recombinase.

V(D)J recombination is a multistep enzymatic process in which the first reaction is recognition of RSSs by recombinase enzymes and alignment of two signal sequences together with the adjacent coding sequence (Schatz, 2004). RAG-1 and RAG-2 make two single-strand DNA breaks at sites just 5′ of each RSS, leaving a free 3′ -OH group at the end of each coding segment. This 3′ -OH group then hydrolyzes the phosphodiester bond on the other strand, sealing the end of the dsDNA to create a 'hairpin' structure at the cut end of the coding sequence and a flush, 5′ –phosphorylated, double-strand break at the signal sequence (Janeway et al., 2001). The DNA hairpin is nicked open by a single-stranded break at various points for the addition of P-region nucleotides, followed by the trimming of a few nucleotides from the coding sequence by a single-strand endonuclease. Terminal deoxynucleotidyl transferase (TdT) allows the addition of N-region nucleotides. Double-strand break repair (DSBR) enzymes then catalyse the ligation of the coding sequences with each other and the signal sequences with each other (Goldsby et al., 2000).

Although the double-strand DNA breaks that initiate V(D)J rearrangement are introduced precisely at the junctions of signal sequences and coding sequences, there is variation in the enzymatic processes in the subsequent joining of the coding sequences (Schatz et al., 1992). This imprecision in the joining process seems to create diversity by contributing to the hypervariability of the antigen-binding site. Another consequence of imprecise joining is nonproductive rearrangement, where the resulting VJ or VDJ unit will lose or gain nucleotides at the junctions such that the triplet reading frame for translation is interrupted (Schatz et al., 1992). When the gene segments are joined in phase, the reading frame is maintained. This generates a productive rearrangement with the resulting VJ or VDJ unit translated in its entirety, yielding a complete antibody. If one allele rearranges non-productively, a B cell may still be able to rearrange the other allele productively.

B cells, like all somatic cells, are diploid and contain both maternal and paternal chromosomes (Albert et al., 2002). In fact, however, each B cell expresses the rearranged H chain genes from only one chromosome and the rearranged L chain genes from only one chromosome. This process, termed allelic exclusion is essential for the antigenic specificity of the B cell since it ensures that functional B cells never contain more than one $V_HD_HJ_H$ and one V_LJ_L unit and accounts for their monospecificity (Mostoslavsky et al., 2004). It is apparent that once a productive $V_HD_HJ_H$ rearrangement and a productive V_LJ_L rearrangement have occurred, recombination stops, so that the H and L chain genes on the homologous chromosomes are not expressed.

A model proposed to explain allelic exclusion suggests that once a productive rearrangement is attained, its encoded protein is expressed and its presence acts as a signal to prevent further gene rearrangement (Yancopoulos and Alt, 1986). According to this model, the presence of μ H chains signals the maturing B cell to turn off rearrangement of the other H chain allele and to turn on rearrangement of the κ L chain genes. If a productive κ rearrangement occurs, κ L chains are produced and then pair with μ H chains to form a complete antibody molecule. The presence of this antibody then turns off further L chain rearrangement. If κ rearrangement is nonproductive for both κ alleles, rearrangement of the λ chain genes begins. If neither λ allele rearranges productively, the B cell ceases to mature and dies by apoptosis.

However, Ig expression sometimes fails to prevent further rearrangement. Some B cells that have made productive H and L chain rearrangements will nevertheless rearrange a second L chain locus (Prak and Weigert, 1995). This process is called receptor editing and allows a B cell to change its receptor specificity. Prak and Weigert, (1995) described a mouse model which recreates a functional rearranged κ locus by replacing the unrearranged J_κ region with a rearranged V_κ-J_κ gene. This L chain replacement (V_κR) simulates the genotype of a normal B cell with a functional V_κ-J_κ L chain gene on one allele. The V_κR models illustrate that a productively rearranged L chain can either terminate further rearrangement or allow further rearrangement.

1.4.3 Generation of antibody diversity

Antibody diversity is generated in four main ways. Two of these are consequences of the recombination process described above. The third is due to the different possible combinations of a H and a L chain in the complete Ig molecule. The fourth is a mutational process that occurs in mature B cells, acting only on rearranged DNA encoding the V regions.

The gene rearrangement that combines gene segments to form a complete V region generates diversity in two ways (Maizels, 2005). First, there are multiple different copies of each type of gene segment. Human germline DNA contains 44 V_H (Matsuda et al., 1998), 27 D (Corbett et al., 1997), 6 J_H (Ravetch et al., 1981), 40 V_κ (Schable and Zachau, 1993), 5 J_κ, 30 V_λ (Williams et al., 1996) and 4 J_λ gene segments (these numbers apply to functional genes, discussed in more detail in section 1.4.5). Different combinations of gene segments can be used in different rearrangement events, however, not all gene segments are used at the same frequency and some are more common than others. This combinatorial V(D)J diversity is responsible for a substantial part of the diversity of the H and L chain V regions.

Second, junctional flexibility is introduced at the joints between the different gene segments. As described above, recombination involves both the joining of RSSs to form a coding joint and the joining of coding sequences to form a coding joint. Although the signal sequences are always joined precisely, joining of the coding sequences is often imprecise. Junctional flexibility leads to many nonproductive rearrangements, but it also several produces productive combinations that encode

alternative amino acids at each coding joint. The third hypervariable region (CDR3) falls between the V-D and the D-J joints. In both H and L chains, the diversity of CDR3 is significantly increased by the addition (P-addition and N-addition) and deletion of nucleotides in the formation of the junctions between gene segments. Since CDR3 makes a major contribution to antigen binding by the antibody molecule, amino acid changes generated by junctional flexibility can make a major contribution to antibody diversity.

A third source of diversity is also combinatorial, arising from the many possible different combinations of H and L chain V regions that pair to form the antigen-binding site in the Ig molecule. Although it is difficult to calculate with precision the number of different antibody combining sites the immune system can generate, the number is very large. Nevertheless, it is not likely that all V_H and V_L will pair with each other. Finally, somatic hypermutation (discussed in more detail in the next section) introduces point mutations into the rearranged V region genes, creating further diversity that can be selected for enhanced binding to antigen.

1.4.4 Somatic hypermutation

All of the antibody diversity mechanisms discussed so far occur during the formation of V regions by gene rearrangement in the pre-B cell (early B cell precursor that does not express functional membrane-bound Ig and is therefore not sensitive to antigen). Following antigen exposure, activated B cells migrate into the primary follicles of peripheral lymphoid organs where they undergo intense proliferation and form germinal centres, which appear towards the end of the first week after exposure and provide the location for both hypermutation and antigen selection (Neuberger and Milstein, 1995). Somatic hypermutation occurs only within germinal centers and is targeted to rearranged V regions located within a DNA sequence which includes the whole of the VJ or VDJ segment (Wagner and Neuberger, 1996). Somatic hypermutation occurs at a frequency approaching 10^{-3} per base pair per generation, a rate which is a hundred thousand-fold higher that the spontaneous mutation rate in other genes and is unique to B cells (Neuberger and Milstein, 1995).

The mechanism of somatic hypermutation is poorly defined. The mutations introduced are predominantly single nucleotide substitutions (rather than insertions or deletions). The base changes are distributed throughout the V region, but not completely randomly; there are certain 'hotspots' of mutation that indicate a

preference for characteristic short motifs (A/G-G-C/T) (Rogozin and Kolchanov, 1992). Gearhart and Bogenhagen (1983) examined six rearranged $V_{\kappa}167$ genes from hybridoma and myeloma cells. The sequences were compared to the germline sequence of the $V_{\kappa}167$ gene, the J_{κ} genes, and the C_{κ} gene to identify sites of mutation. Four of six rearranged genes had extensive mutation which occurred exclusively in a region of DNA centered around the V-J gene. Mutations were mostly due to nucleotide substitutions and their location around each gene indicates that they occur in clusters at random sites (Gearhart and Bogenhagen, 1983).

Experimental evidence demonstrating the importance of somatic hypermutation was obtained from a series of experiments on the response to the phOx hapten (2-phenyl-5-oxazolone) in mice by Berek and Milstein, (1988). They coupled the hapten to a protein carrier, immunised mice with the phOx-carrier and used the mouse spleen cells to prepare hybridomas secreting mAbs specific for the phOx hapten. The 12 hybridomas obtained from mice seven days after a primary immunisation, had very similar sequences to the mouse germline V genes. However, 14 days after primary immunisation, all of the hybridomas included one or more mutations from the germline sequence. The number of mutations in the anti-phOx hybridomas progressively increased following primary, secondary, and tertiary immunisations, as did the overall affinity of the antibodies for phOx (Berek and Milstein, 1988).

The process of somatic hypermutation generates antibodies with varying affinity for antigen. Following exposure to antigen, those B cells with higher affinity receptors will be preferentially selected for survival because of their greater ability to bind to the antigen, a process called affinity maturation which takes place within germinal centers. Mutations at some positions in the sequence will either adversely affect the basic structure of the antibody molecule or reduce its affinity for antigen. B cells expressing antibodies with the highest affinity for the driving antigen are likely to become initially the dominating population. These cells consequently proliferate faster thus eventually dominating the germinal centre. This is termed antigen-driven clonal expansion (Berek and Milstein, 1987). Schlomchik et al., (1987) studied a panel of IgG rheumatoid factor autoantibodies derived from the autoimmune MRL/*lpr* mouse and provided evidence for antigen-driven clonal expansion. Sequence analysis showed that these antibodies were derived from a small number of expanded clones. Clonally related antibodies were recognised by possession of identical VDJ joint sequences. The authors noted that a high replacement (alters amino acid sequence

of antibody) to silent (does not alter amino acid sequence of antibody) mutation ratio occurs in the CDRs but not the FRs. Since the V region CDRs mainly determine the structure of the antigen-binding site, replacement mutations in CDRs are more likely to alter antigen binding properties indicating that the development of a particular antibody sequence was antigen-driven (Shlomchik et al., 1987).

Statistical analysis can be applied to the distribution of replacement and silent mutations in an antibody sequence to determine whether this pattern of somatic mutation is a consequence of antigen-drive. Chang and colleagues performed an evaluation using a binomial distribution model equation (Chang and Casali, 1994). The binomial method involves the use of a mathematical formula for calculating the probability that a particular pattern of mutations (in the Ig V gene CDRs or FRs) could have developed by chance only, in the absence of antigen drive (Chang and Casali, 1994). However, this model is inappropriate for Ig genes in which mutations have four different distribution possibilities (replacement and silent mutations in the CDR and/or FW regions of the gene). Lossos and colleagues proposed a multinomial distribution model for assessment of antigen selection (Lossos et al., 2000). Side-by-side application of multinomial and binomial models on 86 previously established Ig sequences disclosed 8 discrepancies, leading to opposite statistical conclusions about antigen selection. Therefore, the use of the multinomial model is thought to be more accurate (Lossos et al., 2000).

After antigenic stimulation of a B cell, the H chain DNA can undergo a further rearrangement in which the $V_HD_HJ_H$ unit can combine with any C_H gene segment, a process called class switching. It is a DNA recombination reaction specific to the Ig H chain (IgH) locus that increases antibody versatility by associating a given IgH V region with one of several different C regions. The $C\mu$ region is replaced by downstream $C\gamma$, $C\varepsilon$, or $C\alpha$ segments to generate IgG, IgE, and IgA antibodies, respectively. There seems to be a link between hypermutation and class switiching since they occur at a similar point in cell development and IgG antibodies tend to carry more somatic mutations than IgM of the same specificity (Tillman et al., 1992). Furthermore, Huang and Stollar (1993) carried out study to determine whether similar Ig gene segment usage occurs in normal human adult peripheral blood lymphocytes. They found that 48 of 77 IgM clones (62%) shared 99% or more identity with known germline V_H segments, and most of these had no mutations in the CDR3 portion of the J_H segment. In contrast, only two of 28 rearranged V_H genes

from the IgG library showed this degree of homology. Therefore, they concluded that IgG-encoding sequences had more mutations than IgM-encoding sequences (Huang and Stollar, 1993).

It has recently been discovered that deficiency in the enzyme called activation induced cytidine deaminase, blocks the accumulation of somatic hypermutations (Selsing, 2006, Nussenzweig and Alt, 2004). The mechanism by which this enzyme contributes to hypermutation is unknown. Interestingly, deficiency of this enzyme also abrogates the rearrangement of C-region genes that underlies the Ig class switching seen in activated B cells.

A key research question which I have attempted to answer in this thesis is: do the somatic mutations that accumulate in the CDRs of rearranged Ig sequences account for changes in the binding properties of antibodies? The binding properties of antibodies depend on the sequences of their V regions, particularly the CDRs. Antibodies with pathogenic properties may have a tendency to utilize the same V_H or V_L genes or develop the same somatic mutations under the influence of the same antigen. For this reason, it is crucial to have full understanding of the germline human V region repertoire.

1.4.5 The repertoire of human variable region genes

To understand the origin and behaviour of the human immune repertoire it is important to have the complete structure of the human V_κ, V_λ and V_H locus. In humans, the Ig molecule is encoded by three independent gene loci, namely Ig_κ and Ig_λ genes for the L chain and IgH genes for the H chain, which are located on chromosome 2 (Malcolm et al., 1982, McBride et al., 1982), chromosome 22 (McBride et al., 1982, Erikson et al., 1981), and chromosome 14 (Croce et al., 1979), respectively.

1.4.5.1 Human V_H locus

A complete map of the functional V_H locus at chromosome 14q32.3 has been produced (Matsuda et al., 1993). The complete nucleotide sequence of the human Ig V_H region locus is 957-kb in length. The region contains 123 V_H segments classifiable into seven different families, of which 79 are pseudogenes. A V_H segment is defined as functional if it contains an open reading frame (ORF), an intact exon-intron

structure and is arranged in a functional Ig gene (functional VDJ rearrangement). Pseudogenes are nucleotide sequences that are stable components of the genome but are incapable of being expressed. Pseudogenes are thought to have been derived by mutation of active ancestral genes (Goldsby et al., 2000). Of the 44 V_H segments with an ORF, 39 are expressed as H chain proteins and 1 as mRNA, while the remaining 4 are not found in Ig cDNAs (Matsuda et al., 1998). Eight V_H were identified on chromosome 15 and sixteen on chromosome 16, however these are unlikely to be functional since chromosomes 15 and 16 have no D, J or C segments (Cook and Tomlinson, 1995).

Human V_H segments can be classified into seven families, V_H1 - V_H7 on the basis of sequence homology, which are interspersed throughout the locus. Different members of the same family are at least 80% homologous at the nucleotide sequence level and some families are more highly related (V_H4) than others, which are more divergent (V_H5). In terms of the number of functional V_H segments, the V_H3 family makes the largest contribution to the functional repertoire, followed by the V_H1 and V_H4 families (Cook and Tomlinson, 1995).

The exact dimensions of the V_H locus and number of functional segments vary between individuals and V_H genes display a certain amount of polymorphism. Under these circumstances, the question arises whether differences between expressed and germline sequences are true somatic mutations or arise because of a difference between alleles of the same gene. Certain genes occur in some people but not in others as a result of insertion/deletion polymorphism with 75% of people having an extra group of genes located between segments 3-30 and 4-31 which are listed in V-BASE, a sequence directory containing the complete human Ig gene repertoire that is constantly being updated with new alleles (Tomlinson et al., 1996). This database of published alleles as well as data from direct sequencing of the same gene in different individuals (Sanz et al., 1989) show that the majority of alleles differ by one or two nucleotides. For this reason, it is logical to assume that most of the differences expressed and germline sequences result from mutation rather than from polymorphism.

1.4.5.2 Human D_H locus

The complete nucleotide sequence of the human IgD segment locus on chromosome 14q32.3 was determined and a total of 27 D segments were identified (Corbett et al., 1997). These can be grouped into seven families based on sequence homology. Six of these families (DXP, DA, DK, DN, DM and DLR) (Ichihara et al., 1988) have at least four members, whilst the seventh is the unique DHQ52 segment. Nucleotide sequences of D_H genes belonging to different families diverge greatly, while those belonging to the same families are well conserved. Other than these typical D_H genes, however, Ichihara et al., (1988) described another D_H gene (which they termed DIR) the spacer lengths of whose neighbouring signals were irregular. The DIR gene appeared to be involved in DIR-D_H or D_H-DIR joining by inversion or deletion. Nevertheless, V_HCDR3 vary greatly due to versatility of the D_H segments. Various other hypotheses had been proposed to explain this extensive versatility such as the DD recombination (regardless of the 12/23 rule) (Sanz, 1991), inverted D segments, or the use of minor D segments found on chromosome 15 (Cook and Tomlinson, 1995).

However, a systematic analysis which compared the complete sequence of the human Ig D segment locus with a database of rearranged sequences to identify all the germline D segments and any other sequences that contribute to the extreme diversity of V_HCDR3 showed no evidence to suggest that any of the above hypotheses took place (Corbett et al., 1997). In previous studies, the criteria for assigning V_HCDR3 sequences to their germline D segments appear to have been more lenient. For example, others have taken five nucleotides of identity or six nucleotides with one mismatch as their minimum cut-off for matching an expressed sequence to a germline D gene (Brezinschek et al., 1995). Corbett et al., (1997), however, applied more stringent criteria whereby at least ten consecutive nucleotides of identity were generally required to confidently assign a D segment to its germline match, with the precise threshold depending on the CDR3 length.

1.4.5.3 Human J_H locus

The human J_H locus contains nine J-like gene segments, six of which appear to be functional (designated J_H1 to J_H6) and account for most of the known human H chain amino acid sequences whilst three are pseudogenes (Ravetch et al., 1981).

1.4.5.4 Human kappa (κ) locus

The human κ L chain locus has been mapped to chromosome 2p11-12 and contains approximately 40 functional V_κ and five J_κ genes with no D gene segments (Schable and Zachau, 1993). The total number of V_κ genes is 76. The 40 functional V_κ genes are arranged into seven families, two of which are very small (V_κIV and V_κVII) and are interspersed throughout the locus amongst 36 pseudogenes. Additional V_κ genes are located upon chromosomes 1 and 22 although these are pseudogenes (Schable and Zachau, 1993). Allelic polymorphism is rare and where present, alleles differ by only one or two nucleotides (Tomlinson et al., 1996). There is only one human C_κ gene segment (Hieter et al., 1980) therefore no alternative isotypes of the κ chain exist.

1.4.5.5 Human lambda (λ) locus

The human λ locus lies on chromosome 22q11.2. There are 30 functional V_λ (Williams et al., 1996) and four functional J_λ genes (Vasicek and Leder, 1990). Ten V_λ families exist, spread amongst three distinct V_λ gene clusters. The V_λ 1, 2 and 3 families comprise more than half of all the V_λ genes and are more commonly expressed in humans. Once again, allelic polymorphism is relatively rare and where present, alleles differ by only one or two nucleotides just as in the human V_H and V_κ loci (Williams et al., 1996).

The J_λ and C_λ loci are also located upon chromosome 22. There are seven C regions however C_λ 1, 2, 3, and 7 are active genes while C_λ 4, 5, and 6 are pseudogenes. They are organized in a tandem array, and each is preceded by a single J_λ region of which J_λ2 and J_λ3 are identical. As a result, mAb sequences can include only one of three different J_λ sequences (Vasicek and Leder, 1990). The presence of different C_λ genes means λ chains like H chains can exist as one of several isotypes.

1.4.5.6 How is the repertoire defined in the expressed monoclonal antibodies?

The recognition that certain V_H, V_λ, and V_κ genes and families are preferentially rearranged is important in interpreting sequences of human mAbs specific for a particular antigen. Certain genes might be rearranged more often than others and some genes may be present in several copies (Dorner and Lipsky, 2001).

It has been demonstrated using in situ hybridization studies that overexpression of V_H3 genes persists in the adult (Zouali, 1992). Huang and Stollar, (1993) made an IgG H chain cDNA library from peripheral blood lymphocytes (PBL) of healthy adults and have shown that there was preferential use of certain genes within the V_H3 family, particularly V3-23 and V3-30. Furthermore, Brezinschek et al., (1997) have amplified genomic DNA from peripheral B cells expressing surface IgM using using a single cell PCR technique that permits the amplification of both productive and nonproductive V_H rearrangements. They showed that genes from the V_H3 family were more likely to be used in productive rearrangements, with V_H-23 being the most commonly rearranged gene accounting for 13% of all productive rearrangements.

Cox et al., (1994) sequenced 37 human germline V_κ segments from the genomic DNA of a single individual and compiled a comprehensive directory of all germline V_κ segments. Comparison with 236 rearranged sequences revealed about 11 sequences (O2, O8, L8, L12 and A20 from the V_κI family, A3 and A17 from the V_κII, L2, L6 and A27 from the V_κIII, and B3 from the V_κIV) were used frequently and accounted for 90% of the expressed repertoire. These results suggest that the expressed V_κ repertoire is mainly derived from a limited number of segments.

The expressed human Ig V_λ repertoire also demonstrates a strong bias in the use of individual V_λ segments. By hybridisation of 7600 cDNA clones from peripheral blood lymphocytes Ignatovich et al., (1997) have shown that the use of V_λ families is heavily biased in the expressed repertoire. Indeed, of the ten families, only three ($V_\lambda1$, $V_\lambda2$ and $V_\lambda3$) are used to any significant extent. Furthermore, by sequencing 532 λ cDNA clones they have now shown that this bias in family use is mainly due to biases in the use of individual V_λ segments. Indeed, of the 30 functional segments three (2a2, 1e and 2c) encode half the expressed V_λ repertoire (Ignatovich et al., 1997).

1.5 Sequence Analysis of Human Monoclonal Anti-DNA and Antiphospholipid Antibodies

The antigen-binding site of an antibody is determined by the V region of the H chain and the L chain. Within V_H and V_L, the CDRs show particularly high variability of

sequence and have been shown by crystallography to contain most of the sites where antigen and antibody make contact. Sequence changes which affect the CDRs are therefore particularly important in determining affinity and specificity of antigen-antibody interactions. The aim of sequence analysis is to identify sequence features that are found commonly in anti-DNA antibodies or aPL which can cause disease, but rarely in antibodies which are not associated with disease. Sequence analysis studies that differentiate these two groups, may provide some evidence of links between sequence and pathogenicity,

1.5.1 Sequence analysis of murine anti-DNA antibodies

The sequences of over 300 murine anti-DNA mAbs have been published. The antibodies were derived from two types of SLE murine models. The first are autoimmune strains such as MRL/*lpr* and (NZB/W)F_1 that spontaneously develop autoimmune disease closely paralleling the symptoms of human SLE. The MRL/*lpr* mice display the most dramatic symptoms, developing widespread lympadenopathy, a range of autoantibodies (including anti-dsDNA and aPL) and glomerulonephritis. (NZB/W)F_1 mice develop a lupus-like illness characterised by the presence of autoantibodies and glomerulonephritis. The second type of SLE murine models are normal mice that have experimentally induced autoimmune disease similar to human SLE through immunisation with proteins/DNA complexes or antibodies some of which carry public idiotypes associated with autoantibodies (Radic and Weigert, 1994). Of the 283 V_H sequences studied, 183 belong to the J558 V_H gene family, 61 to the 7183 V_H gene family and 39 are members of other families (Radic and Weigert, 1994). V_L gene use in anti-DNA antibodies is more diverse that V_H gene use. Most notable among the recurrent L chains are 8A and 8B, both derived from V_κ genes (Radic and Weigert, 1994).

Sequence analysis of anti-DNA antibodies from various murine models has led to the identification of recurrent characteristics amongst them. Firstly, it has highlighted the importance of clonal expansion and antigen driven accumulation of somatic mutations in determining the ability of these antibodies to bind dsDNA. Secondly, sequence analysis of these murine anti-DNA antibodies has led to the assumption that certain amino acids are present in the CDRs of anti-DNA antibodies at a higher frequency than in antibodies with other antigen specificities. Marion and colleagues analysed the sequences of 117 monoclonal anti-DNA antibodies from (NZB/W)F_1 mice (Marion et al., 1992). In some cases, IgG produced by a single mouse were

derived from the same expanded B cell clone whereas the majority of IgM were not. Within a single clone, the IgG carried more mutations than the IgM, particularly in the CDRs. An increase in the number of mutations led to increased affinity for dsDNA. The mutations that led to high avidity binding to dsDNA were those that increased the number of arginine residues in the CDRs, especially V_HCDR3 (Marion et al., 1992). Radic et al., (1994) also re-iterated that the accumulation of somatic mutations has led to higher frequencies of certain basic residues, including arginine, asparagine and lysine being present at various positions in the CDRs of murine anti-DNA mAbs. Arginine and lysine are positively charged and it has been suggested that these amino acids could therefore interact with the anionic sugar-phosphate backbone of the DNA double helix. The presence of uncharged asparagine residues also appears to enhance binding to DNA by forming hydrogen bonds with adenine in the major groove and guanine in the minor groove (Seeman et al., 1976).

1.5.2 Sequence analysis of murine aPL

In the case of aPL produced from animal models, the animals show consistent serological features of the syndrome, with either the obstetric or thrombotic clinical features but not both (Radway-Bright et al., 1999). MRL/*lpr* mice frequently produce aPL. Kita and colleagues analyzed the V_H and V_κ genes of aPL from MRL/*lpr* mice. Sequence analysis showed that different antibodies from a single mouse tended to be clonally related. 67% used the V_H gene of the J558 family, and 43% used the V_κ gene of the V_κ 23 group (Kita et al., 1993). The same authors analysed the usage of V_H and V_κ genes of aPL in (NZW x BXSB)F_1 mice. The (NZW x BXSB)F_1 mouse may be a better autoimmune model of APS since both male and female mice produce serum aPL of IgG isotype and enhanced binding to CL in the presence of β_2GPI. Also, male mice develop thrombocytopenia and vascular disease, features characteristic of human APS. Sequence analysis of V_H and V_κ genes of aPL showed that the "pathogenic" aPL, which induced thrombosis when injected into mice, had V_H J558 and V_κ 21 or V_κ 23 genes, whereas the other "non-pathogenic" antibodies used mainly the 7183 V_H family and the random V_κ gene group (Kita et al., 1994).

However, the role of gene and family preference in mouse anti-dsDNA and aPL may not be applicable to human antibodies, because the mouse and human Ig gene repertoires are very different (Goldsby et al., 2000). Mice have much larger numbers of V_H and V_κ genes than humans; whereas there are only about 44 functional human

V_H genes, there may be up to 1000 in the mouse. Mice very rarely rearrange V_λ genes; approximately 40% of human antibodies but only 5% of murine antibodies carry λ chains (Goldsby et al., 2000). It is therefore important to study human monoclonal anti-DNA and aPL since murine antibodies are generated from mice that have a disease that is only similar but not identical to human disease.

1.5.3 Sequence analysis of human anti-DNA antibodies

Far fewer human than mouse mAbs have been produced. Whereas laboratory mice can be produced in large numbers to obtain splenocytes for use in the production of mAbs, large numbers of human splenocytes are rarely available. The sequences of 66 human antibodies have been published [reviewed by (Rahman et al., 2002a)]. However, most of these are polyreactive IgM antibodies that bind DNA with low affinity and relatively few human IgG anti-DNA antibodies have been sequenced. Most human IgG anti-DNA antibodies use genes of the V_H3 or V_H4 families and the most commonly utilized gene is V3-23, which is known to be the most commonly rearranged gene in the V_H repertoire (Rahman et al., 2002a).

Amongst the 66 antibodies human anti-DNA antibodies reviewed by Rahman et al., (2002), 52 have known L chain sequences (34 have κ chains and 18 have lambda chains). Certain κ genes (including A27, O2/O12, L2, L12, L6, B3, O8/O18 and A30) and λ genes (such as 2a2, 2b2, 2c, 1b, 1e, 1c and 3h) are used more frequently than other L chain genes. A27 is the most commonly rearranged gene in the V_κ repertoire according to the results of Cox and colleagues (Cox et al., 1994). A single V_λ gene, 2a2, encodes five λ chains shown to occur in IgG anti-DNA antibodies (Rahman et al., 2002). 2a2 is the most commonly used gene in productive λ rearrangements in normal adults (Ignatovich et al., 1997). One of the IgG mAbs studied in this thesis, B3, was derived from an SLE patient and is encoded by the most commonly rearranged human V_H and V_λ genes, V3-23 and 2a2 respectively (Rahman et al., 2001).

1.5.4 Sequence analysis of human aPL

Fifty-one human monoclonal aPL were reviewed by Giles et al., (2006). Twenty-eight IgG aPL were identified. Eleven were derived from patients with SLE (four of whom had APS) and 12 were produced from patients with PAPS. Most of the monoclonal

IgG aPL showed specificity for PL or β_2GPI. The functional activities of 13 IgG monoclonal aPL have been examined in various biological assays and eight were shown to be pathogenic compared to control mAb. When the monoclonal aPL 516 was injected into mice, the mice displayed a significantly higher rate of fetal resorption and a significant reduction in fetal and placental weight (Ikematsu et al., 1998). Similarly, another group has shown that CIC15, an aPL derived a patient with PAPS, induced a significantly higher rate of fetal losses following intravenous injection into pregnant BALB/c mice (Lieby et al., 2004). In addition, five aPL derived from patients with PAPS (IS2, IS3 and IS4) and SLE/APS (CL15 and CL24) were found to be thrombogenic in an *in vivo* model of thrombosis (Pierangeli et al., 2000).

The commonest V_H genes were found to be V3-23, V3-30, V3-07, V4-34, V4-39, V4-59, V1-18, V1-69 and V5-51 encoding 15 of the published IgG aPL. The most commonly used gene amongst IgG aPL is V3-23. All IgG aPL used V_L genes which are members of the three largest and most commonly expressed V_κ (I-III) or V_λ (1-3) families. The most commonly rearranged V_κ genes include A3, A17, A20, 02/12, L8, L12, L6, A27, L2, and B3 while commonly rearranged V_λ genes include 1b, 1c, 1e, 2a2, 2b2, 2c, 3h and 3r.

1.5.5 The importance of somatic hypermutation and charged residues in the CDRs of anti-DNA antibodies and aPL?

Each V region sequence of an antibody can be aligned to the closest germline equivalent in the V-Base directory using DNAplot software. This allows subsequent analysis of nucleotide homology and distribution of somatic mutations. According to the multinomial distribution model for assessment of antigen selection proposed by Lossos et al., (2000), a *P* value of less than 0.05 is evidence for a non-random distribution of mutations. Statistical analysis of IgG anti-DNA antibodies and aPL strongly supports the idea that antigen drive was responsible for the pattern of mutation in the majority of cases (Giles et al., 2006, Rahman et al., 2002a).

The CDRs form most of the contact between antibody and antigen. Comparison of the Ig V region genes expressed in six human hybridomas secreting IgG anti-dsDNA antibodies derived from three SLE patients with known germline genes showed that the V_H and V_L sequences are somatically mutated particularly in the CDRs and the pattern and extent of the observed somatic mutations are suggestive for an antigen-

driven selection (Winkler et al., 1992). MacCallum and colleagues showed that certain residues within CDRs of the H and L chains are more likely than others to contact antigens (MacCallum et al., 1996). Giles et al., (2006) studied the sequences of fifty-one human monoclonal aPL and reported that there is an excess of mutation to arginine, lysine and asparagine in the contact sites of the IgG aPL in comparison to aspartic acid or glutamic acid. IgM aPL also show some evidence of accumulation of arginine, asparagine and lysine at the contact sites. These amino acids are mostly encoded in the germline in IgM and derived from somatic mutation in IgG. Pathogenic aPL have a tendency to bind negatively charged PLs and β_2GPI, which has a potential epitope rich in negatively charged residues within DI. Therefore the presence of arginine, asparagine, and lysine residues in the contact regions of aPL may increase the affinity of binding via electrostatic interactions and hydrogen bonds with negatively charged epitopes on PLs and DI of β_2GPI.

This finding also holds true for anti-DNA antibodies. Sequence analysis of murine and human anti-DNA antibodies has shown that somatic mutation leads to an increase of the amino acid residues arginine, asparagine and lysine (discussed in sections 1.5.1 and 1.5.4). Rahman et al., (2002) by systematic analysis of all published sequences of anti-DNA antibodies to date also confirmed a statistically significant accumulation of somatic mutations encoding these amino acids, particularly in the CDR1 and CDR2 areas of the IgG H and κ L chains of human IgG anti-DNA antibodies. It has been postulated that the presence of positively charged residues might enhance binding to DNA, which carries an array of negative charges. Somatic mutations to arginine are a common feature of high-affinity, anti-dsDNA antibodies and have been predicted to be particularly important in the binding to DNA (Radic et al., 1993).

It is important to note that not all antibodies with arginine residues in their CDRs bind dsDNA since it is the actual positions of the arginine residues within the V region of the H and H chains that determine the binding affinity of the antibody. The results obtained by Stevenson et al., (1993) using two antibodies RT-79 and D5 derived from the gene, V_H4-34, support this argument. The V_H CDR3 sequences for both the antibodies had a predominance of basic amino acids, with RT-79 having five and D-5 two arginine residues respectively. Despite the fact that RT-79 contains five arginine residues in V_H CDR3, it only binds ssDNA. Conversely, D5 contains only two, but binds dsDNA with high affinity (Stevenson et al., 1993). Arginine residues that

have arisen as a result of somatic mutation are present in human IgG anti-DNA antibody, B3 and aPL, IS4 which were studied in this thesis.

1.6 Three-dimensional images of anti-DNA and antiphospholipid antibodies

The importance of individual amino acid residues in determining the binding affinity of an antibody cannot be determined by sequence analysis. For this reason, various groups have produced three-dimensional images of antibodies interacting with their antigen, using X-ray crystallography or computer-generated modelling, to predict the specific amino acid residues in the antigen-binding site of individual antibodies that are most likely to make contact with the antigen. Crystal structures and computer models enable hypotheses to be proposed and it is necessary to test these hypotheses by expressing mutated forms of the Ig cDNA to create slightly altered molecules and examining the effects upon their ability to bind antigen.

1.6.1 X-ray crystallography of anti-DNA antibodies

Few crystal structures of anti-DNA antibodies have been published, because of difficulties in purifying sufficient quantities of a single mAb for X-ray crystallography analysis (Kalsi and Sutton, 1997). Co-crystals show the precise locations at which amino acid residues of the antibody make contacts with the antigen as well as changes in conformation of an antibody on binding to an antigen. The only published co-crystal involving an anti-DNA antibody, has been made by Herron et al., (1991) of an autoantibody BV04-01, with specificity for ssDNA in the presence and absence of a trinucleotide of deoxythymidylic acid, d(pT)3. When complexed with $d(pT)_3$, BV04-01 undergoes conformational changes in order to incorporate the trinucleotide. The co-crystal also showed that the trinucleotide is held in a large irregular groove between the V_H and V_L regions on the surface of the antibody and that all three CDRs of the H and L chains contribute to this binding site (Herron et al., 1991). An arginine residue, which was present in V_HCDR2 at position 52, was seen to form an electrostatic contact with the phosphodiester backbone of the trinucleotide. Despite the fact that other arginine and lysine residues were present in the binding site, they were unable to directly interact with the antigen due to either participating in ionic bonds in order to stabilise the topography of the antigen binding site or due to not being in the appropriate locations to contact the phosphodiester backbone. This

finding highlights the fact that arginines are important but it is their precise position that determines the interaction with the antigen.

1.6.2 Computer-modelling of anti-DNA antibodies and aPL

It is difficult to purify sufficient quantities of a single mAb to make crystals. Therefore to predict the antibody-antigen interaction, sequence data can be employed in computer modelling to produce three-dimensional structures of anti-DNA antibodies and aPL and therefore provide information as to which amino acid groups may enhance or reduce binding to antigen. Computer modelling program create models of the predicted structures of antibody molecules from their V_H and V_L sequences.

This approach has been used to model several human anti-dsDNA mAbs. The computer model of B3 (Kalsi et al., 1996), shown in Figure 1.5, revealed a binding groove along the V_H-V_L axis, which was flanked by three arginines at positions R27a (V_L CDR1) and R54 (V_L CDR2) of the L chain, and position R53 (V_H CDR2) of the H chain. Two of these arginine residues, V_LR27a and V_H R53, were derived by somatic mutation. Interactive docking of the dsDNA helix in the binding groove confirmed that these arginines are present at antibody-antigen contact sites and might stabilise the binding of B3 to dsDNA. Further models have been produced, by replacing B3V_L with 33H11V_L and UK4V_L as shown in Figures 1.6 and 1.7 respectively. According to the computer model shown in Figure 1.6, B3V_H/33H11V_L is likely to bind DNA. The model predicts that this interaction is stabilised by the presence of an arginine residue at position 92 in 33H11V_L. In contrast, the computer model shown in Figure 1.7 predicts that B3V_H/UK4V_L is likely to be a poor binder to DNA. One explanation for this prediction could be that the groove in the B3V_H/UK4V_L model is blocked by various residues from the light chain including a bulky positively charged arginine residue at position 94. In addition, the serine residue at position 29 introduces a possible destabilising electrostatic interaction with the phosphate backbone of DNA.

Barry et al., (1994) modelled six murine anti-DNA mAbs. Three of these antibodies were derived from autoimmune mice, whereas the others were prepared by immunisation of mice with the respective nucleic acid polymer. This procedure allowed the authors to compare autoimmune and induced antibodies on the basis of both sequence and structure to determine whether there were any obvious differences between the antibodies obtained from these two sources. Comparison of three of the models with the respective crystal structures indicated that the

differences between the two at the majority of sites were within 0.1nm. In general, ssDNA-specific antibodies have deep clefts where the antigen might bind, whereas dsDNA-specific antibodies present a relatively flat surface. In addition, on the basis of both sequence and structure, there is little to distinguish autoimmune antibodies from those produced by immunisation (Barry et al., 1994).

The computer model of the IgG aPL IS4 shown in Figure 1.8, supports the idea that arginine residues are important at the binding sites of this aPL. IS4 is likely to be representative of pathogenic aPL since it was isolated from a patient with PAPS and was shown to be pathogenic in a murine model. In addition, IS4 shows β_2GPI dependent binding to CL and contains numerous somatic mutations in an antigen driven pattern. Overall the H chain CDRs of IS4 display an accumulation of predominantly arginine and asparagine residues compared to acidic residues. In particular, IS4V_HCDR3 is 15 amino acids long with a very high content of five arginine residues, four of which are a result of somatic mutation. The model in Figure 1.8 shows these four arginine residues in IS4V_HCDR3 are surface exposed and could therefore be important in contributing to CL-binding specificity. Conversely, IS4V_L shown in Figure 1.8 has no somatic mutations to arginine but contains a germline encoded arginine at position 54.

Computer models such as these have been used to identify particular amino acids that potentially affect the antibody-antigen interaction. These residues need not always be in the binding site itself. Certain hidden residues may control the interaction between different loops of the V region. Alterations in such residues could change the three-dimensional structure of the whole molecule in such a way as to enhance or inhibit its binding ability. Computer models provide a hypothesis and then expression experiments need to be carried out to test the hypothesis and produce variant forms of the antibodies in which these key residues are altered. The effects of wild-type and altered antibodies can be compared upon binding and/or pathogenic properties of the antibody. A number of different systems to express antibody sequences as whole Ig molecules have been developed and are discussed in the next section.

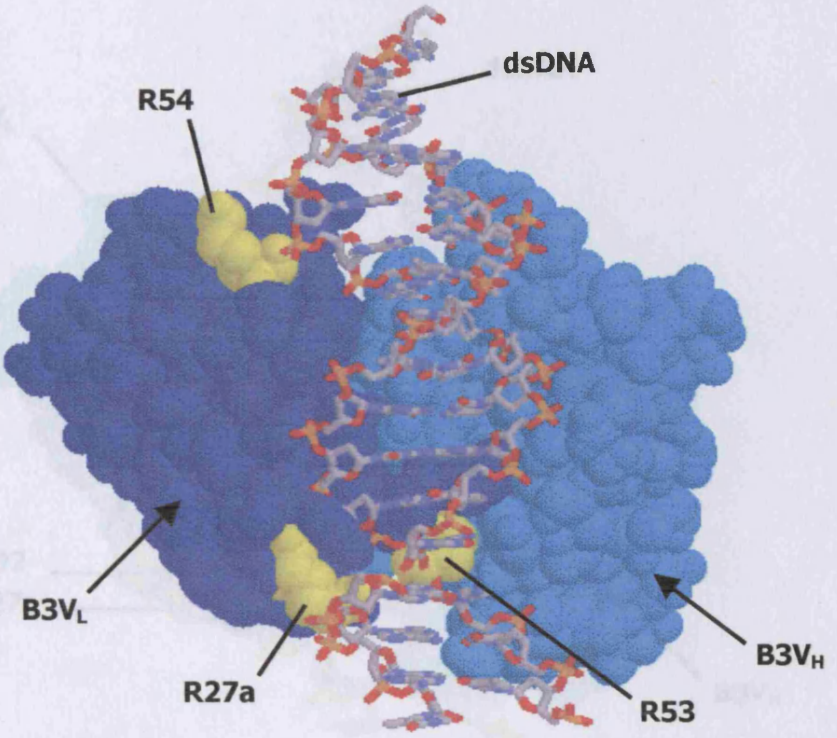


Figure 1.5 Computer generated model of B3V$_H$/B3V$_L$ with dsDNA

B3V$_H$ is shown in light blue whilst B3V$_L$ is shown in dark blue. The double helix of the DNA molecule is shown in the centre of the figure. The three arginine residues at the periphery of the site R27a (V$_L$ CDR1) and R54 (V$_L$ CDR2) of the light chain, and R53 (V$_H$ CDR2) of the heavy chain, predicted by this model to stabilise the interaction with the dsDNA helix, are shown in yellow. R27a and R53 are derived from somatic mutation. Modelled by Dr S. Nagl.

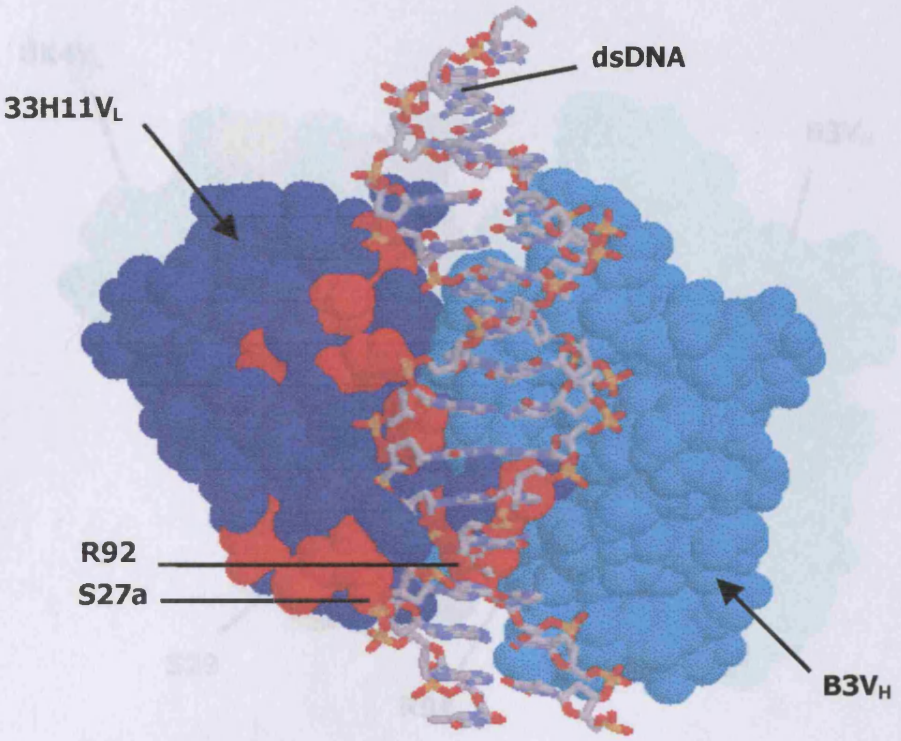


Figure 1.6 Computer generated model of B3VH/33H11VL with dsDNA
The computer model predicts that 33H11VL is able to create a DNA binding site in combination with B3VH. B3VH is shown in light blue whilst 33H11VL in dark blue. The VL residues that differ from those found at the same positions in B3VL are shown in red. Unlike B3VL, 33H11VL does not have an arginine residue at 27a in CDR1, it has the germline-derived serine (27a). However, the presence of an arginine residue at position 92 (R92) in 33H11VL CDR3 may compensate for this, since the model predicts that R92 can interact with the backbone of dsDNA. Modelled by Dr S. Nagl.

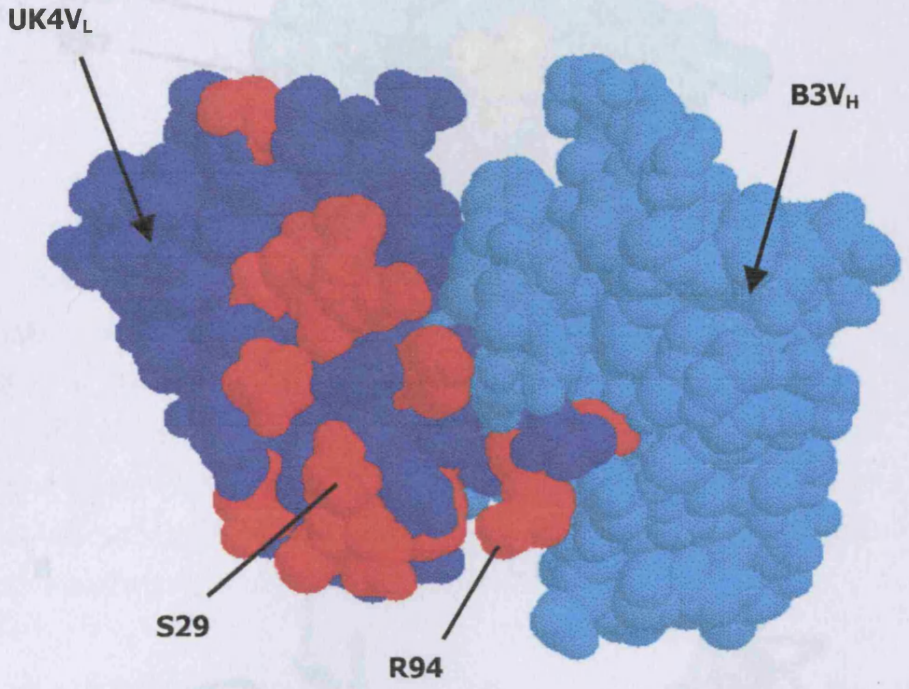


Figure 1.7 Computer generated model of B3V$_H$/UK4V$_L$

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

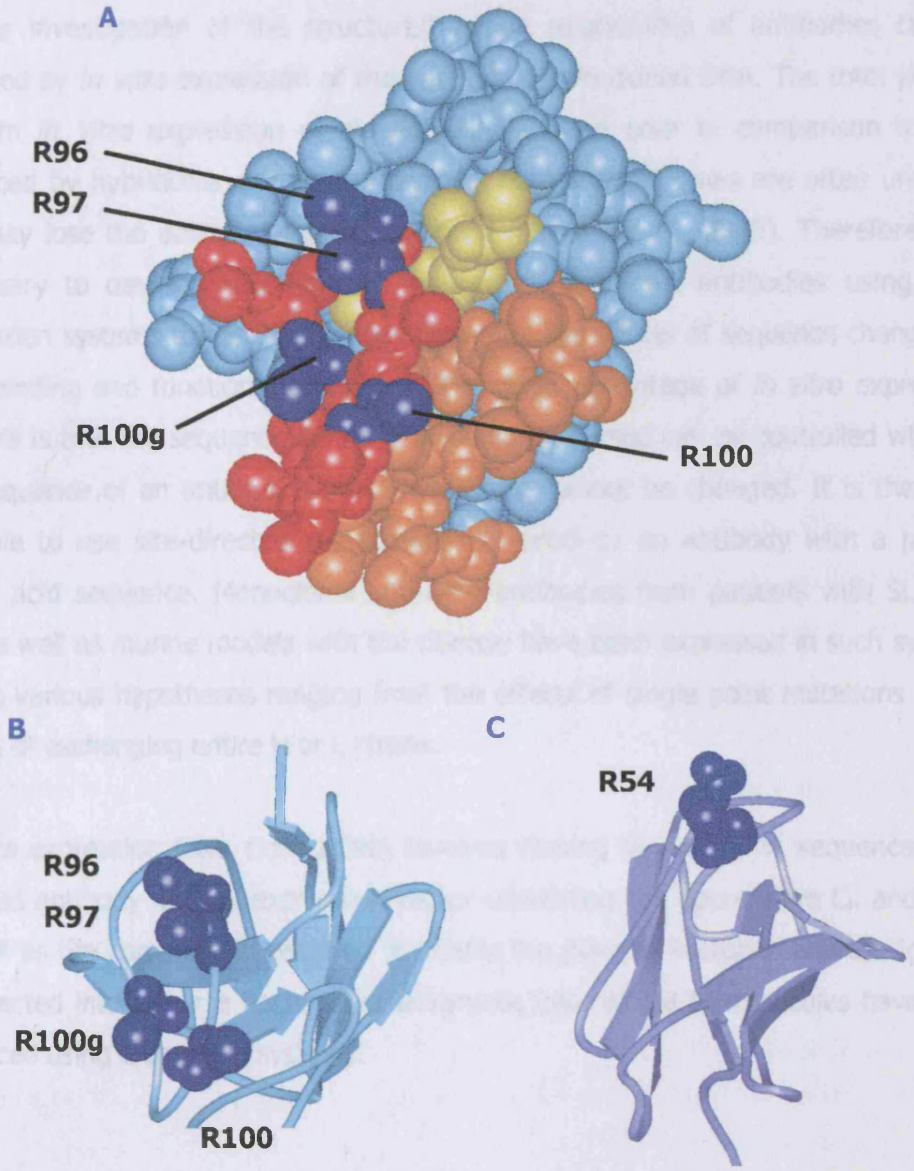


Figure 1.8 Models of IS4V$_H$ and IS4V$_L$

(**A**) Spacefill model of the heavy chain of IS4. The four arginines (96, R97, R100 and R100g) in the large CDR3 loop region of IS4VH are predicted to importantly contribute to cardiolipin-binding specificity. (**B**) Secondary structure model of the heavy chain of IS4. (**C**) Secondary structure model of the light chain of IS4. Modelled by Dr S. Nagl.

1.7 Expression systems for antibodies

Further investigation of the structure/function relationship of antibodies can be achieved by *in vitro* expression of the antibodies from cloned DNA. The total yield of Ig from *in vitro* expression of cloned DNA is often poor in comparison to that produced by hybridoma cell lines. However, human hybridomas are often unstable and may lose the ability to secrete antibody (Barnes et al., 2003). Therefore, it is necessary to develop recombinant monoclonal anti-DNA antibodies using such expression systems to test hypotheses regarding the effects of sequence changes on their binding and functional properties. The great advantage of *in vitro* expression systems is that the sequence of the DNA being expressed can be controlled whereas the sequence of an antibody from a hybridoma cannot be changed. It is therefore possible to use site-directed mutagenesis to produce an antibody with a precise amino acid sequence. Monoclonal anti-DNA antibodies from patients with SLE and APS as well as murine models with the disease have been expressed in such systems to test various hypotheses ranging from the effects of single point mutations to the effects of exchanging entire H or L chains.

In vitro expression from cloned DNA involves cloning V_H and/or V_L sequences of a selected antibody into an expression vector containing the appropriate C_H and/or C_L as well as the components required to enable the plasmid vector to express Ig once transfected into either a bacterial or eukaryotic cell. Whole Ig molecules have been produced using expression systems.

1.7.1 Bacterial expression systems

The disadvantage of bacterial expression of eukaryotic proteins is bacteria do not possess all the enzymes necessary for post-translational modifications such as glycosylation and the molecules may not be folded properly. This misfolding applies particularly to IgG molecules as it has not been possible to express a stable, functional whole antibody molecule in bacteria (Persic et al., 1997). However, since the antigen-binding site of an antibody is encoded by only the V region, smaller fragments of antibodies, such as single chain Fv molecules (scFv) or Fab molecules [which contain the whole L chain (V_L and C_L) covalently bound to half of the H chain (V_H and C_H1)], have been expressed that still bind to antigen.

scFv are the simplest of the expression products (Figure 1.9). They are composed of two domains, an antibody V_L amino acid sequence tethered to a V_H sequence by a designed peptide that links the carboxyl terminus of the V_L sequence to the amino terminus of the V_H sequence (Bird et al., 1988). The flexibility of the polypeptide linker, which is usually rich in glycine and serine, enables the V_H and V_L domains to adopt various conformations to produce an antigen-binding site. One disadvantage is that because scFV molecules have no C region cDNA and consist of only V regions, the structure of scFvs does not resemble any molecule occurring in nature.

In order to study the contribution of the H or L chain of the Ig molecules to the binding and pathogenic properties of anti-β_2GPI, Blank and colleagues converted the anti-β_2GPI mAbs into scFV, replaced H and L chains between the pathogenic and non-pathogenic anti-β_2GPI scFV, and studied their contribution *in vitro* and *in vivo* (Blank et al., 1999). The authors showed that in most cases replacement of the pathogenic V_H domain with the non-pathogenic V_H decreased the binding affinity of the scFv to β_2GPI and completely abrogated the anticoagulant activity. BALB/c mice were immunised with the anti-β_2GPI scFv and developed clinical manifestations of APS such as thrombocytopenia, prolonged activated partial thromboplastin time, and increased fetal resorptions (Blank et al., 1999). Their results showed that scFv of pathogenic antibodies are capable of inducing the same clinical manifestations as the whole antibody molecule upon active immunisation. Brigido and collegues carried out a similar study where they used a plasmid scFv expression vector to express V_H and V_L from the murine monoclonal anti-Z-DNA antibody Z22 (Brigido et al., 1993). Z-DNA is a form of DNA in which the double helix winds to the left in a zig-zag pattern (instead of to the right, like the more common B-DNA form). scFV containing both V_H and V_L of Z22 bound Z-DNA but not B-DNA or ssDNA which were the same binding properties shown by whole Z22 molecules. Replacement of either V_H or V_L by other mouse V_H or V_L genes prevented binding to Z-DNA in most cases (Brigido et al., 1993). These experiments suggested that both V_H and V_L were important in this antibody.

In some scFv expression systems the V_H and V_L DNA are ligated into phagemids rather than plasmid vectors (Rahman et al., 1998a). These vectors are modified bacteriophages (viruses which infect bacteria). The scFvs are expressed linked to a surface protein on a phage particle, which is produced by the transfected bacteria. This technique is known as phage display. If a library of V_H and V_L sequences is

cloned into a phagemid, a library of different phage particles that display a repertoire of different V_H/V_L combinations is produced. This process is called repertoire cloning.
An alternative to scFvs is to express Fab fragments (Figure 1.10). The Fab can be free in solution or displayed on the surface of a phage, allowing repertoire cloning (McCafferty et al., 1990). Bacterial expression of Fab fragments gives lower yields than svFv. However, Fab maintain the structure of an antibody binding site, and therefore are the most appropriate fragments to be used in crystallisation studies, particularly if they can be produced and purified from large scale bacterial cultures. Kumar et al., (2004) investigated the binding characteristics of human autoantibodies B3 and UK4 in their IgG and cloned Fab formats. Although B3 did not show convincing binding to Sm, it exhibited moderate binding to La or Ro and high binding to RNP. UK4 bound little to Ro and weakly to Sm, La and RNP. The results obtained show that the relative binding pattern against nuclear antigens exhibited by the cloned Fab fragments of B3 and UK4 was in general comparable to that observed for these molecules in their IgG format (Kumar et al., 2004).

Studies using bacterial expression systems also illustrate the importance of not just the presence but the precise position of residues such as arginine, lysine and asparagine on DNA-binding. Mockridge et al., (1996) showed that human mAb D5 V_H and V_L could be expressed as phage bound Fab, which bound both ssDNA and dsDNA. When the D5 H chain was replaced with one with no mutations and no basic residues in CDR3, DNA binding activity was lost completely (Mockridge et al., 1996). This study showed that somatic mutations in the FRs, CDR1 and CDR2 of D5 VH were not as essential in binding to DNA whereas the basic residues in V_H CDR3 were more crucial.

The advantage of bacterial expression systems is that bacteria are very easy to culture and relatively inexpensive. Therefore, it is possible to produce large amounts of antibody fragments (Verma et al., 1998). In addition, by using libraries of expression vectors, large numbers of combinations of V_H and V_L sequences can be rapidly screened for the ability to bind a particular antigen. However, because Fab and scFv molecules lack C region domains, they are both unsuitable for use in the investigation of the binding properties affecting the pathogenicity of these antibodies *in vivo*. Whole IgG molecules are required and can be produced by transfecting cultured eukaryotic cells.

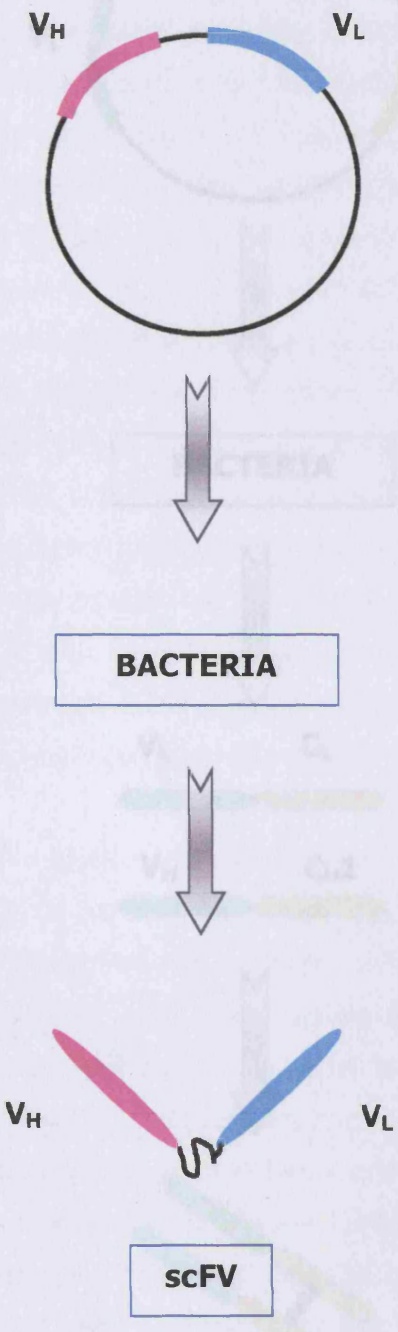


Figure 1.9 Production of scFv in bacterial expression system

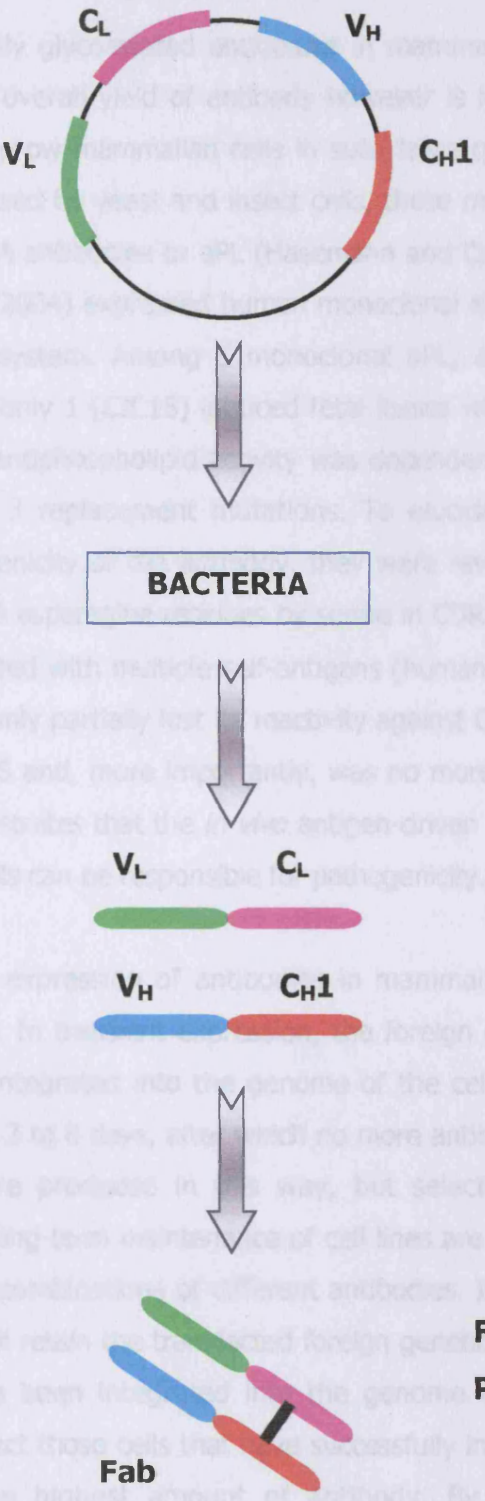


Figure 1.10 Production of Fab antibody fragments in bacterial expression system

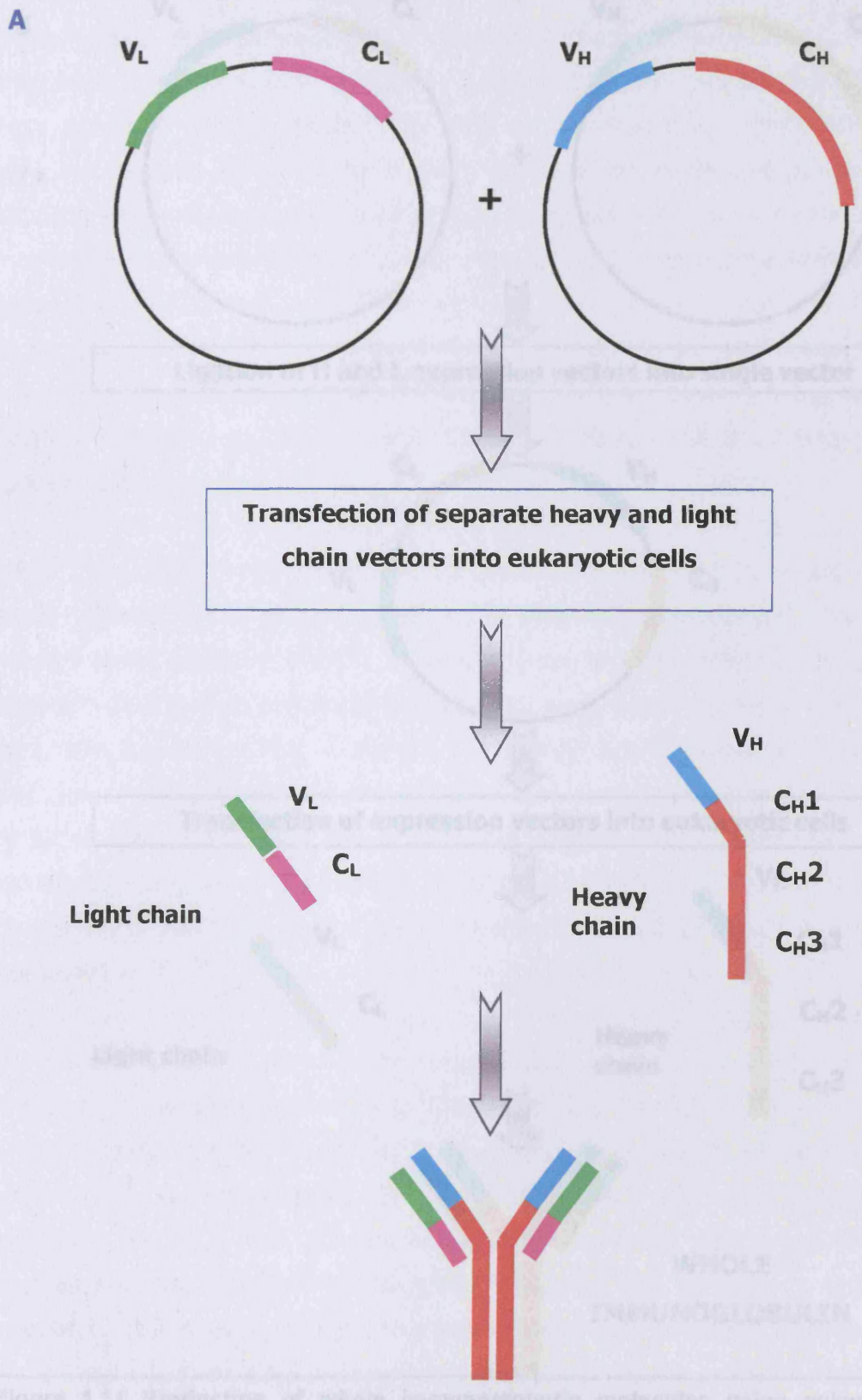
1.7.2 Eukaryotic expression systems

Expression of whole, fully glycosylated antibodies in mammalian cells is the ideal expression system. The overall yield of antibody however is lower than in bacteria because it is difficult to grow mammalian cells in such large quantities. Although Ig molecules can be expressed by yeast and insect cells, these methods have not been used to produce anti-DNA antibodies or aPL (Hasemann and Capra, 1990, Horwitz et al., 1988). Lieby et al., (2004) expressed human monoclonal aPL in insect cells by a baculovirus expression system. Among 5 monoclonal aPL, all originating from a single patient with APS, only 1 (CIC15) induced fetal losses when passively injected into pregnant mice. Its antiphospholipid activity was dependent on annexin A5, and its V regions contained 3 replacement mutations. To elucidate the role of these mutations in the pathogenicity of the antibody, they were reverted to the germline configuration (replacing 3 asparagine residues by serine in CDR1 of V_κ). The resulting "germline" antibody reacted with multiple self-antigens (human thyroglobulin, human myoglobin, ssDNA) and only partially lost its reactivity against CL, but it was no more dependent on annexin A5 and, more importantly, was no more pathogenic (Lieby et al., 2004). This study illustrates that the *in vivo* antigen-driven maturation process of natural autoreactive B cells can be responsible for pathogenicity.

There are two types of expression of antibodies in mammalian cells [reviewed in (Rahman et al., 2002b)]. In transient expression, the foreign genes inserted on the plasmid vector are not integrated into the genome of the cell. Therefore, they are active for a short period, 3 to 6 days, after which no more antibody is produced. Only small amounts of Ig are produced in this way, but selection of cells for drug resistance markers and long-term maintenance of cell lines are not required, allowing rapid screening of large combinations of different antibodies. In stable expression, a small minority of cells will retain the transfected foreign genetic information for many generations since it has been integrated into the genome of the host cell. It is essential however to select those cells that have successfully integrated the DNA and that are expressing the highest amount of antibody. By incorporating genes conferring drug resistance into the expression plasmid, this minority of high producing cells can be isolated since only those that have taken up the plasmid will survive in the selective media used (Fann et al., 2000).

The H chain expression vectors used in both transient and stable eukaryotic expression systems, contain the V_H DNA sequence 5′ to the appropriate C_H DNA

sequence (i.e. encoding the entire H chain C region and the hinge region). The L chain expression vectors contain the V_L DNA sequence 5′ to the appropriate κ or λ C_L DNA sequence. A number of techniques have been described for the transient and stable expression of antibodies in mammalian cells and these fall into three groups: the simultaneous transfection of separate H and L chain expression vectors into eukaryotic cells to express whole fully glycosylated Ig molecules (Figure 1.11A); the transfection of a single expression vector (supervector) containing both H and L chain sequences (V_H, C_H, V_L and C_L DNA sequences) (Figure 1.11B); and the transfection of H chain vector into hybridoma cells which only secrete L chains (a H chain loss variant) [reviewed in (Rahman et al., 2002b)]. The advantage of transfection using a supervector is that more cells may acquire the DNA required to make both chains therefore increasing the yield of Ig produced. However, the simultaneous transfection of separate H and L chain expression vectors does make it easier to produce a wide range of Igs with different H/L chain combinations.



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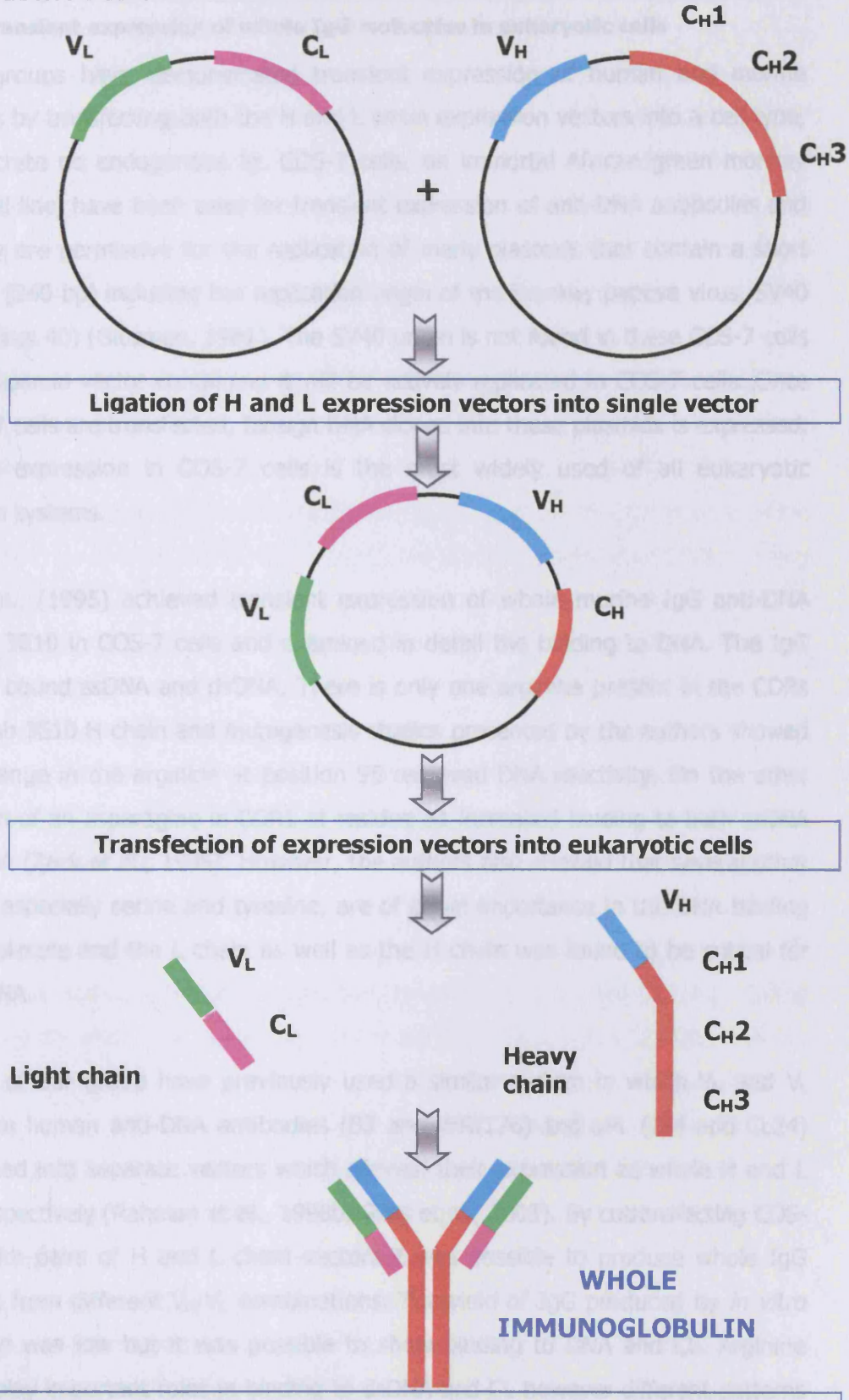

Figure 1.11 Production of whole immunoglobulin molecules using eukaryotic expression systems

A) Transfection of separate heavy and light chain expression vectors into eukaryotic cells (B) Transfection of single expression vectors containing both heavy and light chain cDNA into eukaryotic cells

1.7.2.1 Transient expression of whole IgG molecules in eukaryotic cells

Various groups have demonstrated transient expression of human and murine antibodies by transfecting both the H and L chain expression vectors into a cell type, which secrete no endogenous Ig. COS-7 cells, an immortal African green monkey kidney cell line, have been used for transient expression of anti-DNA antibodies and aPL. They are permissive for the replication of many plasmids that contain a short sequence (340 bp) including the replication origin of the monkey papova virus, SV40 (Simian Virus 40) (Gluzman, 1981). The SV40 origin is not found in these COS-7 cells but any plasmid vector containing it will be actively replicated in COS-7 cells. Once the COS-7 cells are transfected, foreign DNA cloned into these plasmids is expressed. Transient expression in COS-7 cells is the most widely used of all eukaryotic expression systems.

Zack et al., (1995) achieved transient expression of whole murine IgG anti-DNA antibody, 3E10 in COS-7 cells and examined in detail the binding to DNA. The IgG produced bound ssDNA and dsDNA. There is only one arginine present in the CDRs of the mAb 3E10 H chain and mutagenesis studies presented by the authors showed that a change in the arginine at position 95 removed DNA reactivity. On the other hand, gain of an asparagine in CDR1 at residue 31 increased binding to both ssDNA and dsDNA (Zack et al., 1995). However, the authors also showed that several other residues, especially serine and tyrosine, are of equal importance in the DNA binding of this molecule and the L chain as well as the H chain was found to be critical for binding DNA.

Members of our group have previously used a similar system in which V_H and V_L cDNA from human anti-DNA antibodies (B3 and WRI176) and aPL (IS4 and CL24) were cloned into separate vectors which allowed their expression as whole H and L chains respectively (Rahman et al., 1998b, Giles et al., 2003). By cotransfecting COS-7 cells with pairs of H and L chain vectors it was possible to produce whole IgG molecules from different V_H/V_L combinations. The yield of IgG produced by *in vitro* expression was low but it was possible to show binding to DNA and CL. Arginine residues play important roles in binding to dsDNA and CL however different patterns of mutation to arginine are associated with binding to each of these antigens.

1.7.2.2 Stable expression of whole IgG molecules in eukaryotic cells

Establishing a stable expression system can be difficult and time-consuming however, it has the advantage of producing higher quantities of antibodies. The yields obtained enable researchers to purify the expressed antibodies. Moreover, fuctional studies in mice or certain affinity assays such as BIAcore (surface plasmon resonance), which require the use of purified antibody can be performed, to determine the pathogenic properties of sequence alterations in antibodies.

Experiments using H chain loss variants (HCLVs) have been carried out to study the importance of sequence changes particularly arginine and other basic residues in V_HCDR3. Radic et al., (1993) studied the H chain of a murine IgG anti-dsDNA mAb, 3H9 that contains three somatic mutations in CDR2, one of which creates an arginine residue and there is a single arginine in CDR3. Initially they transfected 3H9 V_H into a number of different HCLVs and showed that it could produce anti-DNA antibodies in combination with several different V_L sequences. Mutagenesis of the 3H9 V_H sequence to revert the arginine at position 53 in CDR2 to serine has led to a dramatic reduction of affinity for dsDNA. Similarly, when the arginine in CDR3 was changed to glycine, the ability to bind DNA was abolished. However, reversion of the other somatic mutations in CDR2 that did not involve basic residues had little or no effect. Furthermore, introduction of an extra arginine in 3H9 V_H at sites where they were known to occur in other murine anti-DNA mAb CDRs, led to an increase in affinity for both ssDNA and dsDNA by between 5 and 10-fold. If two or more arginines were added to the same chain then binding was increased by up to 70-fold showing the effects of the mutations can be additive. These data however, do not imply that every arginine will always react with DNA. A case in point is arginine at position 64, which did not bind detectably to DNA. The authors proposed that a neighbouring aspartic acid at position 65, the product of a somatic mutation in 3H9 V_H, reacts with R64 by formation of a salt bridge, in a way that precludes DNA binding. Therefore the nature of amino acid residues as well as the precise position is important in affecting binding affinity. Furthermore, Guth and colleagues found that purified 3H9 binds chromatin and specifically a complex of H2A/H2B/dsDNA, but not dsDNA in solid phase or in solution (Guth et al., 2003). When used in the form of culture supernatant or as a standard protein G preparation, 3H9 binds dsDNA, apparently due to nuclear proteins in the preparation that assemble on target DNA.

The H chains of the murine anti-DNA antibody D42 and the anti-phosphorylcholine antibody 6G6 are both encoded by the same V_H11 germ-line gene (Pewzner-Jung et al., 1996). The two antibodies differ at two sites in V_H CDRs 1 and 2 due to replacement mutations in D42. There are three arginines in CDR3 of D42 V_H whereas 6G6 V_H does not have any arginines. Pewzner-Jung et al., (1996) transfected D42 V_H and 6G6 V_H into HCLVs. Their results indicated that the major contribution to DNA binding affinity is provided by the particular H/L chain pairing and the structure of the V_H CDR3 gene segment. They found that only transfection into loss variants secreting the D42 L chain produced anti-DNA binding, which was only possible if the transfected DNA contained D42 V_H CDR3. The presence of the two replacement mutations increased the DNA binding affinity of D42 and one of these mutations abolished phosphorylcholine binding of 6G6, but did not convert it to an anti-DNA antibody. HCLVs are not well suited to studying the effects of changes in the L chain. Some information can be deduced by transfecting the same H chain into variants secreting different L chains but this does not allow for the design of point mutations in the L chain itself.

To evaluate the role of somatic mutations in DNA binding, Wellmann and colleagues subjected human IgG anti-dsDNA antibody 33.C9 and 33.F12 to side-directed mutagenesis, reverting amino acid substitutions to the germline sequence. The Ig V gene segments from genomic DNA from 33.C9 and 33.F12 hybridomas were cloned into expression vectors and the resulting plasmids were used as a template for the site-directed mutagenesis. The variants produced were expressed in myeloma cells. Geneticin and puromycin were used for neomycin selection and double-resistant clones were screened for IgG production in ELISA. The germline revertants of the V regions of 33.C9 and 33.F12 exhibited no detectable ssDNA or dsDNA binding. The authors showed that arginines enhance antibody affinity for DNA in the case for the human antibody 33.F12. For the antibody 33.C9, however, none of the three amino acid exchanges that are essential and sufficient to create a high-affinity-dsDNA antibody are arginines. In addition, 33.C9 with germline-reverted V genes showed strong to CL, creating an antibody with specificity to PLs (Wellmann et al., 2005).

In another similar study, Katz et al., (1994) used site-directed mutagenesis to produce a range of antibody variants of the V_H sequence of the murine anti-DNA antibody R4A. Mutants were generated with amino acid substitutions in CDR2, CDR3 and FR3 that alter the charge of the V region and tested for binding to DNA and

deposition in the kidneys of SCID mice. Although mutation of an arginine in CDR3 was found to reduce binding, a single amino acid substitution (arginine to serine) in CDR2 resulted in higher binding than R4A to dsDNA, showing that an arginine may be lost within a CDR without a resulting decrease in dsDNA binding. In addition, the glutamic acid to glutamine replacement in FR3 showed a modest increase in dsDNA binding, demonstrating that FR substitutions can also alter DNA binding. The most dramatic finding of this study is that a very few amino acid substitutions can completely abrogate pathogenicity or alter the *in vivo* target of a pathogenic autoantibody. They injected young SCID mice intraperitoneally with either R4A hybridoma cells or one of the HCLVs expressing whole IgG with the altered $R4AV_H$ (Katz et al., 1994). All mice carrying the R4A hybridoma developed proteinuria with glomerular deposition of the antibody in the kidneys. The mutant cell line producing antibody that gave the highest dsDNA binding, described above, showed some glomerular deposition but deposited to a greater extent in the tubules. However, the change in affinity for dsDNA may also be associated with the acquisition of a novel antigenic cross-reactivity. The localisation of renal deposition raises the question of whether DNA binding is central or ancillary to the pathogenicity of these antibodies. In conclusion, these results indicate that changes in the ability to bind dsDNA do not necessarily predict changes in the pathogenic properties of an antibody.

HCLVs have not been used to express whole human anti-DNA antibodies. Human monoclonal anti-DNA antibodies have only previously been stably expressed in eukaryotic cells by one group (Li et al., 2000). In this system F3B6 human/mouse heteromyeloma cells were transfected simultaneously with expression vectors containing H or L chain DNA of the human IgA monoclonal anti-DNA antibody 412.67. The antibody was expressed as IgG rather than IgA, because the expression vector contained a cloned C_γ sequence (not C_α). Neomycin was used as a selecting agent as the L chain vector carried a neomycin resistance gene *neo*. Therefore only those cells successfully transfected with the L chain expression vector survived the neomycin treatment. Those cells expressing both H and L chain whole IgG were detected by ELISA and the IgG purified by ammonium sulphate precipitation and then on a column. The quantities of whole Ig produced were far greater than those produced by transient expression systems thus enabling further binding assays to be carried out, such as inhibition assays (ssDNA inhibited antibody binding to solid-phase DNA) which require a greater yield of Ig than that produced by transient expression. Li et al., (2000) used this stable expression system to show that for

412.67 to bind dsDNA the presence of two V_H CDR3 arginine residues was essential although the presence of either arginine singly was sufficient for ssDNA binding.

1.8 Conclusion:

In conclusion it appears that high affinity anti-dsDNA and aPL IgG are particularly important in patients with SLE and APS respectively. Sequence analysis has shown antigen-driven accumulation of multiple somatic mutations in the V_H and V_L CDRs of these antibodies. Overall the findings from various experiments, using different expression systems support the importance of basic amino acids, particularly arginine residues in V_H CDR3 at the antigen binding site. The importance of these residues has been highlighted using site-directed mutagenesis and tested for binding affinity.

Human monoclonal anti-DNA antibodies and aPL may well be representative of those antibodies most closely related to disease pathogenesis in patients with SLE and APS especially if they are of the IgG isotype. As the pattern of V_H/V_L gene usage is not significantly different in patients with SLE and APS in comparison to that seen in healthy individuals, it is likely that representative anti-DNA antibodies and aPL for all SLE and APS patients are those in which both the V_H and V_L sequences are encoded by the most commonly rearranged genes.

The human monoclonal anti-DNA antibody B3, which I studied in this thesis was derived by members of our group from a patient with active SLE (Ehrenstein et al., 1993a). B3 is relevant to studying SLE for a number of reasons. It has an IgG isotype and a high affinity for dsDNA. The V_H and V_L of B3 are encoded by the two most commonly rearranged human V_H and V_λ genes, V3-23 and 2a2 respectively. Furthermore, when injected into SCID mice, B3 deposited in the kidneys and the mice developed proteinuria (Ehrenstein et al., 1995, Mason et al., 2005). The other mAb that I studied is an aPL IS4, derived from a patient with PAPS (Zhu et al., 1999). IS4 possesses multiple features likely to make this antibody relevant to the pathogenesis of APS and representative of pathogenic aPL found in many APS patients. It is of IgG isotype, binds with high affinity to anionic PL only in the presence of β_2GPI and can bind to β_2GPI alone. Moreover, it has been shown to be thrombogenic in a murine *in vivo* pinch-induced thrombosis model and to cause *in vitro* and *in vivo* EC activation (Pierangeli et al., 2000). There have been few studies of the relative importance of H and L chain sequence changes in the binding of aPL

to PL-β_2GPI. Several groups have studied murine aPL however human aPL are more likely to demonstrate the direct relevance to APS in humans.

The computer-generated model of the B3/dsDNA complex (Kalsi et al., 1996) suggests that binding is stabilised by the interaction of dsDNA with three arginine residues on the periphery of the binding site (R27a [V_L CDR1] and R54 [V_L CDR2] of the L chain, and R53 [V_H CDR2] of the H chain). One of the arginine residues (R27a in V_L CDR1) is the product of a somatic mutation of the germline gene, 2a2 and has been previously studied by our group (Rahman et al., 2001). The arginine residue in V_H CDR2 (R53) is the product of a somatic mutation of the germline gene, V3-23. The computer model of IS4 V_H predicts that four arginine residues are surface-exposed, and therefore potentially important in binding to PL. These are in CDR3 at positions 96, 97, 100 and 100g.

1.9 Aims of this thesis

The general aims of my thesis were to examine which sequence features in B3 and IS4 H chains are important in determining their ability to bind antigen by expressing whole antibodies using a transient and stable expression system. Experiments to produce variant forms of antibodies using site directed mutagenesis were also carried out and their effects upon antibody binding to different antigens examined. In more detail I aimed to:

1. produce variant forms of the V_H chain region of B3 and IS4 by replacing the arginine residues in B3V_H CDR2 and IS4V_H CDR3 using site-directed mutagenesis.

2. develop a COS-7 transient expression system to express different H/L chain combinations as whole IgG molecules and determine their ability to bind dsDNA, nucleosomes, α-actinin, CL, β_2GPI, DI of β_2GPI and PS.

3. develop a system for the stable expression of cloned antibody DNA sequences, derived from human monoclonal aPL and anti-dsDNA antibodies, as whole IgG molecules in Chinese hamster ovary cells (CHO) which lack the dihydrofolate reductase (*dhfr*) gene.

4. purify the expressed antibodies produced from the CHO stable expression system and test their binding affinities to dsDNA, nucleosomes, α-actinin, CL, β_2GPI, DI of β_2GPI and PS.

CHAPTER TWO

Materials and Methods

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MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals used in this project were supplied by BDH, Lutterworth, Leicestershire, UK with the following exceptions:

Absolute Alcohol & Propan-2-ol (Hayman Ltd, Witham, Essex, UK)
Methotrexate (MTX) / Amethopterin (A6770, Sigma, Poole, UK)
Dimethyl Sulfoxide (DMSO) (D-5879, Sigma, Poole, UK)
Trypan Blue solution (0.4%) (T8154, Sigma, Poole, UK)
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in dimethylformamide (Xgal), Isopropyl–β–thiogalactopyranoside (IPTG) (Insight Biotechnology, Wembley, U.K)

All solid chemicals were dissolved in ddH_2O, adjusted to the correct pH with 0.1M HCL, glacial acetic acid or 0.1M NaOH and autoclaved or filter sterilised with 0.22μm syringe driven filter units (Millipore, Bedford, MA, USA) unless otherwise stated.

Deoxynucleotide Triphosphates (dNTPs)- 100mM (pH 7.5) set of dATP, dCTP, dGTP, dTTP (Promega, Southampton, UK) were stored at -20°C.

2.1.2 General materials and equipment

15ml and 50ml sterile tubes (#91015T, Helena Biosciences, Sunderland, U.K)
Bijou tubes (#39740, Bibby Sterilin Ltd, Staffordshire, U.K)
Sterilin tubes (#30593, Bibby Sterilin Ltd, Staffordshire, U.K)
1.5ml and 0.5ml microfuge tubes (Anachem, Luton, Bedfordshire, U.K)
1.5ml thin-walled microfuge tubes (Eppendorf AC, Hamburg, Germany)
Centrifuges: (Kendro Laboratory Products Ltd, Herts, U.K)
Sorvall Biofuge *Pico* (and refrigerated version *Fresco*),
Sorvall RT-7 Plus and
RC26 Plus
PCR Cycler Machine (Eppendorf Mastercycler, Hamburg, Germany)
Gene Pulser II Electroporator (Bio-Rad Laboratories Ltd, Herts, U.K)

Gene Pulser II Cuvettes 0.4cm (#165-2088, Bio-Rad Laboratories Ltd)
Centricon-YM30 centrifugal concentrators (Amicon Bioseparations, Millipore, UK)
Spectrophotometer (BioRad laboratories, CA, USA)

2.1.3 Enzymes

Xho I (#R6161, Promega, Southampton, UK)
Hind III (#R6045, Promega, Southampton, UK)
Age I (#R7251, Promega, Southampton, UK)
Sal I (#R6051, Promega, Southampton, UK)
BamH I (New England Biolabs(NEB), Hertfordshire, UK)
EcoRI (#R6017, Promega, Southampton, UK)
RNase-free, *Dnase I* (776 785, Roche, Lewes, East Sussex, UK)
Taq DNA Polymerase (#M1665, Promega, Southampton, UK)
PfuTurbo® DNA Polymerase (Quikchange™ Site-directed mutagenesis kit Stratagene, California, USA)
T4 DNA Ligase (# M1801, Promega, Southampton, UK)
All enzymes were stored at -20°C.

2.1.4 Reaction buffers

a) 10x *PfuTurbo* DNA polymerase reaction buffer in Quikchange™ Site-directed mutagenesis kit (Stratagene, California, USA)
- 100mM KCl
- 100mM $(NH_4)_2SO_4$
- 200mM Tris-HCl (pH 8.8)
- 20mM $MgSO_4$
- 1% Triton® X-100
- 1mg/ml nuclease-free bovine serum albumin (BSA)

b) DNA ligase buffer (Promega, Southampton, UK)
- 300mM Tris-HCl
- 100mM $MgCl_2$
- 100mM dithiothreitol (DTT)
- 10mM adenosine triphosphate (ATP)

c) Buffer D- for restriction enzymes *Xho I* and *Sal I* (Promega, Southampton, UK)
- 60mM Tris-HCl (pH 7.9)
- 1.5M NaCl

- 60mM $MgCl_2$
- 10mM DTT

d) Buffer E- for restriction enzymes *Hind III* and *BamH I*
(Promega, Southampton, UK)

- 60mM Tris-HCl (pH 7.5)
- 1M NaCl
- 60mM $MgCl_2$
- 10mM DTT

e) Buffer H- for restriction enzyme *EcoR I* (Promega, Southampton, UK)

- 900mM Tris-HCl (pH 7.5)
- 500mM NaCl
- 100mM $MgCl_2$

f) Buffer K- for restriction enzyme *Age I* (Promega, Southampton, UK)

- 100mM Tris-HCl (pH 7.4)
- 1.5M KCl
- 100mM $MgCl_2$

g) Thermophilic DNA Polymerase 10x Buffer B supplied with separate $MgCl_2$
(Promega, Southampton, UK)

- 20mM Tris-HCl (pH 8.0 at 25°C)
- 100mM KCl
- 0.1mM EDTA
- 1mM DTT
- 50% glycerol
- 0.5% Tween 20
- 0.5% Nonidet®-P40.

All reaction buffers were stored at -20°C.

2.1.5 Agarose gel electrophoresis materials and buffers

Agarose and low melting point agarose were supplied by Gibco BRL, Life Technologies, Paisley, Scotland, UK.

Molecular weight markers:

- 1Kb DNA MW marker (Invitrogen, Paisley, UK)
- Lambda/HindIII DNA MW marker (Promega, Southampton, UK)
- 100bp DNA MW marker (Promega, Southampton, UK)

Loading Buffer:

The following components were added to 8.45ml autoclaved distilled water:

- 25mg xylene cyanole
- 1.5g Ficoll 400 (Pharmacia, Biotech, Uppsala, Sweden)

2.1.6 General buffers and solutions

a) Tris Acetate EDTA (TAE buffer)

A stock solution of 50 times working strength TAE buffer was made by dissolving the following components in 1L of distilled water:

- 242g/L Tris base
- 57.1ml glacial acetic acid
- 100ml 0.5M EDTA (pH 8.0)

Dilution to a working strength gave a final concentration of 40mM Tris acetate and 1mM EDTA.

b) Tris-EDTA (TE buffer) (pH 7.5)

- 10mM Tris-HCl (pH 7.5)
- 1mM EDTA

c) Phosphate buffered saline (PBS) (pH 7.4)

One PBS tablet (Gibco, Paisley, UK) was added per 500ml distilled water.

To make PBS/0.1% Tween, 1ml "Tween 20" detergent was added to 1L PBS.

2.1.7 Specific buffers and solutions

a) Plasmid DNA purification from bacterial cultures

The following buffers were supplied with Qiagen (Crawley, West Sussex, UK) plasmid purification kits:

- Buffer P1 (Resuspension Buffer) 50mM Tris-Cl (pH 8.0)
  10mM EDTA
  100µg/ml RNase A
- Buffer P2 (Lysis Buffer) 200mM NaOH
  1% SDS
- Buffer P3 (Neutralisation Buffer) 3.0mM potassium acetate (pH 5.5)
- Buffer QBT (Equilibriation Buffer) 750mM NaCl
  50mM MOPS (pH 7.0)
  15% isopropanol
  0.15% Triton ®X-100
- Buffer QC (Wash Buffer) 1.0M NaCl

	50mM MOPS (pH 7.0)
	15% isopropanol
- Buffer QF (Elution Buffer for maxiprep kit)	1.25M NaCl
	50mM MOPS (pH 7.0)
	15% isopropanol
- Buffer N3 (Neutralisation Buffer)	3.0mM potassium acetate (pH 5.5)
- Buffer EB (Elution buffer for miniprep kit)	10mM Tris-HCl (pH 8.5)

b) Buffers for production of "super" competent DH5α *E.coli* cells:

Transformation buffer 1:	10mM MES (pH 5.8)
	100mM $RbCl_2$
	10mM $CaCl_2$
	50mM $MnCl_2$
Transformation buffer 2:	10mM PIPES (pH 6.5)
	75mM $CaCl_2$
	10mM $RbCl_2$
	15% v/v glycerol

c) Western Blot reagents

Perfect Protein™ Western Markers (Novagen, Nottingham, UK)

Anti-human IgG (γ-chain specific) Peroxidase Conjugate (A8419, Sigma, Poole, UK)

Anti-Domain I Monoclonal Antibody (kind gift from Dr M. Iverson and Dr M. Linnik, La Jolla Pharmaceuticals (LJP), San Diego, USA)

Peroxidase-Conjugated Goat Anti-Mouse Immunoglobulins (P0447, Dako, Glostrup, Denmark)

d) Nucleosome preparation buffers

Dounce buffer:

- 10mM HEPES-NaOH (pH 7.9)
- 1.5mM MgCl2
- 10mM KCl
- 0.5mM Phenylmethylsulphonylfluoride (PMSF)
- 1:100 protease inhibitor cocktail

All reagents made up in ddH_2O except PMSF, which is dissolved in isopropanol and mixed at 55°C until the crystals dissolved. Aliquots of 1ml were frozen and stored at -20°C.

Digestion buffer:

10mM Tris-HCl (pH 7.4)

1mM $CaCl_2$

1mM Phenylmethylsulphonylfluoride (PMSF)

2.1.8 ELISA Reagents and buffers

Bovine serum albumin (BSA) (#A7030, Sigma, Poole, UK)

Goat anti-human IgG (Fc fragment specific) (#I8885, Sigma, Poole, UK)

Cardiolipin (#C1649, Sigma, Poole, UK)

β2-Glycoprotein I (#B2G1-0001, Haematologic Technologies Inc, U.S.A)

Purified human IgG_λ (#I5029, Sigma, Poole, UK)

α-actinin from chicken gizzard (#A9776, Sigma, Poole, UK)

Goat anti-human λ alkaline phosphatase conjugate (#A2094, Sigma, Poole, UK)

Goat anti-human IgG_γ alkaline phosphatase conjugate (#A3150, Sigma, Poole, UK)

p-nitrophenyl phosphate tablets (#104-105, Sigma, Poole, UK)

Sample, enzyme and conjugate dilution buffer (SEC)

- 100mM Tris-HCl (pH 7)
- 100mM Sodium chloride
- 0.02% Tween 20
- 0.2% BSA

Bicarbonate buffer (BIC) (pH 9.6)

- 0.5M Sodium bicarbonate
- 0.05M Sodium dihydrogencarbonate

2.1.9 Bacterial Strains

- *E. coli* of strain DH5α were supplied by Gibco, Paisley, U.K.
- *E. coli* XL1-Blue Supercompetent cells were supplied with Quikchange™ Site-directed mutagenesis kit by Stratagene, California, USA.

2.1.10 Bacterial growth media

Media were made in ddH_20 and sterilised by autoclaving.

Luria-Bertani (LB) medium:

- 1% (w/v) Bacto-tryptone (Duchefa, Haarlem, The Netherlands)
- 0.5% (w/v) Bacto-yeast extract (Duchefa, Haarlem, The Netherlands)
- 1% (w/v) Sodium chloride

The pH was adjusted to pH 7.0 using NaOH.

For LB agar plates 1.5% (w/v) agar (Duchefa, Haarlem, The Netherlands) was added to the LB medium.

When antibiotic was added to the medium, the final concentration used was 50µg/ml ampicillin. Stock solutions of ampicillin at 50mg/ml in sterile water were kept at -20°C in light-tight containers.

NZY+ Broth:

- 1% (w/v) NZ amine (casein hydrolysate) (Sigma, Poole, UK)
- 5% (w/v) Bacto-yeast extract (Duchefa, Haarlem, The Netherlands)
- 5% (w/v) NaCl

This was made up to 1L with distilled water, the pH adjusted to pH 7.5 using Sodium hydroxide and then autoclaved. The following supplements were added to the media prior to use:

- 1.25% 1M $MgCl_2$
- 1.25% 1M $MgSO_4$
- 1% 2M Glucose solution

2.1.11 Human monoclonal antibodies

B3, 33H11, UK4 and IS4 are all human IgG mAbs produced from peripheral blood lymphocytes (PBL) of four different patients.

B3, 33H11, and UK4 were isolated by fusion of PBL from patients with SLE with cells of the mouse heteromyeloma line CB-F7. 33H11 is specific for dsDNA, B3 binds ssDNA, dsDNA, nucleosomes and histones. UK4 binds negatively charged (but not neutral) PL in the absence of β_2GPI and does not bind DNA.

IS4 was derived from a patient with PAPS by EBV transformation of peripheral blood mononuclear cells and fusion with the human-mouse heterohybridoma K6H6/B5 cell line. IS4 binds to CL in the presence of bovine and human β_2GPI and to human β_2GPI alone.

B3 and UK4 were produced by our group at UCL in London. 33H11 was a kind gift from Dr Thomas Winkler (Erlangen, Germany). The hybridoma cell lines of IS4 were produced and kindly donated by Dr P. Chen, Department of Medicine, Division of Rheumatology, University of California at Los Angeles, Los Angeles, USA.

2.1.12 Eukaryotic cell lines

COS-7 cells and CHO*dhfr*$^-$ were both kind gifts from Mrs Alison Levy, AERES Biomedical, MRC Collaborative Unit, Mill Hill, London.

The COS-7 cells were originally derived from American Type Culture Collection, Ref No. CRL 1651.

CHO*dhfr*$^-$ cells used are from the cell line DXB11 originally derived from CHO-K1 in 1978 by Professor Lawrence Chasin (Professor of Biological Sciences, Columbia University, USA). These CHO-K1 cells were derived from Ted Puck and Fa-ten Kao at the Eleanor Roosevelt Cancer Institute in Denver, Colorado, USA in 1970. One *dhfr* allele was deleted in DXB11 and the other allele carries a mis-sense mutation resulting in a single amino acid substitution.

2.1.13 Growth media and solutions for maintenance of eukaryotic cell lines

- Pre-Electroporation COS-7 Growth Medium 1

Dulbecco's Modified Eagle Medium (DMEM) (41966-029, Invitrogen, Paisley, UK)
Supplemented with:
10% (v/v) Fetal calf serum (FCS) (10099-133, Invitrogen, Paisley, UK)
580µg/ml L-glutamine (25030-024, Invitrogen, Paisley, UK)
10000Units/ml penicillin/10000µg/ml streptomycin
(15140-122, Invitrogen, Paisley, UK)

- Post-Electroporation COS-7 Growth Medium 2

Dulbecco's Modified Eagle Medium (DMEM) (41966-029, Invitrogen, Paisley, UK)
Supplemented with:
10% (v/v) Ultra low IgG FCS (16250-078, Invitrogen, Paisley, UK)
580µg/ml L-glutamine (25030-024, Invitrogen, Paisley, UK)
10000Units/ml penicillin/10000µg/ml streptomycin
(15140-122, Invitrogen, Paisley, UK)

- CHO*dhfr*$^-$ (Non-selective) Growth Medium A

MEM α-Medium(α-MEM) with ribonucleosides and deoxyribonuclosides

(32571-028, Invitrogen, Paisley, UK)

Supplemented with:

10% (v/v) FCS (10099-133, Invitrogen, Paisley, UK)

10000Units/ml penicillin/10000µg/ml streptomycin

(15140-122, Invitrogen, Paisley, UK)

- CHO*dhfr*$^-$ (Selective) Growth Medium B

MEM α-Medium (α-MEM) without ribonucleosides and deoxyribonuclosides

(32571-029, Invitrogen, Paisley, UK)

Supplemented with:

10% (v/v) Dialysed FCS (Hyclone, UK)

10000Units/ml penicillin/10000µg/ml streptomycin

(15140-122, Invitrogen, Paisley, UK)

- Hank's Balanced Salt solution, containing no magnesium and no calcium (HBS)

(14175- 053, Invitrogen, Paisley, UK)

- Trypsin-EDTA (1x) in HBSS w/o CA&MG, liquid - 0.25% Trypsin 1mM EDTA•4Na

(25200-056, Invitrogen, Paisley, UK)

2.1.14 Expression constructs

The expression vector (neSLE122H45.4) that encodes a human V_H3-23, D6-25, JH5 IgH chain (CDR3: DGGGYSSSFDP) was a kind gift from Dr H. Wardemann, The Rockefeller University, New York, USA. It comes from a non-DNA-reactive, non-self-reactive germline IgM antibody from a naive B cell originally associated with a Vk3-15, Jk2 light chain. The vector has an Amp resistence cassette and the VDJ was cloned into the vector using a 5' *Age I* restriction site and a 3' *Sal I* site. The VDJ was originally amplified from a single cell using nested PCR.

pG1D1, pG1D210, pLN10 and pLN100 are expression vectors and were all kind gifts from Dr C.A. Kettleborough and Dr T. Jones at AERES Biomedical, MRC Collaborative Unit, Mill Hill, London. Each expression vector contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and either the bacterial *neo* gene (in pLN10 and pLN100) or mouse *dhfr* gene (in pG1D1 or pG1D210) coding sequences driven by the SV40 early promoter to act

as dominant selectable markers during stable transformation. The SV40 promoter however that drives the *dhfr* is crippled by the presence of a defective SV40 promoter-enhancer sequence so that expression is poor thus allowing for the selection of high expression level clones using comparatively low levels of MTX. Each vector also contains an ampicillin resistance gene driven by an internal promoter to enable it to be cultured in *E.coli*. In the recombinant expression vectors pG1D210 and pLN100, the CMV IEI promoter contains a regulatory sequence intron A, which has been shown to increase the levels of expression of glycoprotein from vectors containing this additional sequence. Plasmids pG1D1 and pLN10 are "old" versions of pG1D210 and pLN100 respectively which lack intron A.

2.1.14.1 Heavy chain expression vectors

Recombinant expression vector pG1D1 and pG1D210 containing human Ig V_H ($B3V_H$/pG1D1 and $B3V_H$/pG1D210 respectively) cDNA was constructed by Drs A. Rahman and J. Haley. The construct $IS4V_H$/pG1D210 was constructed by Dr I. Giles. The vector map of pG1D210 is shown in Figure 2.1.

pG1D1 contains a human cDNA version of the gamma-1 constant region (Human C_H), 5` to which is a multiple cloning site (MCS) allowing the insertion of a variable domain gene immediately followed by a splice donor site. The human C_H is immediately preceded by a splice acceptor site hence the DNA between the variable (V) domain and constant (C) region is treated like a V::C intron by mammalian cells. The C_H is followed by a termination sequence to prevent read through. The C_H is followed by a termination sequence to prevent read through. The promoter driving the *dhfr* gene is crippled so that expression is poor making selection by MTX amplification during stable transfection possible. pG1D210 is identical to pG1D1 apart from for the removal of the V::C intron and the replacement of the multiple cloning site (MCS) with a new sequence which also encodes the last few amino acids of a heavy chain J region, i.e. GTLVTVSS. V_HJ_H sequences can be cloned into the vector directly adjacent to C_H and in frame with it.

2.1.14.2 Light chain expression vectors

The following expression constructs were prepared by Dr A. Rahman: B3V_L/pLN10, 33H11V_L/pLN10 and UK-4V_L/pLN10 (Figure 2.2). The construct IS4V_L/pLN100 was constructed by Dr I. Giles.

The light chain expression vectors pLN10 (Figure 2.2) and pLN100 (Figure 2.3) contain a human cDNA version of the λ-2 constant region sequence DNA (C_λ), 5` to which is a MCS allowing the insertion of a variable domain gene immediately followed by a splice donor site. The constant region cDNA is immediately preceded by a splice acceptor site hence the DNA between the V domain and C region is treated like a V::C intron by mammalian cells and not represented in the expressed light chain peptide. A termination sequence follows the human C_L to prevent read through. Each of the V_λ region sequences were ligated into expression vector pLN10 5' to the C_λ human DNA sequence to produce the constructs, B3V_L/pLN10, 33H11V_L/pLN10 and UK4V_L/pLN10.

2.1.14.3 Supervectors

The three supervectors IS4V_H&IS4V_L/PG1D210, IS4V_H&B3V_L/PG1D210 and IS4V_H&UK4V_L/PG1D210 were made by Dr I. Giles. The four supervectors B3V_H&B3V_L/pG1D1, B3V_H&B33V_L/pG1D1, B3V_H&B3(R27aS)V_L/pG1D1 and B3V_H&BUV_L/pG1D1 were made by Drs J. Haley and L. Mason. The method used is outlined in Figure 2.4.

2.1.15 Hybrid V_L chain constructs

Each hybrid V_L chain construct contained the CDR1 of one of the human monoclonal IgG antibody and the CDR2 and CDR3 of a different antibody. These hybrid sequences were named by combining the names of the parent antibodies such that the first letter represented the antibody from which the CDR1 was derived and the last letter represented the antibody from which both the CDR2 and 3 were derived. Thus, hybrid B33 contains CDR1 from B3 and CDR2 and 3 from 33H11 whereas hybrid BU contains CDR1 from B3 and CDR2 and 3 from UK4. The hybrid V_L chain BU had previously been made by Dr J. Haley (Haley et al., 2004), and B33 was made by a similar method by Dr L. Mason.

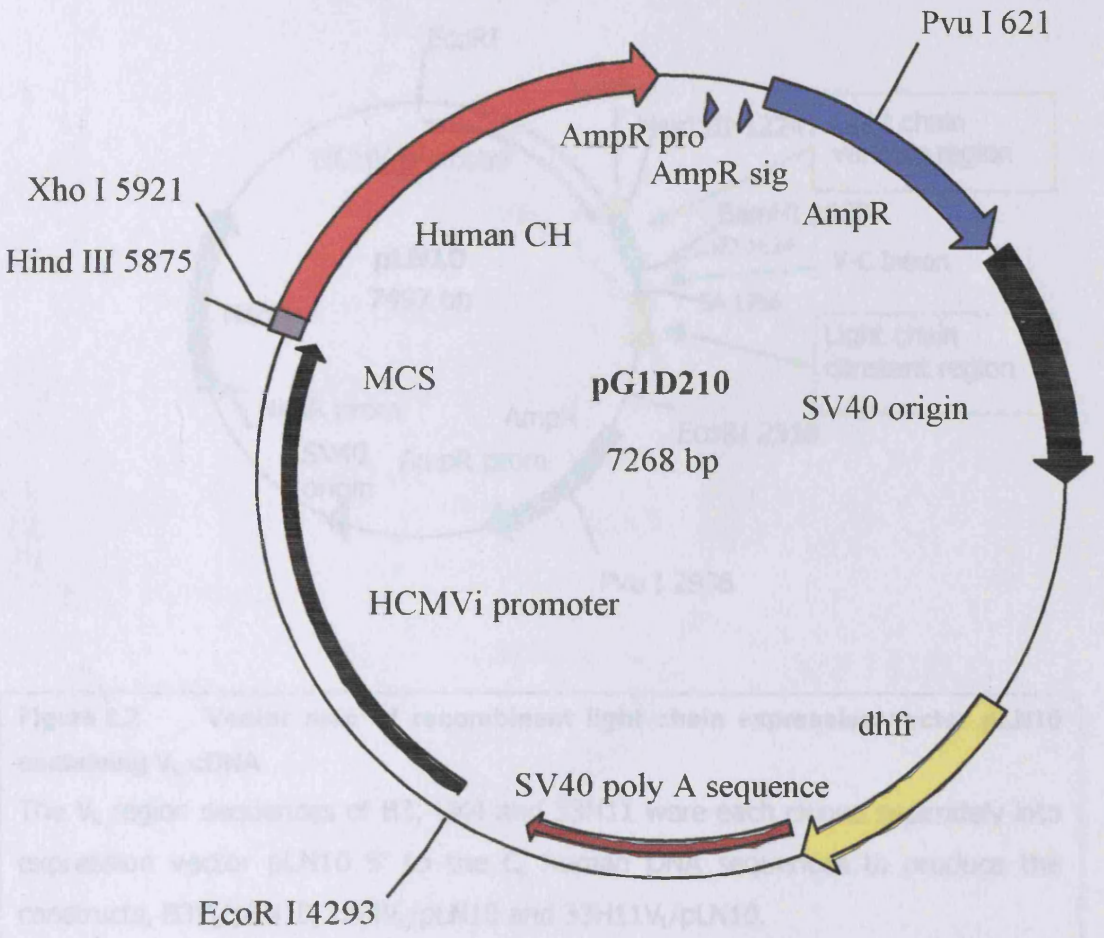


Figure 2.1 Plasmid map of expression vector pG1D210

Abbreviations: - HCMVi – improved human cytomegalovirus promoter containing intron A; Human CH - cDNA version of the gamma-1 constant region; MCS - multiple cloning site; AmpR pro – ampicillin internal promoter; AmpR sig – ampicillin signal sequence; AmpR - Ampicillin resistance gene; dhfr - dihydrofolate reductase gene.

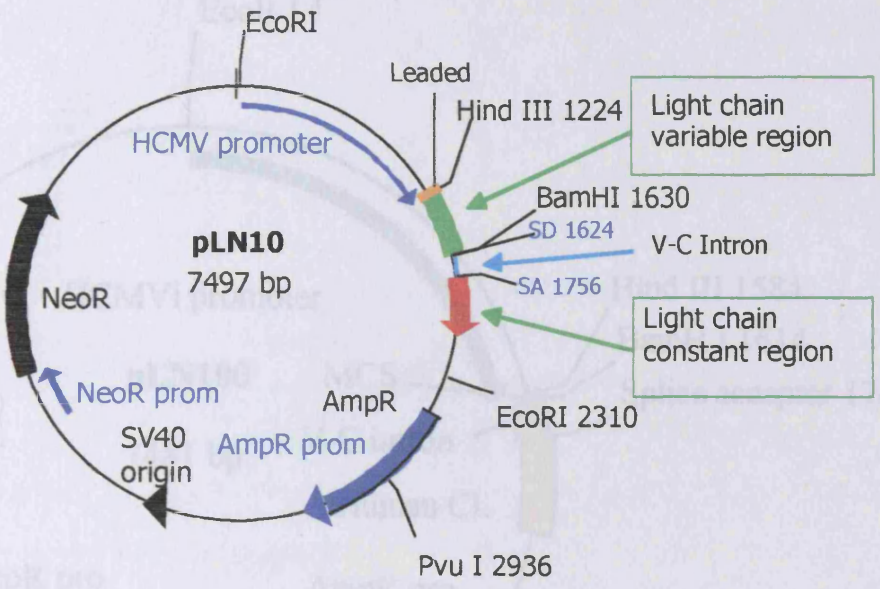


Figure 2.2 Vector map of recombinant light chain expression vector pLN10 containing V_L cDNA

The V_L region sequences of B3, UK4 and 33H11 were each cloned separately into expression vector pLN10 5' to the C_L human DNA sequences to produce the constructs, B3V_L/pLN10, UK4V_L/pLN10 and 33H11V_L/pLN10.

pLN10 contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant Ig gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and the bacterial *neo* gene coding sequence driven by the SV40 early promoter to act as a dominant selectable marker during stable transformation. pLN10 also contains an ampicillin resistance gene (AmpR) driven by an internal promoter to enable it to be selected in *E. coli*. The Ig V region sequence is immediately followed by a splice donor (SD) site whilst the Ig C region sequence is immediately preceded by a splice acceptor (SA) site. The purpose of these splice sites was to facilitate the transfer of the V region cassette (V region sequence and Ig leader sequence) into the expression vectors.

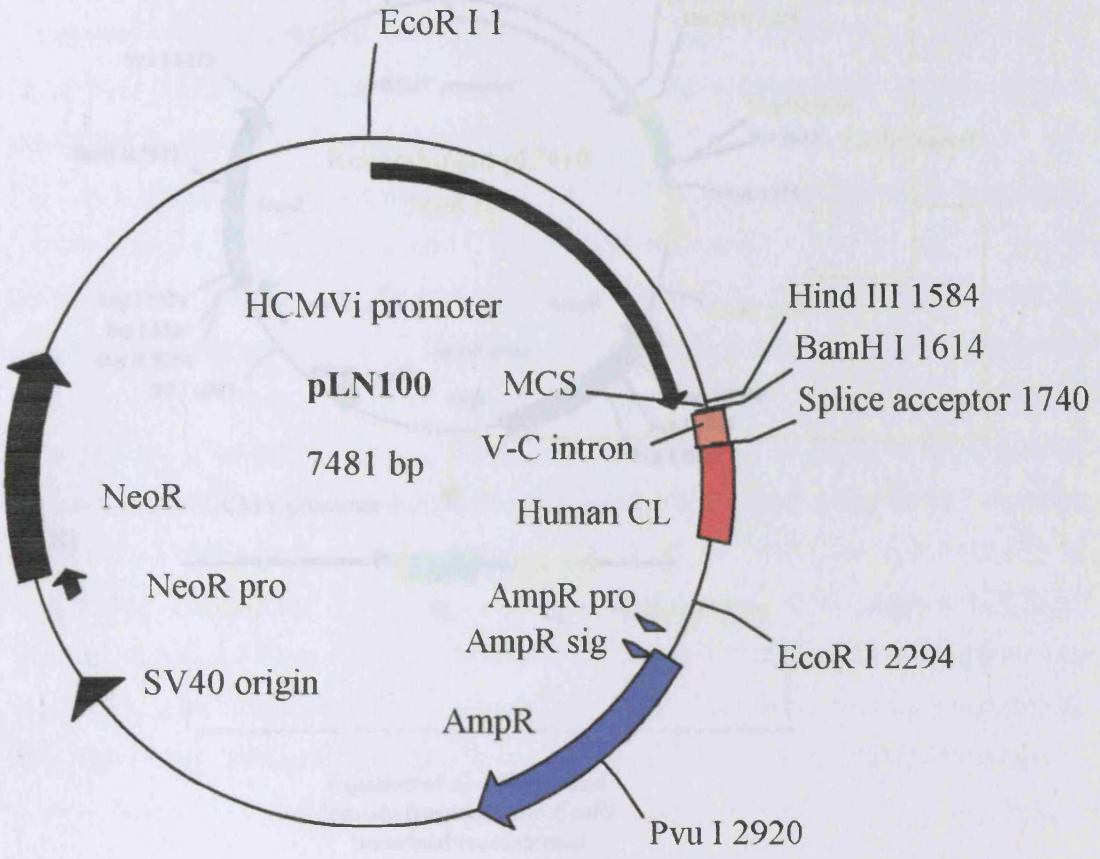


Figure 2.3 Plasmid map of expression vector pLN100

Abbreviations: - HCMVi – improved human cytomegalovirus promoter containing intron A; Human CL - cDNA version of the human lambda-2 constant region; MCS - multiple cloning site; V::C intron - region of plasmid DNA between variable (V) domain insert and constant (C) region treated like a V::C intron by mammalian cells; AmpR pro – promoter for ampicillin resistance gene; AmpR sig – signal sequence for ampicillin resistance gene; AmpR - ampicillin resistance gene; NeoR pro – promoter for neomycin resistance gene; NeoR – neomycin resistance gene.

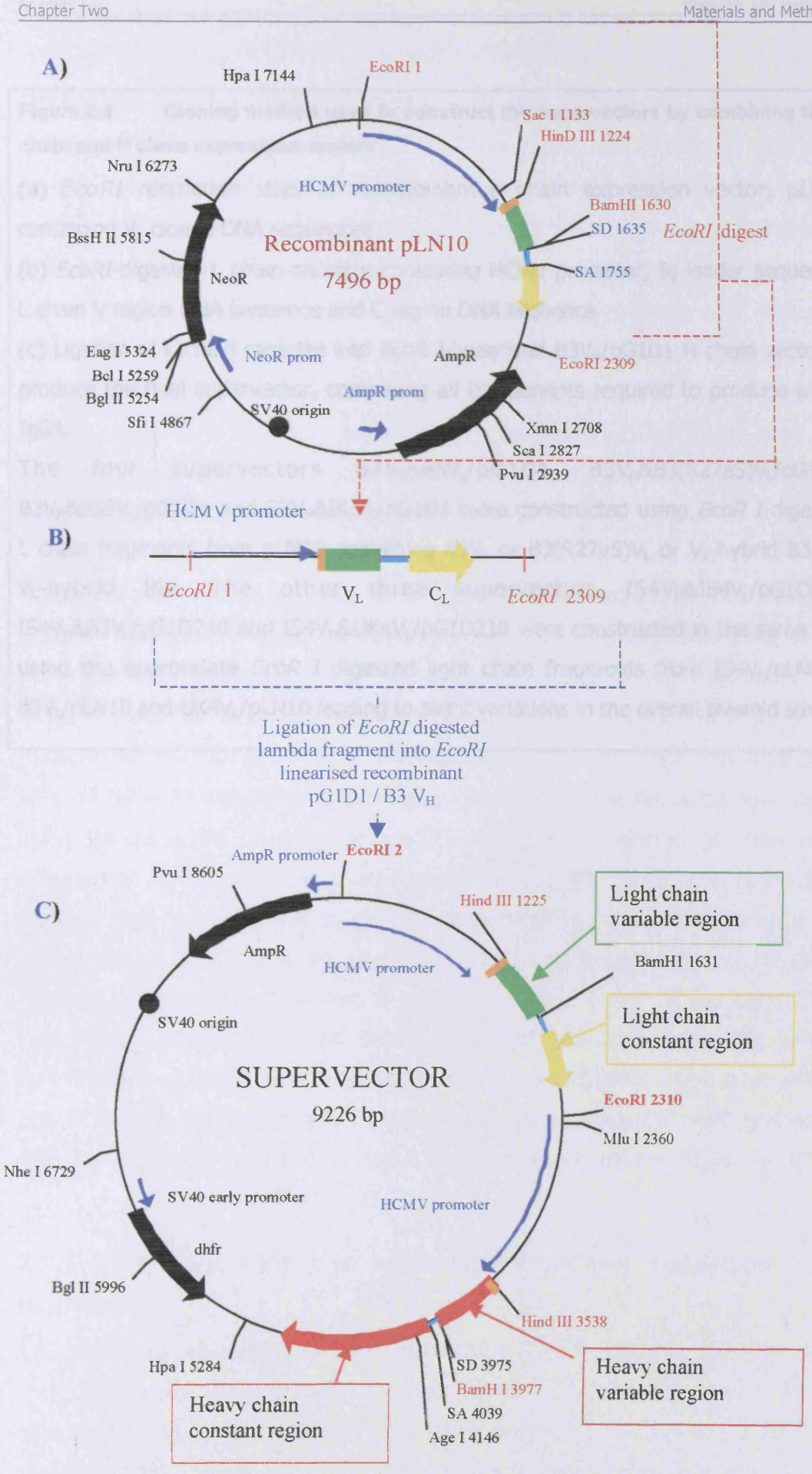

A)
Hpa I 7144
EcoRI 1
Sac I 1133
HinD III 1224
Nru I 6273
HCMV promoter
BamHI 1630
SD 1635
EcoRI digest
BssH II 5815
Recombinant pLN10
7496 bp
NeoR
SA 1755
Eag I 5324
NeoR prom
AmpR
EcoRI 2309
Bcl I 5259
Bgl II 5254
AmpR prom
SV40 origin
Sfi I 4867
Xmn I 2708
Sca I 2827
Pvu I 2939
HCMV promoter
B)
EcoRI 1
VL
CL
EcoRI 2309
Ligation of EcoRI digested
lambda fragment into EcoRI
linearised recombinant
pG1D1 / B3 VH
C)
AmpR promoter
EcoRI 2
Pvu I 8605
Hind III 1225
Light chain
variable region
AmpR
HCMV promoter
BamH1 1631
SV40 origin
Light chain
constant region
SUPERVECTOR
9226 bp
EcoRI 2310
Mlu I 2360
Nhe I 6729
SV40 early promoter
HCMV promoter
dhfr
Bgl II 5996
Hind III 3538
Hpa I 5284
SD 3975
Heavy chain
variable region
BamH I 3977
SA 4039
Heavy chain
constant region
Age I 4146

Figure 2.4 Cloning method used to construct the supervectors by combining the L chain and H chain expression vectors

(a) *EcoRI* restriction sites in recombinant L chain expression vector, pLN10 containing V_λ cloned DNA sequences

(b) *EcoRI*-digested L chain cassette containing HCMV promoter, Ig leader sequence, L chain V region DNA sequence and C region DNA sequence

(c) Ligation of L chain cassette into *EcoR I*-linearised $B3V_H$/pG1D1 H chain vector to produce the final supervector, containing all components required to produce whole IgG1.

The four supervectors $B3V_H$&$B3V_L$/pG1D1, $B3V_H$&B3(R27aS)V_L/pG1D1, $B3V_H$&$B33V_L$/pG1D1 and $B3V_H$&BUV_L/pG1D1 were constructed using *EcoR I* digested L chain fragments from pLN10 containing $B3V_L$ or B3(R27aS)V_L or V_L-hybrid B33 or V_L-hybrid BU. The other three supervectors $IS4V_H$&$IS4V_L$/pG1D210, $IS4V_H$&$B3V_L$/pG1D210 and $IS4V_H$&$UK4V_L$/pG1D210 were constructed in the same way using the appropriate *EcoR I* digested light chain fragments from $IS4V_L$/pLN100, $B3V_L$/pLN10 and $UK4V_L$/pLN10 leading to slight variations in the overall plasmid size.

2.2 *Small-scale extraction of recombinant V_H/V_L vectors*

DH5α-strain *E.coli* containing the recombinant V_H/V_L vectors was stored in media containing 15% (v/v) glycerol at -80°C. Under sterile conditions (i.e. next to a Bunsen burner flame), a sterilised wire loop was used to streak the glycerol-stored cultures onto a Luria-Bertani (LB) agar plate supplemented with 50μg/ml ampicillin and incubated at 37°C in a dry incubator overnight. The plates were transferred to the refrigerator and kept at 4°C. 5ml LB medium supplemented with 100μg/ml ampicillin in a 50ml falcon tube was inoculated with a colony using a sterile wire loop and incubated at 37°C in a shaking incubator overnight. 5ml of LB medium (supplemented with 100μg/ml ampicillin) that had not been inoculated were also incubated under the same conditions. This aliquot was a control to ensure that irrelevant bacteria did not contaminate the LB medium.

Recombinant vector was extracted from overnight *E. coli* bacterial cultures using the QIAGEN QIAprep® Miniprep kit (Crawley, West Sussex, UK) according to the manufacturer's instructions. A bacterial pellet was obtained by centrifugation (Sorvall RT-7) at 3000rpm for 10 minutes at 4°C and dried by inversion. The pellet was resuspended in 250μl of buffer P1 and transferred to a 0.5ml microfuge tube. Then 250μl of buffer P2 was added and the tube inverted six times. Following this 350μl of buffer N3 was added, mixed by inversion and then centrifuged for 10 minutes. All subsequent centrifugation steps were carried out at 13000rpm in a microcentrifuge (Biofuge *Pico*) for one minute at room temperature (RT). Supernatant from this step was decanted into a QIAprep spin column and centrifuged. DNA bound to the QIAprep spin column was washed by adding 0.75ml of Buffer PE and centrifuged. Flow through was discarded and the column centrifuged. After placing the QIAprep spin column in a clean 1.5ml microfuge tube recombinant DNA was eluted by adding 50μl of autoclaved water to the centre of each QIAprep column, which was left to stand for one minute before being centrifuged. Eluted DNA was then stored at -20°C.

2.2.1 Restriction digest of recombinant vectors containing V_H/V_L fragments

$B3V_H$ and $IS4V_H$ were digested with *Hind III/Xho I.* $B3V_L$, $33H11V_H$, $33H11V_L$, $IS4V_L$ and $UK4V_L$ were digested with *Hind III/BamH I.* V_H3-23/neSLE122H45.4 was digested with *Sal I/Age I* and V_H3-23/pG1D210 was digested with *Hind III/Xho I.* For the confirmation digests 1μg of plasmid DNA and 10 units of each restriction enzyme

were usually incubated in a total volume of 20μl for one hour with the appropriate buffer, at 37°C as recommended by the manufacturer (Promega, Southampton, UK).

The recipe for a typical small-scale restriction digest would be as follows:

Plasmid DNA	5μl (1μg DNA)
Enzyme	1μl (10u/μl)
10x Buffer	2μl
ddH_2O	to 20μl

2.2.2 Separation of DNA fragments by agarose gel electrophoresis

In order to separate the DNA fragments resulting from the above restriction digests, the products were run on a 1% agarose gel. One litre of TAE buffer was prepared, 100mls of which were then removed and mixed with 1g of low melting point agarose. The solution was heated in a microwave oven until all the agarose had dissolved and then allowed to cool until hand hot when 2μl of 10mg/ml ethidium bromide was added. Ethidium bromide molecules intercalate between base pairs in the DNA double helix rendering it fluorescent when viewed under ultraviolet (UV) light.

The open ends of a gel tray (Bio-Rad) were closed off with tape and the agarose solution poured in. Plastic combs were then placed in the gel tray to create wells in the agarose as it solidified and any air bubbles were removed. Once the gel had set the comb and tape were carefully removed and the gel tray placed in an electrophoresis tank (Bio-Rad) to which the remaining 900mls of TAE buffer was added.

To each 10μl of sample to be loaded onto the gel 2μl of loading buffer were added. This buffer contains Ficoll which increases the density of the sample, ensuring the DNA in each sample sinks evenly into the well. The buffer also colours the sample with a dye (xylene cyanole) that moves towards the anode at a predictable rate, allowing progress through the gel to be monitored. Between 5 and 10μl of each sample were then added to each well and a voltage of 100 Volts applied for one hour. The gel was then removed from the electrophoresis tank and examined under UV light. The position of the band is visualised to assess the size and the relative intensity of each band which reflects the amount of DNA present by comparison with

a standard DNA molecular weight marker (1Kb DNA ladder and the λ/*Hind III* marker) loaded into another well in the same gel. Photographs of the gel were taken using the Genesnap programme (Syngene, Cambridge, UK) and printed onto paper.

2.2.3 Ethanol Precipitation of DNA

In the presence of salt (monovalent cations such as Na^+) and at a temperature of -20°C or less, absolute ethanol efficiently precipitates nucleic acids. Therefore 0.1 volume of 3M Sodium Acetate, pH 5.2 and 2.5 volumes of 100% ethanol were added to the DNA preparation in a 1.5ml microcentrifuge tube. This tube was then placed in a –80°C freezer for one hour to precipitate the DNA.

To collect the precipitate the preparation was firstly spun in a microcentrifuge (Biofuge *Fresco*) for 10 minutes at 13000rpm at 4°C. Supernatant was then discarded and the pellet washed with 1ml 70% ice-cold ethanol. The solution was centrifuged again for 10 minutes in a microcentrifuge at 13000rpm at 4°C and then air dried on the bench for 10-30 minutes. Care was taken to ensure that no ethanol remained as this could affect subsequent procedures such as ligations, restriction digests or transfections.

Plasmid DNA intended for transfection into eukaryotic cells was resuspended in sterile autoclaved ddH_2O at a concentration of 2μg/μl under sterile conditions in a tissue culture hood and stored at -20°C.

2.3 Cloning of VH3-23 sequence into pG1D210 Vector

2.3.1 Amplification of V_H by PCR

In this step, template DNA encoding the V_H3-23 is selectively amplified by specific PCR primers.

At the 5′ end the following primer was used to create a *Hind III* site:

5′ GAGCTAAGCTTGCCGCCACCATGGGTTGGTCATGTATCATCCTT 3′

At the 3′ end the following primer was used to create a *Xho I* site:

5′ GGTCGACGCTCTCGAGACGGTGACCAG 3′

The following reaction mixture was made in a thin-walled PCR tube: 1μl template DNA, 10μl DNA Polymerase 10x Buffer B, 8μl 25mM $MgCl_2$, 1μl of a mixture of dATP, dCTP, dGTP and dTTP containing each dNTP at a concentration of 25mM, 1μl forward primer (final concentration 1μM), 1μl reverse primer (final concentration 1μM), autoclaved ddH_20 to 80μl and 1μl (5u/μl) *Taq* Polymerase.

As a negative control no template DNA was added to one reaction mixture. Each mixture was then pulse spun and placed into a PCR machine programmed as follows:
Initial denaturation at 95°C for 10 minutes
Then 30 cycles as follows:
94°C for 1 minute
Annealing temperature at 42°C for one minute
72°C for one minute
Then a final elongation phase at 72°C for 5 minutes.

The presence of PCR product of the expected length (420-440bp) was confirmed by gel electrophoresis through a 2% agarose gel (see section 2.2.2).

2.3.2 Purification of amplified V_H DNA from agarose gels

PCR sample was purified from agarose gel using the QIAquick gel extraction (Qiagen, Crawley, West Sussex, UK) kit. An excised band of DNA of up to 0.4g was placed in a 1.5ml microfuge tube and 1ml of buffer QG added. The mixture was then incubated at 55°C for 10 minutes until all agarose had dissolved. 700μl of this solution was then added to a spin column placed in a collection tube and centrifuged (Biofuge *Pico*) at 13000rpm for 10 seconds. Flow through was discarded from the collection tube and the procedure repeated until all of the initial mixture had been passed through the spin column. A further 500μl of buffer QG was added to each spin column and centrifuged at 13000rpm for 10 seconds. After discarding the flow through the column was washed twice with 500μl of buffer PE and centrifuged as above. Each column was then placed into a fresh 1.5ml microfuge tube and dried under a lamp for 5 minutes. Elution of DNA from each column was performed by addition of 50μl of solution EB (10mM Tris-HCl pH 8.5), allowing the column to stand for 1 minute followed by centrifugation as above. The product was then stored at -20°C.

2.3.3 Ligation of amplified DNA into pG1D210 Vector

Purified PCR product from step 2.3.2 was then ligated into (50ng) pG1D210 Vector by TA cloning at a range of 3:1 to 1:3 insert:vector molar ratios. To 10μl of pG1D210 Vector: purified PCR product mix, 2μl of 10x ligase buffer, 7.4μl of autoclaved water and 0.6μl (3u/μl) of T4 DNA ligase were added. Samples were incubated for 3 hours at RT or overnight at 4°C. The ligation product was used to transform DH5α cells made competent by the following method.

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

Bacterial cultures were prepared from glycerol stocks of DH5α-strain *E.coli* as described in section 2.2 except that no antibiotic was added to agar plates or LB medium. This culture was then grown overnight in a shaking incubator (250rpm) at 37°C. 100μl of this overnight culture were added to 100ml LB medium in a 2.5L sterile (autoclaved) conical flask and then returned to the shaking incubator (250rpm) for approximately three hours.

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

The culture was then divided between two 50ml sterile falcon tubes, placed on ice for five minutes and centrifuged at 3000rpm for 10 minutes in a Sorvall RT-7 Plus at 4°C. The supernatant was discarded and the pellet in each tube was resuspended in 20mls ice-cold transformation buffer 1 and incubated on ice for 5 minutes. Each tube was again centrifuged at 3000rpm for 10 minutes at 4°C and the supernatant discarded. Each pellet was then resuspended in 1ml of transformation buffer 2 and incubated on ice for 15 minutes. 100μl of this suspension were then aliquoted into pre-chilled 1.5ml microcentrifuge tubes and stored at -80°C or used directly for transformations.

2.3.5 Transformation of competent E.coli cells

5μl of ligation mixture was added to 100μl of competent E.coli cells and incubated on ice for 30 minutes. A heat shock at 42°C for 60 seconds was then applied, following which the cells were incubated in 800μl of LB at 37°C for one hour. The cell suspension was then centrifuged (Biofuge *Pico*) at 3000rpm for 10 minutes, most of the supernatant poured off and the pellet resuspended in the remaining supernatant. This suspension was then spread on an appropriate antibiotic containing LB agar plate. After drying on the bench for 15 minutes the plates were then incubated at 37°C overnight and finally stored at 4°C. The following day antibiotic resistant clones were picked, grown in 5ml LB with appropriate antibiotic and miniprep plasmid DNA produced as in section 2.2.

2.4 Site directed PCR mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene) was found to be a convenient method to produce the following:

Arg to Ser mutations-

at position 53 in B3V_H CDR2

at 96, 97, 100 and 100g in IS4V_H CDR3

at position 94 in UK4V_L

Arg to Asp mutation-

at position 53 in B3V_H CDR2

Arg to Lys mutation-

at position 53 in B3V_H CDR2

Ser to Arg mutation-

at position 53 in V_H3-23 CDR2

The basic procedure requires a dsDNA vector containing an insert of interest and the design of two specific oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo* DNA polymerase. This enzyme has a 6-fold higher fidelity in DNA synthesis than *Taq* DNA polymerase; hence has a low potential for generating further mutations during PCR. Incorporation of the oligonucleotide primers generates a mutated plasmid. Following PCR the product is treated with *DpnI*, an endonuclease specific for methylated DNA produced by DH5α *E.coli*, which thus digests the parental DNA template but not the mutated plasmid.

Vector DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells.

The mutagenic oligonucleotide primers were designed according to manufacturers specifications. Primers were between 25 and 45 bases in length with a melting temperature (T_m) greater than or equal to 78°C. The formula used to calculate T_m is:

$T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$

Where - N is the primer length in bases

Values for %GC and %mismatch are whole numbers

% GC = the percentage of GC bases in the primer sequence

% mismatch = the number of deletions / primer sequence x 100

The specific primers used designed according to the formula are shown in Table 2.1.

Purified plasmid DNA was obtained as described in section 2.2. A series of PCR reactions were then set up containing 5ng, 10ng, 20ng, and 50ng concentrations of plasmid DNA with the appropriate mutagenic primers (Table 2.1) and reaction solutions provided in the QuikChange site-directed mutagenesis kit (Stratagene). 1μl (5-50ng) of plasmid DNA was mixed with 5μl 10x reaction Buffer, 1μl of dNTP mix, 5μl forward primer (125ng), 5μl reverse primer (125ng), 1μl *Pfu Turbo* DNA Polymerase (2.5 units/μl) and autoclaved ddH_2O to a final volume of 51μl.

As a negative control no plasmid DNA was added to one reaction mixture. Each mixture was then pulse spun and placed into a PCR machine programmed according to the manufacturers instruction with the extension time of each reaction calculated at two minutes per kb of plasmid length. Cycling parameters used were as follows:

Initial denaturation at 95°C for 30 seconds

Then 12 cycles as follows:

- Denaturation- 95°C for 30 seconds
- Annealing- 55°C for 1 minute
- Extension- 68°C for 18 minutes for sample reactions and 12 minutes for control reaction

The reaction was then held at 4°C until the samples were removed.

Once the PCR step had been carried out the methylated template DNA containing the undesired sequence was digested by the addition of 1μl (10u) of *Dpn* I restriction

enzyme directly to each amplification reaction. The resultant reaction mixture was mixed gently by pipetting up and down several times, after which it was pulse spun in a bench centrifuge and incubated at 37°C for one hour.

	PRIMER SEQUENCE
1.	Forward 5`GCTCATATACAAACAGCGTGTTTTTCGGCGGAGGG 3`
2.	Back 5`CCCTCCGCCGAAAAACACGCTGTTTGTATATGAGC 3`
3.	Forward 5`GGGTCTCAACTATTAGTGGTAGTGGTAGTAGCACATAC 3`
4.	Back 5`GTATGTGCTACTACCACTACCACTAATAGTTGAGACCC 3`
5.	Forward 5`GGGTCTCAACTATTAGTGGTAATGGTAGTAGCACATAC 3`
6.	Back 5`GTATGTGCTACTACCATTACCACTAATAGTTGAGACCC 3`
7.	Forward 5`GGGTCTCAACTATTAGTGGTAAGGGTAGTAGCACATAC 3`
8.	Back 5`GTATGTGCTACTACCCTTACCACTAATAGTTGAGACCC 3`
9.	Forward 5`CTCAGCTATTAGTGGTCGTGGTGGTAGCACATAC 3`
10.	Back 5`GTATGTGCTACCACCACGACCACTAATAGCTGAG 3`
11	Forward 5`GCGAGAGGAAGTAGTGACGTTAGTGGAGTTCTTTGGAGGGGCAGTCATGACTAC 3`
12.	Back 5`GTAGTCATGACTGCCCCTCCAAAGAACTCCACTAACGTCACTACTTCCTCTCGC 3`
13.	Forward 5`GCGAGAGGAAGTCGAGACGTTAGTGGAGTTCTTTGGAGGGGCAGTCATGACTAC 3`
14.	Back 5`GTAGTCATGACTGCCCCTCCAAAGAACTCCACTAACGTCTCGACTTCCTCTCGC 3`
15.	Forward 5`GCGAGAGGACGTAGTGACGTTAGTGGAGTTCTTTGGAGGGGCAGTCATGACTAC 3`
16.	Back 5`GTAGTCATGACTGCCCCTCCAAAGAACTCCACTAACGTCACTACGTCCTCTCGC 3`
17.	Forward 5`CGCGAGAGGAAGTAGTGACGTTCGGG 3`
18.	Back 5`CCCGAACGTCACTACTTCCTCTCGCG 3`
19.	Forward 5`CTGCGCGAGAGGAAGTCGAGACGTTCGG 3`
20.	Back 5`CCGAACGTCTCGACTTCCTCTCGCGCAG 3`
21.	Forward 5`CGCGAGAGGACGTAGTGACGTTCGGGGAG 3`
22.	Back 5`CTCCCCGAACGTCACTACGTCCTCTCGCG 3`
23.	Forward 5`GGACGTCGAGACGTTAGTGGAGTTCTTTGGAGG 3`
24.	Back 5`CCTCCAAAGAACTCCACTAACGTCTCGACGTCC 3`
25.	Forward 5`CTTTGGAGGGGCAGTCATGACTACTTTGAC 3`
26.	Back 5`GTCAAAGTAGTCATGACTGCCCCTCCAAAG 3`

Table 2.1 Sequences of primers used in site directed mutagenesis

Mutagenic primers with sites of point mutation shown in bold. Primers 1+2 were used to mutate Arg94 to Ser in UK4V_L. Primers 3+4 were used to mutate Arg53 to Ser in B3V_H. Primer 5+6 were used to mutate Arg53 to Asn in B3V_H. Primers 7+8 were used to mutate Arg53 to Lys in B3V_H. Primers 9+10 were used to mutate Ser53 to Arg in V_H3-23. Primers 11-26 were used to mutate Arg to Ser in IS4V_H at the following positions; primers 11+12 mutated Arg 96, 97, 100 and 100g to create V_Hx; primers 13+14 mutated Arg 96, 100 and 100g to create V_Hi,iii&iv; primers 15+16 mutated Arg 97, 100 and 100g to create $V_H$ii,iii&iv; primers 17+18 mutated Arg 96 and 97 to create V_Hiⅈ primers 19+20 mutated Arg 96 to create V_Hi; primers 21+22 mutated Arg 97 to create $V_H$ii; primers 23+24 mutated Arg 100 to create $V_H$iii; primers 25+26 mutated Arg 100g to create $V_H$iv.

A separate 50μl aliquot of XL1-Blue supercompetent cells was then transformed with 1μl of the *Dpn I* treated DNA from each control, sample reaction and transformation control plasmid supplied by Stratagene. Each reaction was kept on ice for 30 minutes and then a heat shock of 42°C was applied for 45 seconds with recovery on ice for a further two minutes; 0.5ml of NZY^+ medium preheated to 42°C was added to each transformation reaction and incubated at 37°C with shaking.

The purpose of the control was to test the efficiency of mutant plasmid generation. The control plasmid contains a stop codon (TAA) at the position where a glutamine codon (CAA) would normally appear in the β-galactosidase gene. XL1-Blue supercompetent cells transformed with this control plasmid appear white on LB-ampicillin agar plates, containing IPTG and X-gal, because β-galactosidase gene activity has been obliterated. The oligonucleotide control primers create a point mutation on the control plasmid that reverts the T residue of the stop codon (TAA) at amino acid 9 of the β-galactosidase gene to a C residue, to produce the glutamine codon (CAA) found in the wild-type sequence. Following transformation, colonies can be screened for the β-galactosidase (β-gal^+, blue) phenotype.

Colonies were picked from the sample transformation plates into 5ml LB cultures containing 50μg/ml of ampicillin and kept overnight in a 37°C shaking incubator. The following day glycerol stocks were made and plasmid DNA purified (see section 2.2) and screened for the presence of the desired mutation by sequencing. The primers used for sequencing the constructs are shown in Table 2.2.

PRIMERS	SEQUENCES
NEO A (binds to pG1D1 and pLN10 30bp 5` of insert)	5`CTCCATAGAAGACACCG3`
HCMV1 (binds to pG1D210, pLN100 and pKN100 50bp 5` of insert)	5`GACTAACAGACTGTTCC3`
HUG1 (binds to pG1D210 90bp 3` of insert)	5`TTGGAGGAGGGTGCCAAG3`
DONOR (binds to pLN100 80bp 3` of insert)	5`CAAAGTTCTGCCCTTGG3`

Table 2.2 Primers used in sequencing of mutations

2.5 Large scale extraction of DNA for transfection into COS-7 cells

The yield of plasmid DNA from a miniprep was insufficient for electroporation into COS-7 cells therefore large-scale extraction to produce 100-500μg of DNA per preparation was carried out using the Qiagen Plasmid Maxi purification buffers and protocol. A single colony of recombinant DNA in final expression vector was picked from a freshly prepared LB/Ampicillin plate to inoculate a starter culture of 5ml LB/Ampicillin medium. This culture was incubated for approximately eight hours at 37°C with vigorous shaking at 300rpm. One ml of the starter culture was then diluted into 500ml of LB/Ampicillin medium and incubated at 37°C for 16 hours overnight with vigorous shaking at 300rpm. Following which bacterial cells were harvested by centrifugation at 9000 rpm in a Sorvall RC26 Plus (SLA 3000 rotor) for 15 minutes at 4°C. Supernatant was discarded and the bacterial pellet resuspended in 10mls of ice cold buffer P1 and 10mls of buffer P2 was added. After mixing and incubation at RT for 5 minutes 10mls of chilled buffer P3 was added. The mixture was kept on ice for 20 minutes then centrifuged at 12000rpm in a Sorvall RC26 Plus (SS-34 rotor) for 30 minutes at 4°C. Supernatant containing plasmid DNA was promptly removed and re-centrifuged for 15 minutes at 4°C. A Qiagen tip 500, containing DNA binding resin, was equilibrated by the addition and free gravity flow of 10mls Buffer QBT. Supernatant was then applied to the Qiagen tip and allowed to enter and bind DNA to the resin by free gravity flow while all other components pass through. The DNA was then washed by passing 30 ml of buffer QC through the tip twice and eluted with 15 ml of buffer QF. To precipitate the DNA 10.5 ml (0.7 volumes) of RT isopropanol was added to the eluted DNA, mixed and then centrifuged at 3000rpm (in a Sorvall RT7 Plus) for 45 minutes at 4°C. Following which supernatant was carefully decanted and the DNA pellet washed with 5 ml of RT 70% ethanol and centrifuged at 3000rpm (in a Sorvall RT7 Plus) for 15 minutes at 4°C. Then supernatant was discarded, the pellet dried under a vacuum for 2-5 minutes and then redissolved in 1ml of autoclaved water.

2.5.1 Quantification of dsDNA concentration

dsDNA concentrations were quantified in two ways; either by running the linearised DNA samples alongside molecular weight markers of known DNA concentration on an agarose gel and comparing the intensity of the bands or by using ultraviolet absorbance spectrophotometry. The amount of ultraviolet light absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample.

Absorbance was measured at 260nm, at which wavelength an absorbance (A_{260}) of 1.0 corresponds to 50μg of double-stranded DNA per ml.

Ultraviolet absorbance was also measured at the wavelength 280nm in order to check the purity of the DNA preparation. The ratio of the absorbance at 260nm and 280 nm (A_{260}/A_{280}) of a pure sample of DNA would be 1.8. A ratio of less than 1.8 indicated that the preparation was contaminated with protein.

2.6 Tissue culture

All tissue culture preparation was carried out in a sterile tissue culture hood. Any buffers, solutions and materials required were kept in the tissue culture laboratory and solely used for this work. All plastic ware was supplied by Nunc, VWR International, Leicester, UK. All protocols used for tissue culture and transfection were obtained from AERES Biomedical, Mill Hill, UK.

2.6.1 Transient Expression System in COS-7 cells

COS-7 cells are immortal monkey kidney cells, which express the T antigen of the human polyoma virus SV40 constitutively. The T antigen binds to the SV40 origin to stimulate replication. Plasmids used in this study all contain the SV40 origin (which is not otherwise present in COS-7 cells) hence will be replicated actively in COS-7 cells and achieve a high copy number. This expression system is thus ideal for achieving efficient transient expression of heavy and light chains.

2.6.2 Defrosting COS-7 cells aliquots stored in liquid nitrogen

When using COS-7 cells in a transient expression system, efficiency is optimised by transfecting the cells at as low a passage number as possible. Therefore a master cell bank of the COS-7 cell line stored in liquid nitrogen was essential.

Aliquots of these cells were frozen in growth medium 1 containing 10% DMSO (D-5879, Sigma, Poole, UK) which is cryoprotective during freezing but toxic to the cells once they have thawed. Therefore once defrosted at 37°C, the cell aliquot was immediately added to 10ml fresh, pre-warmed COS-7 growth medium 1 and spun down at 700rpm (in a Sorvall RT7 Plus) for 10 minutes at RT. The medium was

discarded whilst the pellet was resuspended in 10ml COS-7 growth medium 1 and transferred to an 80cm^2 culture flask containing a further 10ml of the same medium.

2.6.3 Maintenance of COS-7 cells in culture

COS-7 cells were maintained in a continuous culture by changing the growth medium 1 every 3-4 days. All solutions and media to be used were initially placed in a 37°C water bath for 10 minutes. The 80cm^2 or 175cm^2 tissue culture flask containing COS-7 cells was removed from the incubator. Old medium was removed by aspiration and the remaining cells adherent to the floor of the flask washed by adding 10mls sterile HBS to remove any remaining fetal calf serum. HBS was then removed and 3mls of Trypsin-EDTA solution added. The flask was shaken gently to allow Trypsin-EDTA to fully cover the floor of the flask. Trypsin-EDTA was then left in contact with the cells for two minutes in a 37°C incubator to release all cells adherent to the floor of the flask. At the end of this time the flask was tapped and viewed under the microscope to make sure at least 80% of the cells had become detached. 10mls of growth medium 1 were then added to the flask and gently shaken to inactivate the Trypsin-EDTA solution. This mixture was then split from an 80cm^2 to a 175cm^2 flask or from one 175cm^2 to three new 175cm^{2}flasks and growth medium 1 added to a final volume of 20ml for an 80cm^2 flask or 25ml for a 175cm^2 flask. The flasks were then placed horizontally in a 37°C incubator in which the concentration of CO_2 was 5% and checked daily to assess growth.

2.6.4 Freezing down COS-7 cells for storage in liquid nitrogen

To create a cell bank, cells of a low passage number growing in an exponential phase were trypsinised as above then spun at 700 rpm (in a Sorvall RT7 Plus) for 10 minutes at RT. Following a cell count (carried out as described below) the cells were resuspended in the appropriate volume of growth medium 1 containing 10% DMSO, to give a concentration of 1 x 10^7 cells/ml. 1.5ml aliquots were then put in cryovials, immediately placed at -80°C overnight and then transferred to liquid nitrogen.

2.6.5 Preparation of COS-7 cells for electroporation

The COS-7 cell line was maintained as detailed in section 2.6.3 and used in a transfection at as low a passage number as possible. The cells were split 24 hours prior to transfection to ensure exponential growth as follows:

24 hours prior to the electroporation, the COS-7 cells were split from one 175cm^2 to two new 175cm^2 flasks, as described above. The cells were incubated overnight at 37°C in 5% CO_2. The number of flasks of COS-7 cells that were prepared was dependent on the number of electroporations that were planned (approximately one 175cm^2 flask per electroporation).

The following day the cells in each flask were washed with HBS and trypsinised as described above. Ten mls of warm medium 1 were added to each flask and the entire contents then transferred to a sterile 50ml centrifugation tube. The cells were then centrifuged at 700rpm (in a Sorvall RT7 Plus) for five minutes at RT. The supernatant was discarded and the pellet of COS-7 cells resuspended in 20-50 mls of autoclaved PBS according to the size of the cell pellet. A cell count was performed upon a 200µl aliquot of the suspension to estimate the number of cells present.

2.6.6 Trypan blue viable cell count

Trypan blue is one of several stains that can be used in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes whilst dead (non-viable) cells do. The following protocol derived from Sambrook et al. (2001) was used:

The cells were re-suspended in HBS. 0.5ml of 0.4% Trypan blue solution (w/v) was transferred to a 5ml bijou tube. 0.3ml of HBS and 0.2ml of the cells suspension were then added to the bijou, therefore producing a dilution factor of five. The preparation was mixed thoroughly and then allowed to stand for 5 to 15 minutes. (If cells are exposed to Trypan blue for extended periods of time, viable cells as well as non-viable cells may begin to take up the dye).

A haemocytometer was used to count the cells. Each square of the haemocytometer with the cover slip in place represented a total volume of 0.1mm^3 or 10^{-4}cm^3. Since

$1cm^3$ is equivalent to 1ml, the subsequent cell concentration per ml and the total number of cells were determined as follows:

CELLS per ml = the average count per square x dilution factor x 10^4 (count 10 squares)
TOTAL CELLS = cells per ml x the original volume of fluid from which cell sample was removed

This procedure was repeated in order to ensure accuracy.

The remainder of the suspension was again centrifuged at 600rpm for 10 minutes at RT. Supernatant was carefully removed by aspiration and the cell pellet resuspended in the appropriate volume of PBS to give a final concentration of 1 x 10^7 cells per ml.

2.6.7 Transfection of COS-7 cells with recombinant expression vectors by electroporation

Recombinant expression vector DNA was removed from storage at –20°C and thawed at RT. 10μg of H chain and 10μg of λ L chain expression vector DNA were added to a Gene Pulser® (0.4cm gap) disposable electroporation cuvette. Alternatively, 20μg of single expression vector containing both the H and L chain sequence ("supervector") was added to a cuvette. A separate cuvette was used for each supervector or combination of heavy and light chain vectors. 7 x 10^6 washed COS-7 cells (700μl) were then added to each and gently mixed with the DNA by slowly pipetting the contents up and down three times. To ensure that no arcing could occur during the electroporation, it was ensured that there was neither liquid on the side of the cuvette nor air bubbles nor clumps of cells in the cuvette. Using the Bio-Rad Gene Pulser® apparatus a 1.9kV and 25μFarad capacitance pulse was delivered to each cuvette. A negative control cuvette containing COS-7 cells only and no DNA was prepared and subjected to an electroporation pulse in exactly the same way.

The transfected COS-7 cells were allowed to recover at RT for 10 minutes. The contents of each cuvette were then gently transferred into a 10cm diameter tissue culture dish containing 8ml of fresh pre-warmed (post-electroporation) COS-7 growth medium 2. The cells were incubated in 5% CO_2 at 37°C for 72 hours.

2.6.8 Treatment of COS-7 cell supernatants with *DNase I* (RNase –free)

After 72 hours post-electroporation the supernatants were removed from the cells and transferred to sterile 15ml falcon tubes. The supernatants were centrifuged for 10 minutes at 2000rpm in a Sorvall RT7 Plus at RT in order to pellet all the cell debris present. Following centrifugation, the COS-7 cell supernatants were immediately transferred to fresh 15ml falcon tubes and treated with *RNase-free DNase I* as follows:

DNase I (RNase-free) was added to the COS-7 cell supernatants to produce a final concentration of 7.5u *DNase I* per ml of supernatant (i.e. 6µl of 10u/µl *DNase I* was added to 8ml supernatant). The tubes were inverted to mix and then incubated at 37°C in a dry incubator for one hour. After the one-hour incubation, EDTA (pH 8.0) to a final concentration of 15mM was then added to inactivate the enzyme plus 5µl of 1M sodium azide solution (final concentration 0.5mM). The function of the EDTA is to chelate the magnesium ions in the solution required by the *DNAse I* to function. Sodium azide was added to the supernatants to prevent fungal growth. The supernatants were then stored at 4°C for testing by ELISA.

The treatment of the supernatants with *DNase I* (*RNase*-free) was essential since 80% of cells die during electroporation, thus releasing their contents (including DNA) into the supernatant. Whole IgG antibody molecules that were produced due to the transfection may then have bound this DNA, forming immune complexes. Thus making them undetectable by whole IgG ELISA (see section 2.7.1) as had been demonstrated previously by (Rahman et al., 1998). Therefore the *DNase I* was used to digest this DNA thus breaking down the immune complexes leaving the antibody molecules free to be detected by ELISA.

2.6.9 Concentration of whole IgG in cell supernatants

Due to the relatively low concentrations of IgG produced by the transient expression system, the IgG levels in the COS-7 supernatants were concentrated prior to ELISA analysis. The supernatants were concentrated by using Centricon-YM30 centrifugal concentrators (30 000 MW cut-off) according to the manufacturer's instructions. Two mls of supernatant were added to each sample reservoir coupled with its filtrate vial and centrifuged at 5000rpm in a Sorvall RC26 Plus (SS-34 rotor) at RT for 30 minutes. Further supernatant was then added to the sample reservoir and

subsequent centrifugation steps performed for 40-50 minutes. This process was repeated until all but the final one ml of supernatant had passed through the membrane of the sample reservoir. A retentate vial was then placed over the sample reservoir the unit inverted and centrifuged at 3000rpm for 2 minutes in a Sorvall RC26 Plus (SS-34 rotor) to transfer concentrated antibody from the membrane into the remaining supernatant. Concentrated samples were then stored at 4°C.

2.7 Analysis of COS-7 supernatant IgG by immunoassays

In all of the different ELISAs performed the 96 well plates were supplied by Nunc, VWR International, Leicester, U.K. and OD measured by the Genios plate reader (Tecan, Reading, U.K.). Each plate was marked vertically to divide it into two halves; the test half and the control half. The 36 wells at the edge of the plate were not used and henceforth all descriptions of the plate refer to the inner 60 wells only.

2.7.1 Detection of total whole IgG molecules in COS-7 supernatants by ELISA

A "Sandwich" ELISA (Enzyme-linked Immunosorbent assay) was used to detect the total whole IgG molecule concentration of each supernatant. Goat anti-human IgG (Fc fragment specific) was dissolved in bicarbonate (BIC) buffer to give a working concentration of 400ng/ml. 50µl of this solution were added to each well of a *Maxisorp* plate in the left (test) half of the plate. 50µl of BIC buffer alone were added to the wells in the right (control) half of the plate. Each plate was then covered and incubated overnight at 4°C. After this the plates were washed three times with PBS/0.1% Tween and 100µl of PBS containing 2% BSA were added to each well to block non-specific binding of Ig to the plastic. The plates were incubated for one hour at 37°C and then washed three times with PBS/0.1% Tween.

In order to dilute COS-7 cell supernatant in SEC buffer serially, such that for each well in the test half of the plate there was a well in the control half containing the same supernatant at the same dilution the following procedure was performed. 50µl of SEC buffer was added to each well in both halves of the plate except the top wells. 100µl of neat COS-7 cell supernatant was added to the top well and diluted down the column such that each well contained supernatant at half the

concentration of the well above. Purified human IgG_λ of known concentration (100ng/ml or 10ng/ml) was loaded and diluted serially in the same way upon each plate to allow the construction of a standard curve relating optical density (OD) to Ig concentration. The plates were incubated for one hour at 37°C and then washed three times with PBS/0.1% Tween.

To detect bound antibody, 50μl goat anti-human λ alkaline phosphatase conjugate (diluted 2500-fold in SEC dilution buffer) was added to the wells and incubated for one hour at 37°C. After one hour, the plates were washed three times with PBS/0.1% Tween and once with BIC buffer. One tablet of *p*- nitrophenyl phosphate substrate was dissolved in 5ml BIC buffer supplemented with 10μl of 2mM $MgCl_2$. 50μl of this solution was added to each of the wells on the plate. The plates were then incubated at 37°C to allow a yellow colour to develop in each well. The optical density of the reaction was read after 60 minutes at 405nm. The final reading was calculated by subtracting the OD value of each control well from the OD value of the same sample in the corresponding test well. This ensures that only conjugate bound directly to IgG in the supernatant that had been captured by the anti-Fc IgG, would contribute to the result.

The binding of the whole IgG molecules in the supernatants to the plate depended on the presence of the Fc region whereas detection by the alkaline phosphatase conjugate depended on the presence of the λ light chain, therefore this method only detected whole IgGλ antibodies.

2.7.2 Detection of anti-dsDNA activity in COS-7 supernatants by ELISA

Direct ELISAs were used to detect the anti-dsDNA activity of the whole IgG antibody molecules in the supernatant.

To prepare the pure DNA, calf thymus DNA (Sigma, Poole, UK) was dissolved in TE buffer (pH 7.5) on a rotator at RT overnight. The DNA solution was then subjected to a phenol: chloroform: isoamyl alcohol (25:24:1) extraction and ethanol-precipitated (as before, see section 2.8.3). The dried pellet of DNA was resuspended in TE buffer (pH 7.5) and then sonicated at high frequency for one minute. ssDNA was removed by passing the solution through a 0.45μg Millex-HA filter that contained methyl-cellulose esters. The sonication of the DNA produces a more reproducible coating of

the plate whilst there is less chance of the binding detected being due to ssDNA rather than dsDNA.

A 96 well *Maxisorp* ELISA plate was marked vertically into two halves, the test half and the control half. The test half of the plate was coated with 50μl of 10mg/ml purified dsDNA dissolved in PBS, pH 7.4, whilst the control half was coated with PBS, pH 7.4 only. The plates were incubated overnight at 4°C and then washed three times with PBS/0.1% Tween. Non-specific binding to the plate was blocked by adding 150μl of 2% casein in PBS to each well. The plates were then incubated at 37°C for one hour. The plates were washed three times with PBS/0.1% Tween.

The concentrated COS-7 supernatants were serially diluted in SEC buffer and identical concentrations of supernatant added to each half of the plate. As a positive control for experiments described in this thesis, human serum (known to contain anti-dsDNA IgG) was serially diluted from 1/400 to 1/32000 onto the plate. The plates were incubated for one hour at 37°C and then washed six times with PBS/0.1% Tween.

To detect bound antibody, a goat anti-human IgG alkaline phosphatase conjugate (A3150, Sigma, Poole, UK) was diluted to 1/1000 in SEC buffer and 50μl were then added to each well. The plates were incubated for one hour at 37°C and then washed three times with PBS/0.1% Tween and once with BIC buffer. Bound antibodies were detected using the same substrate as described for the human IgG ELISA (section 2.6.1).

2.7.3 Detection of anti-cardiolipin antibodies in COS-7 supernatants by ELISA

Wells in the test half of a *Polysorp* ELISA plate were coated with 50μl of CL (diluted to 50μg/ml in ethanol) and 50μl of ethanol alone were added to wells in the control half. The plates were incubated uncovered overnight at 4°C. Each plate was then washed three times with PBS and 100μl of PBS containing 10% FCS (Invitrogen) were added to each well to block non-specific binding of Ig to the plastic. After incubation at 37°C for one hour, plates were washed three times with PBS.

Each concentrated COS-7 supernatant to be tested was serially diluted in PBS containing 10% FCS. An identical concentration of supernatant was loaded into a well in the test half and a corresponding well in the control half of the plate. Serum containing polyclonal antibodies known to bind CL was loaded as a positive control to each plate. The total volume in each well was 50μl. Plates were then incubated for 90 minutes at 37°C after which they were washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in PBS containing 10% FCS and 50μl of this solution added to each well. After one hour at 37°C the plate was washed four times with PBS and once with BIC buffer. Addition of substrate and incubation were carried out as in section 2.7.1 with additional OD measurements taken at 30 minutes.

The standard CL ELISA was modified to remove β2GPI from the assay by replacing 10% FCS in each relevant step with 10% BSA.

2.7.4 Detection of anti-α-actinin antibodies in COS-7 supernatants by ELISA

A 96 well *Maxisorp* ELISA plate was marked vertically into two halves, the test half and the control half. The test half of the plate was coated with 50μl of 20μg/ml α-actinin dissolved in BIC, pH 9.6, whilst the control half was coated with BIC, pH 9.6 only. The plates were incubated overnight at 4°C and then washed three times with PBS/0.1% Tween. Non-specific binding to the plate was blocked by adding 150μl of 2% (w/v) BSA in PBS/0.1% Tween to each well. The plates were then incubated at 37°C for one hour. The plates were washed three times with PBS/0.1% Tween.

The concentrated COS-7 supernatants were serially diluted in the PBS/0.1% Tween and identical concentrations of supernatant added to each half of the plate. As a positive control for experiments described in this project, human serum (known to have anti-α-actinin activity) was serially diluted from 1/400 to 1/32000 onto the plate. The plates were incubated for one hour at 37°C and then washed six times with PBS/0.1% Tween.

To detect bound antibody, a goat anti-human IgG alkaline phosphatase conjugate (A3150, Sigma, Poole, UK) was diluted to 1/1000 in SEC buffer and 50μl were then added to each well. The plates were incubated for one hour at 37°C and then

washed three times with PBS/0.1% Tween and once with BIC buffer. Bound antibodies were detected using the same substrate as described for the human IgG ELISA (section 2.7.1).

2.7.5 Detection of anti-β_2GPI antibodies in COS-7 supernatants by ELISA

A *Maxisorp* ELISA plate was coated with 50μl of β_2GPI (SCIPAC), diluted to 10μg/ml in PBS, on wells in the test half and 50μl of PBS alone on the control half. Plates were covered and incubated overnight at 4°C. Following that each plate was washed three times with PBS. In order to block non-specific binding 50μl of 0.25% gelatin/PBS was added to each well. After incubation at 37°C for one hour plates were washed three times with PBS.

Antibody samples were serially diluted in 0.1% BSA/PBS and then loaded upon each half of the plate, as well as a positive control of serum containing polyclonal anti-β2GPI antibodies leaving a final volume in each well of 50μl. Plates were then incubated for two hours at 37°C after which they were washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in PBS containing 0.1% BSA/PBS and 50μl of this solution added to each well. A final incubation for one hour at 37°C was performed and the plate washed four times with PBS and once with bicarbonate buffer. Addition of substrate and incubation were carried out as in section 2.7.1 with additional OD measurements taken at 30 minutes.

2.7.6 Detection of monoclonal aPL binding to purified recombinant his_6-tagged DI

DI of human β_2GPI with a C-terminal his_6-tag was expressed in bacteria by Dr Y. Ioannou. By virtue of the incorporated C-terminal his_6-tag, a direct ELISA could be performed by recombinant DI binding to a nickel-coated microwell plate. This process would orientate DI correctly and allow binding to various aPL to be assayed (Iverson et al., 1998).

Recombinant his_6-tagged DI was diluted to a concentration of 75μg/ml using PBS and 50μl added to the wells of a nickel chelate-coated microwell plate. The test half was coated with DI and the control half with PBS. Plates were incubated at RT for

two hours and then washed three times with PBS, blocked with 100μl 0.25% gelatin (Sigma, Poole, UK) in PBS, incubated for a further one hour at RT and washed three times with PBS.

Each monoclonal aPL sample to be tested, either affinity purified antibody or derived from CHO supernatant, was serially diluted in SEC. An identical concentration of primary antibody was loaded into a well in the test half and a corresponding well in the control half of the plate. Serum containing polyclonal antibodies known to bind CL and β_2GPI was diluted 100x with SEC buffer and loaded as a positive control to each plate. The total volume in each well was 50μl. Plates were then incubated for one hour at 37°C after which they were washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in SEC buffer and 50μl of this solution added to each well. After one hour at 37°C the plate was washed three times with PBS and one time with BIC. Addition of substrate was carried out as in section 2.7.1, the plates incubated at RT and OD measurements taken at 30 minutes and one hour.

2.7.7 Detection of monoclonal aPL binding to PS or PC

Wells in the test half of a *Polysorp* ELISA plate were coated with 50μl of PS or PC (diluted to 50μg/ml in methanol and chloroform 4:1) and 50μl of methanol and chloroform alone were added to wells in the control half. The plates were incubated uncovered overnight at 4°C. Following that, the plates were washed twice with PBS and blocked with 100μl of 10% FCS/ PBS at RT for one hour. The plates were then washed twice with PBS. Test samples were serially diluted with 10% FCS/ PBS. An identical concentration of supernatant was loaded into a well in the test half and a corresponding well in the control half of the plate. Polyclonal serum was applied to each plate as a standard. The total volume in each well was 50μl. The plates were then incubated for 90 minutes at RT and washed with PBS three times. Goat anti-human IgG alkaline phosphatase conjugate (Sigma A3150) was diluted 1 in 1000 in 10% FCS/PBS, 50μl of this solution added to each well and incubated for 1 hour at RT. The plates were washed with PBS three times and once with BIC. 50μl/well substrate of 1 tablet PNP in 5ml BIC and 10ul 2M $MgCl_2$ was applied per plate and incubated at 37°C. Plates were read at 405nm at 15, 30, 45 and 60 minutes.

2.7.8 Detection of anti-PS-PT antibodies by standard ELISA

Each well of a *Polysorp* ELISA plate was coated with 30µl of PS (diluted to 50µg/ml in methanol:chloroform 4:1) and the plates were incubated uncovered overnight at 4°C. Following that, the plates were washed with TBS/Tween 0.05%/5mM $CaCl_2$ three times and blocked with 150µl of TBS containing 1% BSA and 5mM $CaCl_2$ (BSA-Ca) at RT for one hour. The plate was washed with TBS/Tween 0.05%/5mM $CaCl_2$ three times. 50µl human PT (10µg/ml) in BSA-Ca was added to the test half of the plate and BSA-Ca alone to the control half and the plate incubated for one hour at 37°C. The plates were then washed three times with TBS/Tween 0.05%/5mM $CaCl_2$ and test samples were serially diluted in BSA-Ca, 50µl/well. Polyclonal serum was applied to each plate as a standard. The plates were incubated for 60 minutes at RT and washed three times with TBS/Tween 0.05%/5mM $CaCl_2$. Goat anti-human IgG alkaline phosphatase conjugate (Sigma A3150) was diluted 1 in 1000 in BSA-Ca, 50µl of this solution added to each well and incubated one hour at RT. The plates were then washed with TBS/Tween 0.05%/5mM $CaCl_2$ three times and once with BIC. 50µl/well substrate of 1 tablet PNP in 5ml BIC and 10ul 2M $MgCl_2$ was applied per plate and incubated for 30-60 minutes at RT. Plates were read at 405nm at 15, 30, 45 and 60 minutes.

2.8 Stable expression system in CHOdhfr⁻ cells

The CHO cell line used lacks a functional *dhfr* gene. The enzyme dihydrofolate reductase is responsible for the intracellular reduction of dihydrofolic acid to tetrahydrofolic acid, which is an important co-factor in the synthesis of nucleic acid precursors. To survive the CHO*dhfr*⁻ cell line required exogenous hypoxanthine (adenine), glycine, proline and thymidine for survival. All of these nutritional requirements were provided via non-selective medium.

If however, these CHO*dhfr*⁻ cells were successfully transfected with expression vectors (e.g. a supervector) containing a functional *dhfr* gene the cells would be transformed to a *dhfr*⁺ phenotype. Thus, allowing them to grow in selective medium that was depleted of ribonucleosides and deoxyribonucleosides (but not proline as all CHO cells are proline-dependent even in the presence of *dhfr* gene). Consequently CHO*dhfr*⁻ cells were used to provide an expression system in which the genes required to produce IgG could be co-transfected with the d*hfr* gene.

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

2.8.1 Maintenance of CHO*dhfr*$^-$ cells prior to electroporation

The CHO*dhfr*$^-$ cells were grown in pre-warmed non-selective growth medium A (described in section 2.1.13) in 175cm^2 flasks prior to transfection and split every 3 – 4 days keeping their passage number to a minimum prior to transfection. A master cell bank was created and the cells were defrosted and frozen down as described in section 2.6.2 and 2.6.4 using growth medium A containing 10% DMSO.

2.8.2 Preparation of CHO cells for electroporation

The cells were split 24 hours prior to transfection to ensure exponential growth. Flasks containing confluent CHO cells were trypsinised and then divided equally between three 175cm^2 flasks, each containing 25ml of fresh, pre-warmed CHO*dhfr*$^-$ (non-selective) growth medium A. One flask was required for each supervector to be tested. The cells were incubated overnight at 37°C in 5% CO_2.

The following day the cells in each flask were washed, trypsinised, pelleted and resuspended in sufficient autoclaved PBS to give a final concentration of 1 x 10^7 cells per ml.

2.8.3 Transfection of recombinant expression supervector into CHOdhfr$^-$ cells

The CHO*dhfr*$^-$ cells were electroporated (using the same method as that used for the COS-7 cells, see section 2.6.7). A 700µl aliquot of the washed CHO*dhfr*$^-$ cell suspension was pipetted into a disposable electroporation cuvette to which 20µg of thawed recombinant supervector was added. A 1.9kV and 25µF capacitance pulse was then delivered to each experimental transfection and a control containing CHO*dhfr*$^-$ cells only, using a Bio-Rad Gene Pulser® II electroporator. The cells were

then allowed to recover at RT for 10 minutes and the contents of each cuvette transferred to a separate 10cm tissue culture dish containing 8ml of pre-warmed medium A. Each dish was then incubated overnight in 5% CO_2 at 37°C.

2.8.4 Selection of transfected CHOdhfr$^-$ cells post electroporation

From this point onwards the cells were maintained in selective growth medium B (described in section 2.1.13) that lacks ribonucleosides and deoxyribonucleosides, which are absent from dialysed FCS. Thus only those cells which contain supervector, hence a functional *dhfr* gene will then be able to grow.

Following overnight growth of the transfected CHOdhfr$^-$ cells in non-selective medium A the cells in each 10cm dish were washed with 10mls of HBS, trypsinised and then pelleted at 700rpm (in a Sorvall RT7 Plus) for five minutes at RT. Cells from each transfection and control were resuspended separately in 100mls of prewarmed selective medium B before being divided equally between ten 10cm diameter tissue culture dishes.

Incubation of the cells at 37°C in 5% CO_2 then took place for 10-14 days changing the selective medium B every 3-4 days. After 10-14 days the cells on the no DNA control dish were all dead and discrete foci of transfected cells were clearly visible on all the test culture dishes without the aid of a microscope. In order to pick these foci they were visualised under a microscope and using a sterile pipette tip 200µl of foci plus medium was aspirated under direct vision and transferred into individual wells of a 24 well tissue culture plate containing 0.5ml of prewarmed medium B. A total of 48 foci were picked from each transfection (ten plates). The picked cells were then grown in the selective medium B until almost confluent (between 7-14 days), changing the media every seven days.

Once almost confluent, the medium from the individual wells of each 24 well plate was tested for whole IgG antibody production by ELISA (see section 2.6.1). Those clones producing the highest levels of antibody were selected for expansion in CHO*dhfr*$^-$ (selective) growth medium B until the cells were growing in 175cm^2 tissue culture flasks.

2.8.5 Assay of antibody production in transfected CHO*dhfr*⁻ cells

Following growth of the selected cells to just below confluent level in selective media in 175cm^2 flasks the cells were washed with 10ml HBS, trypsinised with Trypsin-EDTA and pelleted at 700rpm (in a Sorvall RT7 Plus) for five minutes at RT. Each clone of cells was resuspended in 20ml of prewarmed selective medium B and a viable cell count performed (as in section 2.5.6). The cells were then pelleted again (as above) and resuspended in sufficient prewarmed medium B to produce a viable cell count of 1x10^6 cells/ml. One ml of this solution was then added to a 10cm diameter tissue culture dish containing 9ml of prewarmed medium B and incubated at 37°C in 5% CO_2 for three days. After three days, the supernatant of these cells was collected from each 10cm dish and the concentration of intact whole IgG antibody assayed using an ELISA, as described in section 2.7.1. Cells in each 10cm dish were washed with 5ml HBS, trypsinised (2ml Trypsin-EDTA) and pelleted at 700rpm (in a Sorvall RT7 Plus) for five minutes at RT. Following this the cells were resuspended in one ml of prewarmed medium B and a viable cell count was performed as above.

From the new viable cell count and the antibody concentration in the decanted supernatants, the level of antibody production was calculated and expressed as ng/10^6 cells/day. A cell bank of the clones with the highest antibody production rates was created by suspending 1 x 10^7 cells/ml in medium B containing 10% DMSO and stored in liquid nitrogen (as in section 2.6.4) for further selection using MTX amplification.

2.8.6 Amplification of transfected CHO*dhfr*⁻ cells following electroporation and selection

Following the growth of the selected CHO*dhfr*⁻ cells in CHO*dhfr*⁻ (selective) growth medium B in a 175cm^2 flask, the cell lines producing the highest levels of IgG were trypsinised and centrifuged at 700rpm for two minutes at RT in a Sorvall RT7 Plus. The cells were resuspended in CHO*dhfr*⁻ (selective) growth medium B to produce a final viable cell count of 1 x 10^6 cells/ml. 0.5ml (i.e. 5 x 10^5 cells) of this solution was diluted in a further 49.5ml of pre-warmed 1 x 10^{-8}M amplification medium (i.e. CHO*dhfr*⁻ (selective) growth medium B supplemented with 1 x 10^{-8}M MTX). The resulting culture was then divided equally between five 10cm-diameter tissue culture

dishes, (i.e. 1 x 10^5 cells/culture dish). This dilution was required in order to produce pure foci that could be easily identified from one another on the plate.

The cells were incubated at 37°C in 5% CO_2 for 10-14 days. The amplification medium (supplemented with the appropriate concentration of MTX) was changed every 3-4 days. After 10-14 days, foci of transfected cells were easily visible in most of the culture dishes.

Approximately 24 foci were picked for each transfection (as in section 2.8.4) and were then transferred into individual wells of a 24-well tissue culture plate containing 1ml of pre-warmed CHO*dhfr*$^-$ amplification medium. These cells were allowed to grow in the amplification medium until almost confluent (usually after 7-14 days), whilst the medium was changed every 7 days or as required. Once almost confluent, the medium from the individual wells was tested for antibody production as before, using the whole IgG ELISA (see section 2.7.1).

Those clones producing the highest levels of antibody were selected for expansion in CHO*dhfr*$^-$ (selective) growth medium B with MTX until the cells were growing in 175cm^2 tissue culture flasks. The antibody production rates of individual clones (see section 2.8.5) were then determined. A cell bank of selected amplified cell lines was created. When creating a cell bank, selective pressure was maintained when freezing cells i.e. concentration of MTX was kept the same on freezing as that used in culture.

2.9 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Each gel consisted of 3mls 5% stacking gel layered on top of 5mls 15% resolving gel. The resolving gel was poured and immediately layered with isopropanol. After the gel had set, the isopropanol was removed and the stacking gel added to form the upper layer. A suitable comb was added prior to the polymerisation of the stacking gel. The composition of the resolving and stacking gels is described in Table 2.3.

Total protein samples were separated into polypeptide units by SDS-PAGE according to the method of (Laemmli, 1970). Protein samples were added in a 1:1 ratio to sample buffer (2% (w/v) SDS, 50mM Tris-HCl pH6.8, 0.1% bromophenol blue, 10% (w/v) glycerol, 100mM dithiothreitol (DTT) and denatured at 100°C for 3 minutes immediately before loading on to the gel. 20μl of sample or 5μl of molecular weight

protein marker (Prestained Protein Marker, Broad Range, NEB, Herts, UK or Rainbow™ Coloured Protein Molecular Weight Markers, AmershamBio, Bucks, UK) were loaded. SDS-PAGE gels were prepared in a vertical gel, one-dimensional, electrophoresis system (Gibco-BRL, Paisley, UK) and run in 1x running buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS pH 8.3) at 40mA/gel at RT until the dye reached the bottom of the plate.

	10mls 10% Resolving gel	**8mls 5%Stacking gel**
ddH_2O	2.3mls	5.5mls
30% acrylamide mix[1]	5.0mls	1.3mls
1.5M Tris, pH 8.8	2.5mls	-
1.0M Tris-HCl, pH 6.8	-	1.0ml
10% SDS	0.1ml	0.08ml
10% AmPS[2]	0.1ml	0.08ml
TEMED[3]	0.004ml	0.008ml

Table 2.3. Composition of resolving and stacking gels for SDS-PAGE
[1]Acrylamide bis = N,N'-Methylene-bis-acrylamide. [2]AmPS = ammonium persulfate. [3]TEMED = NNNN-tetramethylethalinediamine.

2.9.1 Coomassie Stain

This stain was used to visualise the protein bands run on an SDS-PAGE gel. The gel was transferred from the electrophoresis apparatus to a plastic container and covered with 0.2% Coomassie gel stain (0.2% (w/v) Coomassie, 7.5% acetic acid, 40% methanol, dH_2O to a volume of 1L) for eight minutes. The gel was then destained with Coomassie destain (7.5% acetic acid and 5% methanol made up to 1L with dH_2O) for three hours. The gel was then dried on a gel dryer at 60-80°C for 30 minutes and kept as a permanent record.

2.9.2 Transfer of protein to nitrocellulose and western blotting

Protein separated on SDS-PAGE gels were transferred to Hybond C membranes (Amersham, Bucks, UK) using a wet-transfer method described by Towbin, 1979. The gel and the nitrocellulose membrane were pre-soaked in transfer buffer (50mM Tris, 180mM glycine, 0.1% (w/v) SDS and 20% methanol) and blotted over one hour at 400mM / RT in a Trans-blot™ Cell (Bio Rad) according to manufacture's instructions. An ice-block was placed in the cell to avoid over-heating. The membrane was subsequently removed and dried.

2.9.3 Immunodetection of proteins on western blots

The membrane was blocked in PBS/0.1% polyxyethylene sorbitan monolaurate Tween 20/5% (w/v) skimmed milk for one hour at RT on a shaking platform. The primary antibody solution (in PBS/0.1% Tween 20/5% milk) was added and incubated for a further one hour, shaking at RT. The unbound primary antibody was then rinsed off by two 10-minute washes with PBS/0.1% Tween 20, and the appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody (in PBS/0.1% Tween 20/5% milk) added for one hour, RT on a shaker. Unbound secondary antibody was then rinsed off with two washes of 10 minutes with PBS/0.5% Tween 20. The bound HRP was then detected using chemiluminescence (ECL™, Amersham, Bucks, UK) and the resultant light emissions exposed to X-ray film for one second to one hour, depending on the strength of the signal.

2.10 Nucleosome preparation

Jurkat cells were grown in 40 ml medium until 70% confluent (7 X 10^7 cells are needed for the nucleosome preparation). The cells were pelleted in a 50 ml Falcon by centrifugation at 700rpm (Sorvall RT7 Plus) at RT for 8 minutes. The supernatant was discarded and gently resuspended in 10 ml ice cold PBS. The cells were again centrifuged at 700rpm for 8 minutes the supernatant discarded and resuspended in 2 ml ice cold Dounce buffer (adding fresh protease inhibitor at 1:100 each time) and transferred to Dounce tube. The cells were incubated in the Dounce tube on ice for 10 minutes. The cell membrane was disrupted using a fine tissue homogenizer that enables release of the nucleus without destroying it. The cells were assessed to identify those with a disrupted membrane from which nuclei could be released. When sufficiently homogenized, the vast majority of cells stain blue with trypan blue,

with the nucleus only remaining. The homogenised cells were transferred into 1.5ml eppendorf tubes and the nuclei were pelleted by centrifugation at 3000rpm (Biofuge *Fresco*), 4°C for 10 minutes. Nuclei were resuspended in a minimal volume of 10mM Tris-HCl buffer, pH 8.0, with 50mM NaCl, 5mM $MgCl_2$. The nuclei were either stored in 50% glycerol at –80°C or directly processed as described below to produce oligonucleosomes or mononucleosomes.

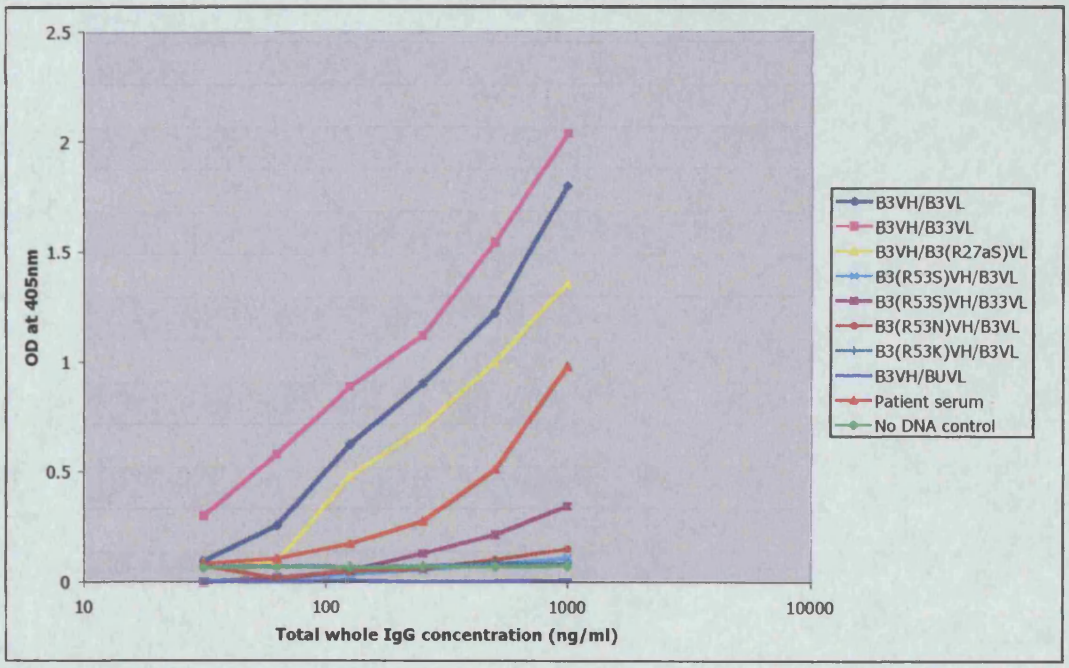
Stored nuclei were rapidly thawed in a 37°C water bath and a minimal volume of digestion buffer was added, spun at 3000rpm (Biofuge *Fresco*) for 20 mins at 4°C and the supernatant was discarded. The wash and cetrifugation steps were repeated, the nuclei were gently resuspended by flicking the tube, and 100µl ice-cold digestion buffer was added. The DNA concentration was checked by taking an aliquot in 2% SDS (ultra-pure quality) and measuring absorbance at 260nm (A_{260}) on a spectrophotometer. Further digestion buffer was added until the DNA concentration was approximately 5µg/µl.

The suspension was quickly warmed to RT and micrococcal nuclease was added (Worthington Biochem, USA) to a final concentration of 200 units/ml. Digestion was continued for 45-60 minutes on a rotator. Digestion was terminated by making up the suspension to a concentration of 2mM in EDTA (pH 8.0). Nuclei were pelletted by centrifugation in an eppendorf centrifuge at 1000rpm for 5 minutes at 4°C and the supernatant was transferred to a fresh tube on ice without disturbing the pellet. Products of this procedure were then confirmed to give the ladder of bands expected from oligonucleosomes on a gel.

2.11 Reproducibility of direct binding assays

In the results chapters to follow, single representative graphs from ELISA experiments are presented. All experiments were carried out in triplicate in separate occasions to demonstrate reproducibility and diluted serum from a patient was run on every plate as a positive control and cells transfected with no DNA, as a negative control. As an example, Figure 2.5 illustrates duplicate experiments carried on separate occasions. Overall, OD values vary in repeat experiments however, the same pattern of binding with the different antibody combinations is observed.

(A)



(B)

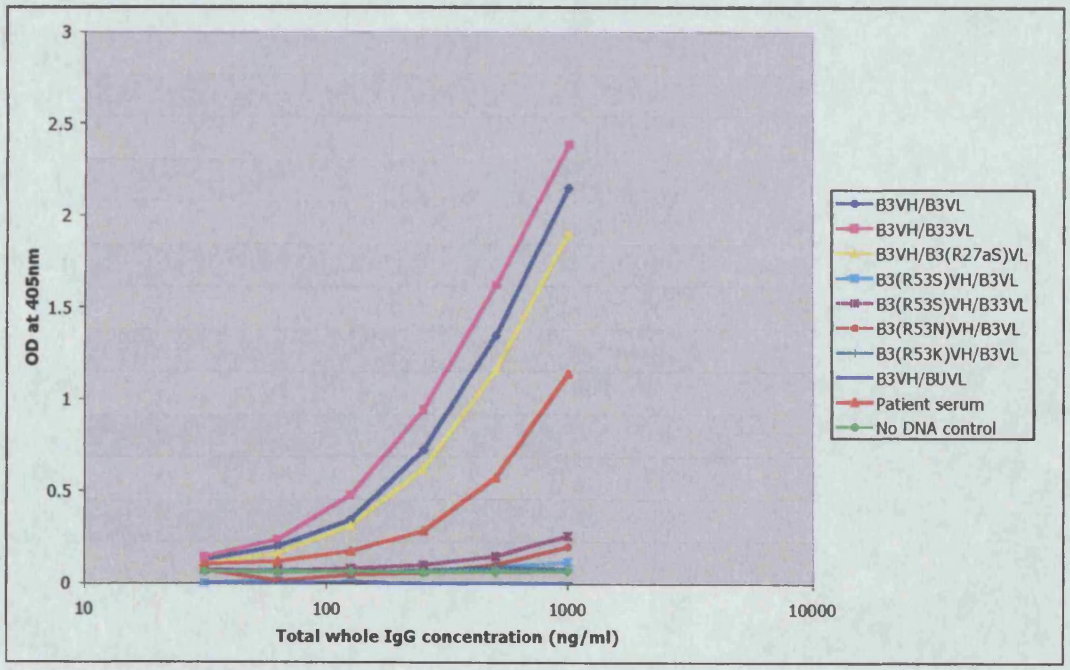


Figure 2.5 Binding of purified human IgG molecules to nucleosomes

In order to confirm the findings of the first experiment (A), this experiment was repeated on a separate occasion (B). Further discussion on the binding of the antibodies shown in this figure is detailed in Chapter 5.

CHAPTER THREE

The importance of sequence features of the heavy chain of B3 and the light chain of UK4 in binding to different antigens

CHAPTER THREE.

THE IMPORTANCE OF SEQUENCE FEATURES OF THE HEAVY CHAIN OF B3 AND THE LIGHT CHAIN OF UK4 IN BINDING TO DIFFERENT ANTIGENS

3.1 Introduction and aims of this chapter

The human monoclonal IgG1 anti-DNA antibody B3, derived from a patient with active SLE, is known to be pathogenic *in vivo* in SCID mice and contains V_H and V_L encoded by the two most commonly rearranged human V_λ and V_H genes, V_λ2a2 and V_H3-23. Therefore, it is likely to be representative of those antibodies related to disease pathogenesis in patients with SLE.

Sequence analysis of both murine and human monoclonal anti-DNA antibodies, suggests that high affinity for dsDNA is associated with the presence of certain amino acid residues within the CDRs, notably arginine, asparagine and lysine, which frequently arise at sites of somatic mutation. Expression and modification of murine and human anti-DNA antibodies in vitro has shown that substitution of arginine residues, especially those in V_HCDR3, often leads to a decrease in affinity for dsDNA.

The original computer model of the B3/dsDNA complex, previously published by our group (Kalsi et al., 1996) (shown in Figure 1.5) suggests that the dsDNA double helix lies in a groove on the surface of B3 between the V_H and V_L and that this interaction is stabilised by the presence of three arginine residues at the periphery of the antigen-binding site (R27a and R54 of the L chain and R53 of the H chain). Since R53 in B3V_H is to be found in the CDR2 region, it is important to determine whether replacing arginine by another amino acid would this hinder or block DNA binding. In this chapter, site-directed mutagenesis was used to determine whether the arginine residue at position 53 (R53) in B3V_H CDR2, which is the product of a somatic mutation of the V_H germline gene V3-23, is important in enabling B3 to bind DNA, as predicted by the model. The hypothesis is that mutation of R53 will reduce binding to DNA. Which leads to another interesting question: can binding be 'created' by adding this arginine? To investigate this question, a construct that has the wild-type

sequence of the V3-23 gene was made and the serine at position 53 was mutated to arginine.

Previous studies showed that the critical arginine residues for DNA binding in the 2a2-derived L chains of human anti-DNA antibodies B3 and 33H11, were those in $B3V_L$ CDR1 and $33H11V_L$ CDR3, whilst an arginine in $UK4V_L$ CDR3 appeared to block DNA binding of this aPL (Rahman et al., 2001). This latter finding was predicted by the computer model (shown in Figure 1.7) because the groove in the $B3V_H/UK4V_L$ model is blocked by various residues from the L chain including a bulky positively charged arginine residue at position 94 (R94). This finding leads to the question: if the arginine in $UK4V_L$ CDR3 is replaced by another amino acid, would it exhibit a positive effect on binding to DNA? To answer this question, a mutant form of $UK4V_L$ was created by site-directed mutagenesis and tested for binding to DNA.

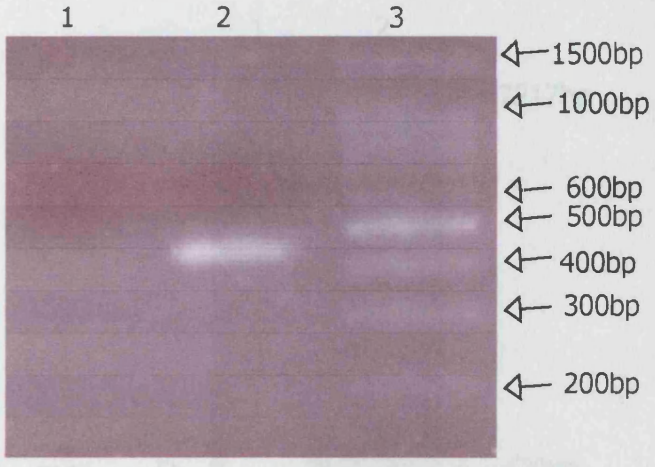
The principal aim of the experiments described in this section was to use a COS-7 transient expression system to produce a variety of IgG1 molecules and test: (1) the effect of the mutations created by site-directed mutagenesis in the H chain of B3 in order to investigate the contribution of the arginine residue in V_HCDR2 to the binding properties of the antibody; (2) the effect of the mutation created by site-directed mutagenesis in the V_H3-23 construct in order to investigate the role of the arginine residue introduced by mutagenesis in V_HCDR2; and (3) the effect of the mutation of the L chain of UK4. The effect of the $B3V_H$ mutation on binding to other clinically relevant antigens such as nucleosomes, α-actinin, and CL was tested, since previous studies have suggested cross-reactivity between DNA and these antigens. Two groups have reported that pathogenic murine anti-DNA monoclonals bind directly to α-actinin (Deocharan et al., 2002, Mostoslavsky et al., 2001).

However, the quantities of IgG produced by the transient expression system are insufficient to affinity purify the antibodies and determine their pathogenic properties. This chapter describes the production of four stable cell lines expressing some of the H/L combinations reported previously in the transient expression system.

3.2 The importance of sequence features of B3V_H in binding to dsDNA, nucleosomes, cardiolipin and alpha-actinin

3.2.1 Transfer of V_H3-23 cloned PCR fragment from neSLE122H45.4 to pG1D210

As explained in more detail in section 2.3, the appropriate V_H3-23 cloned PCR fragment was transferred from the original expression vector neSLE122H45.4, to the vector pG1D210 containing C_H sequence. Through the design of specific primers, a *Hind III* and a *Xho I* site was introduced into V_H3-23/neSLE122H45.4 so that the V_H3-23 region DNA sequence could be extracted from neSLE122H45.4 and transferred to pG1D210 with *Hind III* and *Xho I*. The V_H3-23 sequence was successfully amplified as shown in Figure 3.1 and once ligated into pG1D210, a *Hind III/Xho I* restriction digest was used to verify the presence of V_H3-23 DNA sequence in the new vector. Successful ligation of V_H3-23 into pG1D210 produced two bands of 7217 and 434 bp in length when digested with *Hind III/Xho I* upon gel electrophoresis, as shown in Figure 3.2. DNA sequencing ensured that no additional mutations had been introduced into the sequence due to PCR error and Figure 3.3 shows the representative sequencing plot.



Lanes

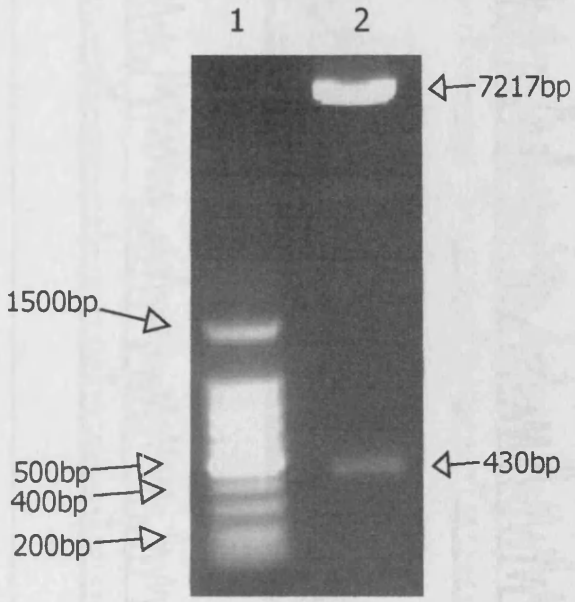
1 = Control (PCR mix without template cDNA)

2 = Template V_H3-23 cDNA

3 = 100bp DNA Ladder

Figure 3.1 PCR amplification of V_H3-23 sequence cDNA

The V_H3-23 region DNA sequence was extracted from neSLE122H45.4 and amplified by using specific PCR primers (see section 2.3.1). A 430bp band of cDNA consistent with V_H3-23 is seen in lane 2 and no band is seen in the control lane 1.



Lanes

1 = Lambda DNA/Hind III marker

2 = *Hind III/Xho I* digested VH3-23/pG1D210

Figure 3.2 Confirmation *Hind III/Xho I* restriction digest of vector pG1D210 containing V_H3-23 insert

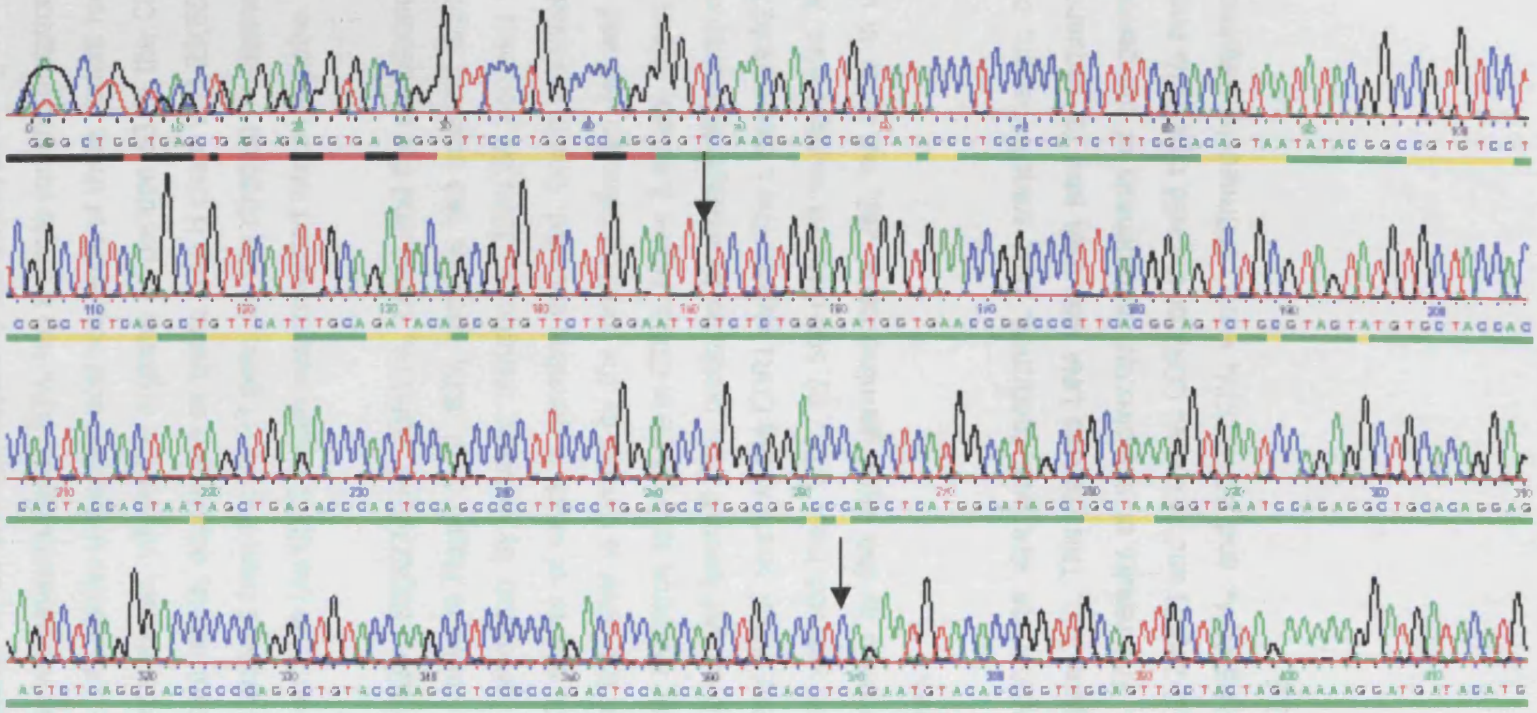


Figure 3.3 Sequencing plot confirming the cloning of V_H3-23 sequence into the pG1D210 plasmid

The sequence of the V_H3-23 insert into pG1D210 was confirmed by automated sequencing. The chromatograph trace is shown here with the black arrows indicating the beginning and the end of the V_H3-23 insert.

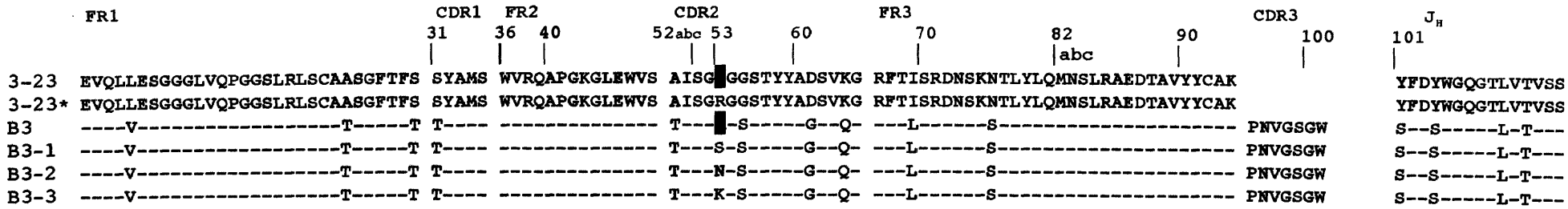
3.2.2 Sequences of heavy chains and light chains expressed and results of site-directed mutagenesis

The amino acid sequence of $B3V_H$, $B3(R53S)V_H$, $B3(R53N)V_H$, $B3(R53K)V_H$, the corresponding germline gene, V3-23 and V_H3-23(S53R) are displayed in Figure 3.4A (highlighted in red are the arginines mutated). $B3V_H$ has a single somatic mutation to arginine in CDR2. Figure 3.4A shows the amino acid sequence of three variant forms of $B3V_H$ produced by mutagenesis, where the arginine at position 53 in the CDR2 region was converted into a serine, asparagine or lysine. The H chain VH3-23(S53R) shown in the figure, was derived from the V3-23 gene, by site directed mutagenesis, where the serine at position 53 in the CDR2 region was converted into an arginine.

Amino acid sequences of $B3V_L$, $B3(R27aS)V_L$, $33H11V_L$, $UK4V_L$ and the corresponding germline gene, 2a2 are shown in Figure 3.4B. $B3V_L$ contains two adjacent arginine residues in CDR1 both produced by somatic mutations. $33H11V_L$ contains two arginine residues in CDR3, neither of which is germline encoded. One is the result of a somatic mutation whilst the other is formed by the junction between 2a2 and $J_\lambda2$. $UK4V_L$ has a single somatic mutation to arginine in CDR3. Figure 3.4B also shows the amino acid sequence of a variant form of $B3V_L$ produced by mutagenesis converting the arginine at position 27a into a serine in the CDR1 region. The L chain B3(R27aS) shown in the figure, was derived from $B3V_L$, by site-directed mutagenesis. All L chains expressed are encoded by the same germline gene, 2a2, but differ in their pattern of somatic mutation.

Plasmid DNA of the appropriate size was confirmed, post transformation, by a *HindIII/XhoI* digest (Figure 3.5). This plasmid DNA was then sent for sequencing analysis (MWG-Biotech). The results of site-directed mutagenesis of the germline V_H3-23 and its variant V_H3-23(53) and of the V_H CDR2 of the wild type $B3V_H$ and its variants $B3(R53S)V_H$, $B3(R53N)V_H$, and $B3(R53K)V_H$ were confirmed by sequencing and are shown in Figure 3.6.

A V_H



B V_L

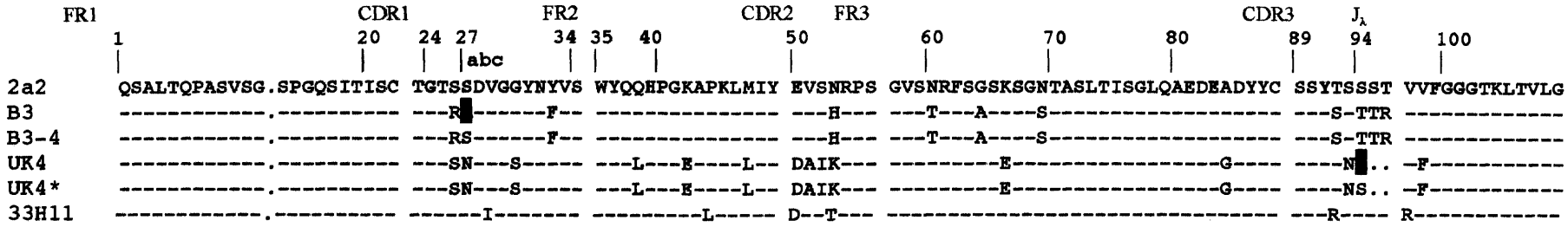
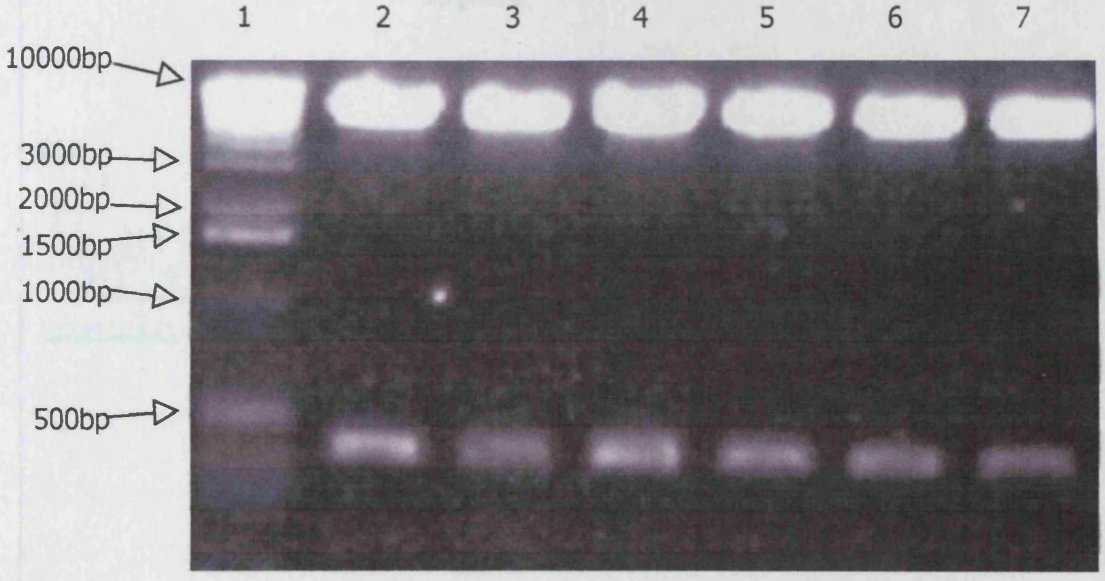


Figure 3.4 Sequence alignment of expressed V_H and V_L regions

(A) Sequences of expressed V_H regions compared to 3-23. (B) Sequences of expressed V_L regions compared to 2a2. The amino acids altered by site-directed mutagenesis are highlighted in red. The amino acids are numbered according to Kabat (Wu and Kabat, 1970). A dash indicates homology with the corresponding germline sequence. Abbreviations: FR – Framework region; CDR – Complementarity determining region; 3-23* – V_H3-23(53); B3-1 – B3(R53S)V_H ; B3-2 – B3(R53N)V_H ; B3-3 – B3(R53K)V_H; B3-4 – B3(R27aS)V_L; UK4* – UK4(R94S)V_L.



Lanes

1 = 1kb DNA ladder

2 = *Hind III/Xho I* digested B3V_H/pG1D210

3 = *Hind III/Xho I* digested B3(R53S)V_H/pG1D210

4 = *Hind III/Xho I* digested B3(R53N)V_H/pG1D210

5 = *Hind III/Xho I* digested B3(R53K)V_H/pG1D210

6 = *Hind III/Xho I* digested V_H3-23/pG1D210

7 = *Hind III/Xho I* digested V_H3-23(53)/pG1D210

Figure 3.5 *Hind III/Xho I* restriction digest of pG1D210 vector containing B3V_H and B3V_H variant constructs, V_H3-23 and V_H3-23(53) variant construct on 1% agarose gel

B3V_H/pG1D210, B3(R53SV_H/pG1D210, B3(R53N)V_H/pG1D210, B3(R53K)V_H/pG1D210, V_H3-23/pG1D210 and V_H3-23(53)/pG1D210 were digested with *Hind III* and *Xho 1* and the fragments separated on a 1% agarose gel. The actual agarose gel is shown above, indicating the expected band sizes. It can be seen from the gel that DNA bands of 7222 and 431 bp are present in each.

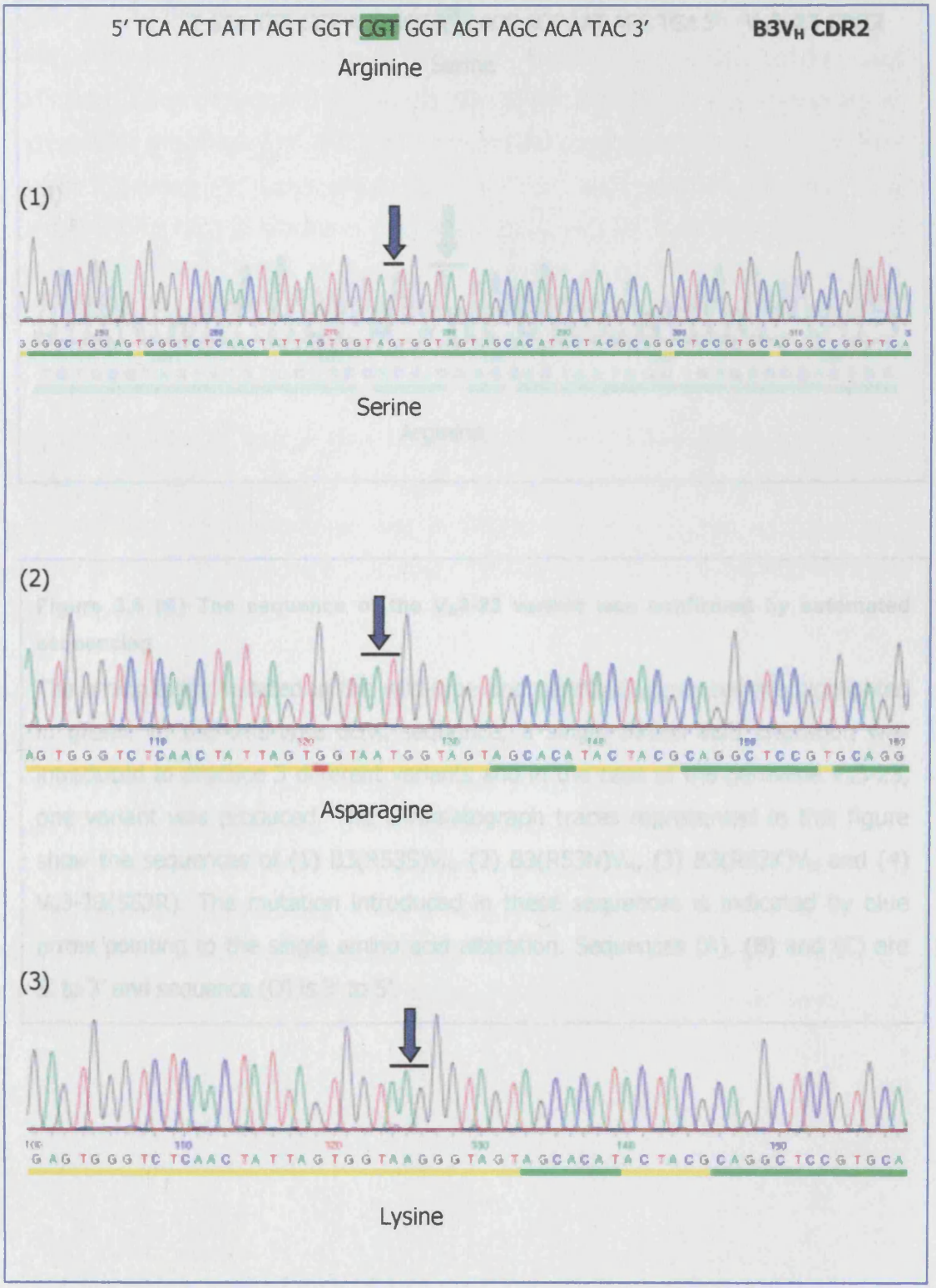


Figure 3.6 (A) The sequences of the B3V$_H$ variants were confirmed by automated sequencing (see over for legend)

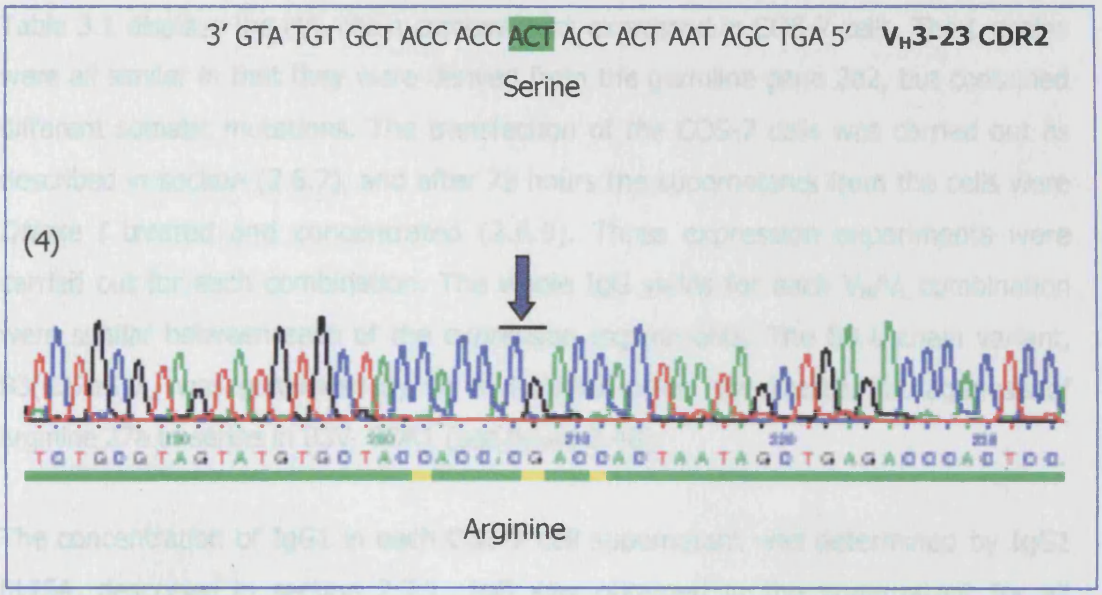


Figure 3.6 (B) The sequence of the V_H3-23 variant was confirmed by automated sequencing

The amino acids mutated in the wild-type and germline sequences are highlighted in green. In the wild-type B3V_H sequence, a single amino acid alteration was introduced to produce 3 different variants and in the case of the germiline V_H3-23, one variant was produced. The chromatograph traces represented in this figure show the sequences of (1) B3(R53S)V_H, (2) B3(R53N)V_H, (3) B3(R53K)V_H and (4) V_H3-23(S53R). The mutation introduced in these sequences is indicated by blue arrow pointing to the single amino acid alteration. Sequences (A), (B) and (C) are 5′ to 3′ and sequence (D) is 3′ to 5′.

3.2.3 Transient expression of whole IgG1 molecules in COS-7 cells

Table 3.1 displays the H/L chain combinations expressed in COS-7 cells. The L chains were all similar in that they were derived from the germline gene 2a2, but contained different somatic mutations. The transfection of the COS-7 cells was carried out as described in section (2.6.7), and after 72 hours the supernatants from the cells were *DNase I* treated and concentrated (2.6.9). Three expression experiments were carried out for each combination. The whole IgG yields for each V_H/V_L combination were similar between each of the expression experiments. The B3 L chain variant, B3(R27aS)V_L, was generated by Dr A. Rahman using site-directed mutagenesis of arginine 27a to serine in B3V_L CDR1 (see figure 3.4B).

The concentration of IgG1 in each COS-7 cell supernatant was determined by IgG1 ELISA, described in section 2.7.1. IgG was obtained in the supernatant for all combinations. Each combination was expressed in three separate occasions and similar yields of antibody were produced. The range of IgG concentrations obtained are shown in Table 3.2.

In each experiment, the negative control sample in which COS-7 cells were electroporated without any plasmid DNA contained no detectable IgG1. Combinations containing UK4V_L produced noticeably higher yields of whole IgG than the other combinations. There is no clear reason for the variable expression seen with different V_H and V_L combinations and the differences in antibody levels produced and this is well documented in this and other expression systems (Rahman et al., 1998, Rahman et al., 2001). This variation could have occurred for a number of reasons, such as variable post-electroporation COS-7 cell growth, or transfection efficiency.

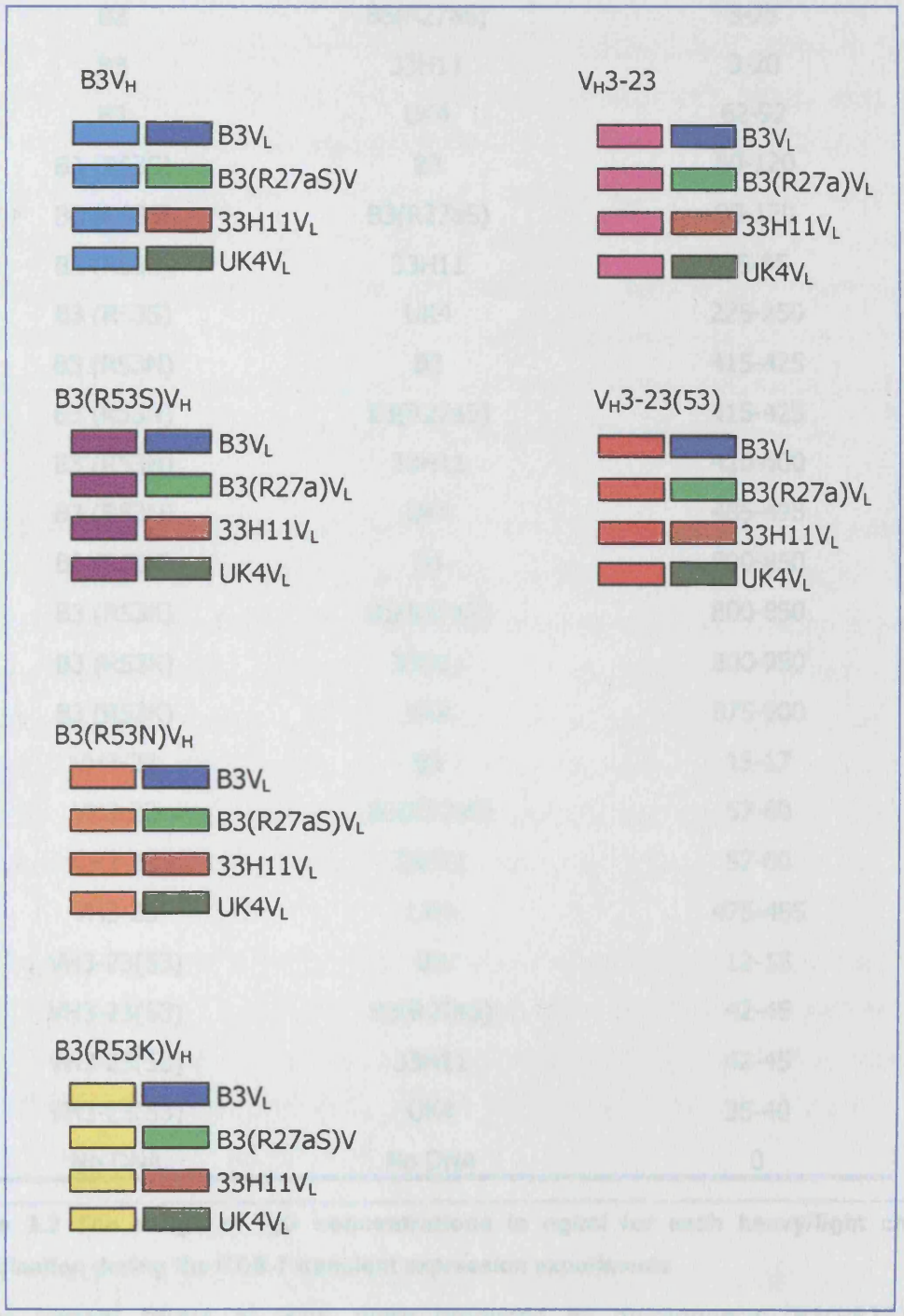


Table 3.1 The heavy/light chain combinations expressed in COS-7 cells

This schematic diagram illustrates the six groups of V_H/V_L combinations in which a different H chain is paired with the four 2a2-encoded V_L chains.

Heavy Chain	2a2-derived Light Chain	IgG concentration (ng/ml)
B3	B3	3-18
B3	B3(R27aS)	3-25
B3	33H11	3-20
B3	UK4	62-92
B3 (R53S)	B3	80-120
B3 (R53S)	B3(R27aS)	90-120
B3 (R53S)	33H11	75-95
B3 (R53S)	UK4	225-250
B3 (R53N)	B3	415-425
B3 (R53N)	B3(R27aS)	415-425
B3 (R53N)	33H11	420-600
B3 (R53N)	UK4	485-495
B3 (R53K)	B3	800-850
B3 (R53K)	B3(R27aS)	800-850
B3 (R53K)	33H11	800-950
B3 (R53K)	UK4	875-900
VH3-23	B3	13-17
VH3-23	B3(R27aS)	57-60
VH3-23	33H11	57-60
VH3-23	UK4	475-485
VH3-23(53)	B3	12-15
VH3-23(53)	B3(R27aS)	42-45
VH3-23(53)	33H11	42-45
VH3-23(53)	UK4	35-40
No DNA	No DNA	0

Table 3.2 The range of IgG concentrations in ng/ml for each heavy/light chain combination during the COS-7 transient expression experiments

Three variant forms of B3V_H were produced by mutagenesis, B3(R53S)V_H, B3(R53N)V_H, and B3(R53K)V_H, where the arginine at position 53 in the CDR2 region was converted into a serine, asparagine or lysine respectively. The H chain V_H3-23(S53R) was derived from the V3-23 gene, by site-directed mutagenesis, where the serine at position 53 in the CDR2 region was converted into an arginine. Each value in the table was derived, by reference to a standard curve of human IgG of known concentration, after the background OD values had been subtracted.

3.2.4 Results of anti-nucleosome and anti-dsDNA ELISA with COS-7 supernatants

Binding of each of the H/L chain combinations to nucleosomes is shown in Figure 3.7A and to dsDNA in Figure 3.7B. In each case, similar binding results were seen from each of the expression experiments and the figures show results of a single representative experiment. The negative control sample in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither anti-DNA activity nor anti-nucleosome activity on testing by ELISA. The positive control in each case was polyclonal serum from a patient with SLE known to bind dsDNA and nucleosomes. The same aliquot of polyclonal serum was used for all the anti-dsDNA and anti-nucleosome ELISAs.

The strongest binding to both antigens was seen with the combinations containing the wild-type sequence of $B3V_H$ when paired with $B3V_L$ or $33H11V_L$ ($B3V_H/B3V_L$ and $B3V_H/33H11V_L$). Both of these H/L chain pairs produced very small amounts of whole IgG yet clearly bound to both these antigens. The introduction of the single R to S mutation in CDR1 led to a reduction in binding to nucleosomes or dsDNA (i.e. $B3V_H/B3(R27aS)V_L$). The results of the DNA ELISA were consistent with those obtained in previous studies of expression studies of these combinations in COS-7 cells (Rahman et al., 2001, Giles et al., 2003, Haley et al., 2004).

Despite being tested at a range of concentrations much higher than those that gave maximal nucleosome and DNA binding for the other wild-type combinations, the variant forms of $B3V_H$ showed no binding to nucleosomes or dsDNA. Also, none of the other combinations containing V_H3-23 or V_H3-23(53) or $UK4V_L$ showed any binding nucleosomes or dsDNA at all.

3.2.5 Anti-alpha-actinin binding of expressed whole IgG molecules

The α-actinin binding ability of each of the V_H/V_L combinations was tested by ELISA and is shown in Figure 3.8. From the three expression experiments, similar results were seen. Figure 3.8 shows the α-actinin binding results of a single, representative, experiment. The negative control sample in each case contained neither IgG1 nor anti-α-actinin activity on testing by ELISA. The positive control in each case was polyclonal serum from a patient with SLE known to bind α-actinin. The same aliquot of polyclonal serum was used for all the α-actinin ELISAs. $B3V_H/33H11V_L$ was the

only combination that bound α-actinin. All other combinations did not bind, despite being tested at a range of concentrations higher than those that gave binding with the $B3V_H/33H11V_L$ combination.

3.2.6 Anti-cardiolipin binding of expressed whole IgG molecules

Binding of each of the H/L chain combinations to CL is shown in Figure 3.9. In each case, similar results were seen from each of the expression experiments and the figure shows results of a single representative experiment. The negative control sample contained neither IgG1 nor anti-CL activity in an ELISA test. The positive control in each case was polyclonal serum from a patient with APS known to bind CL. The same aliquot of polyclonal serum was used for all the CL ELISAs.

The strongest binding was seen with the $B3V_H/B3V_L$ (wild-type) chain combination. The reversion of the arginine residue to a serine residue at position 27a (R27aS) in V_L CDR1 led to a reduction in binding to CL. The introduction of a mutation at position V_H CDR2 (R53S, R53N and R53K) completely abolished binding to CL irrespective of the L chain it was paired with.

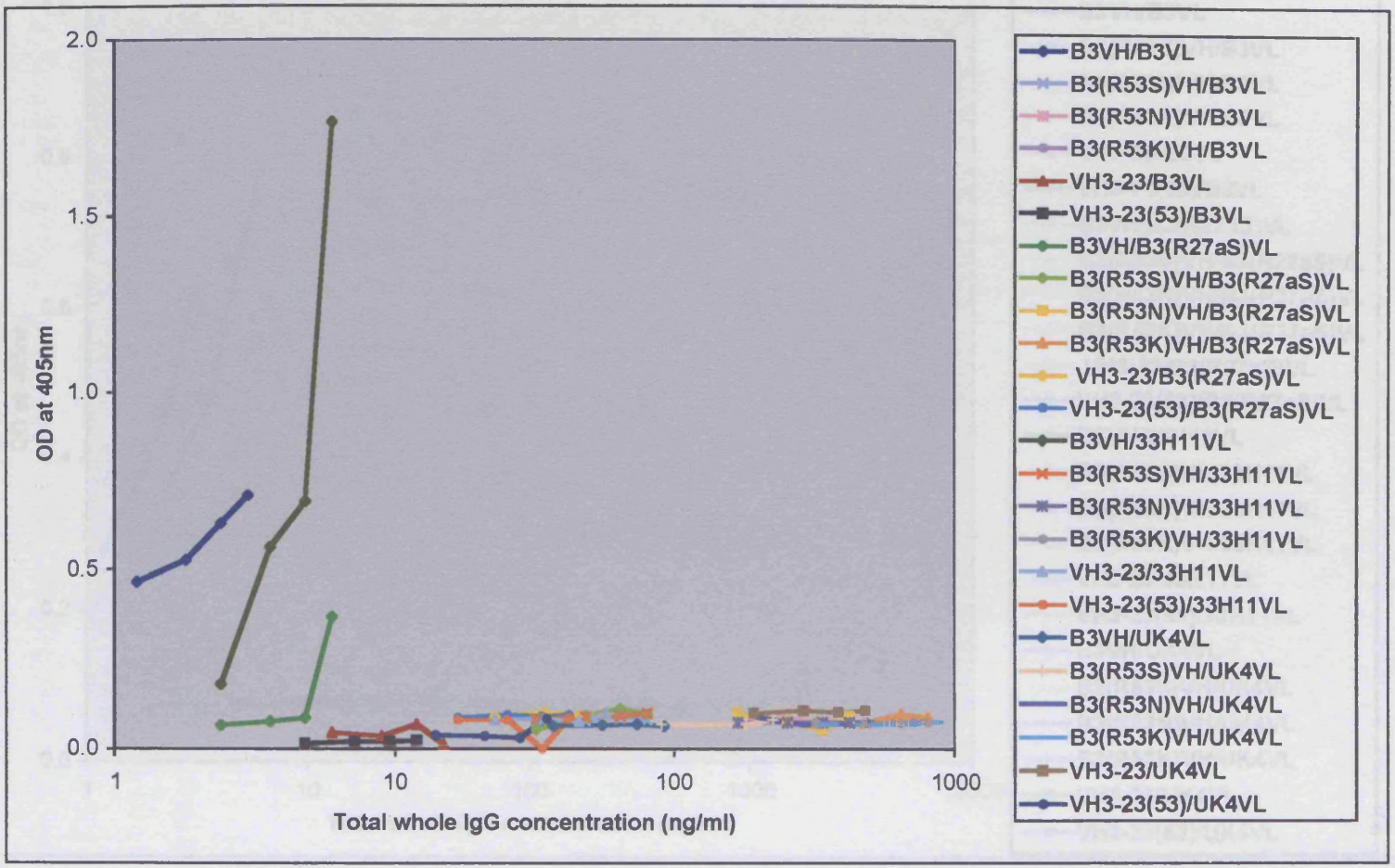


Figure 3.7 (A) Effect of point mutation in B3V_H and V_H3-23 in binding to nucleosomes (See figure legend)

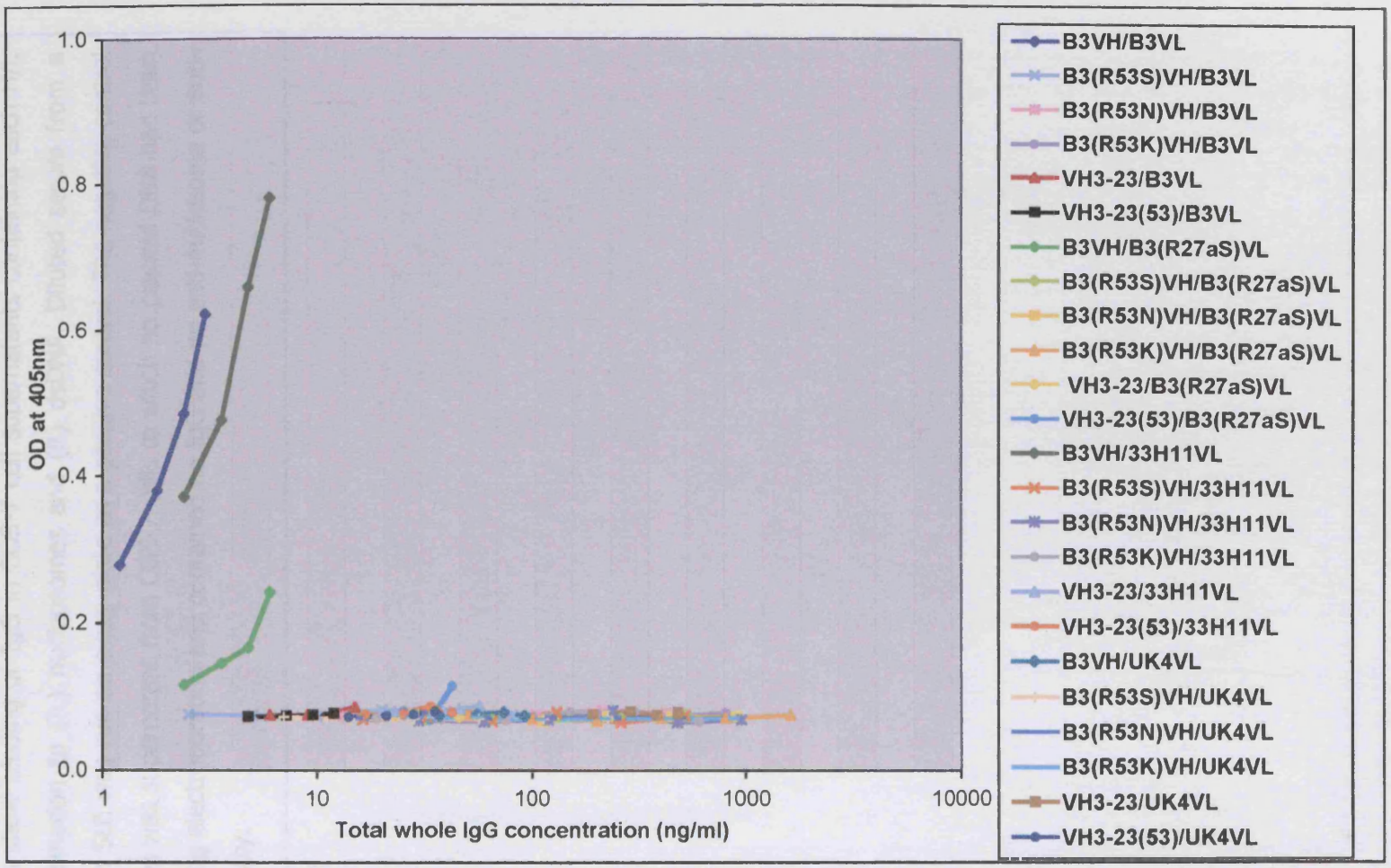


Figure 3.7 (B) Effect of point mutation in B3V_H and V_H3-23 in binding to dsDNA (See figure legend)

Figure 3.7 Effect of point mutation in B3V_H and V_H3-23

The graphs show binding of IgG in COS-7 cell supernatants containing each H/L chain combination to (A) nucleosomes and (B) dsDNA. Diluted serum from a patient with SLE was run on every plate as a positive control. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained no IgG and no anti-nuclesome or anti-dsDNA activity.

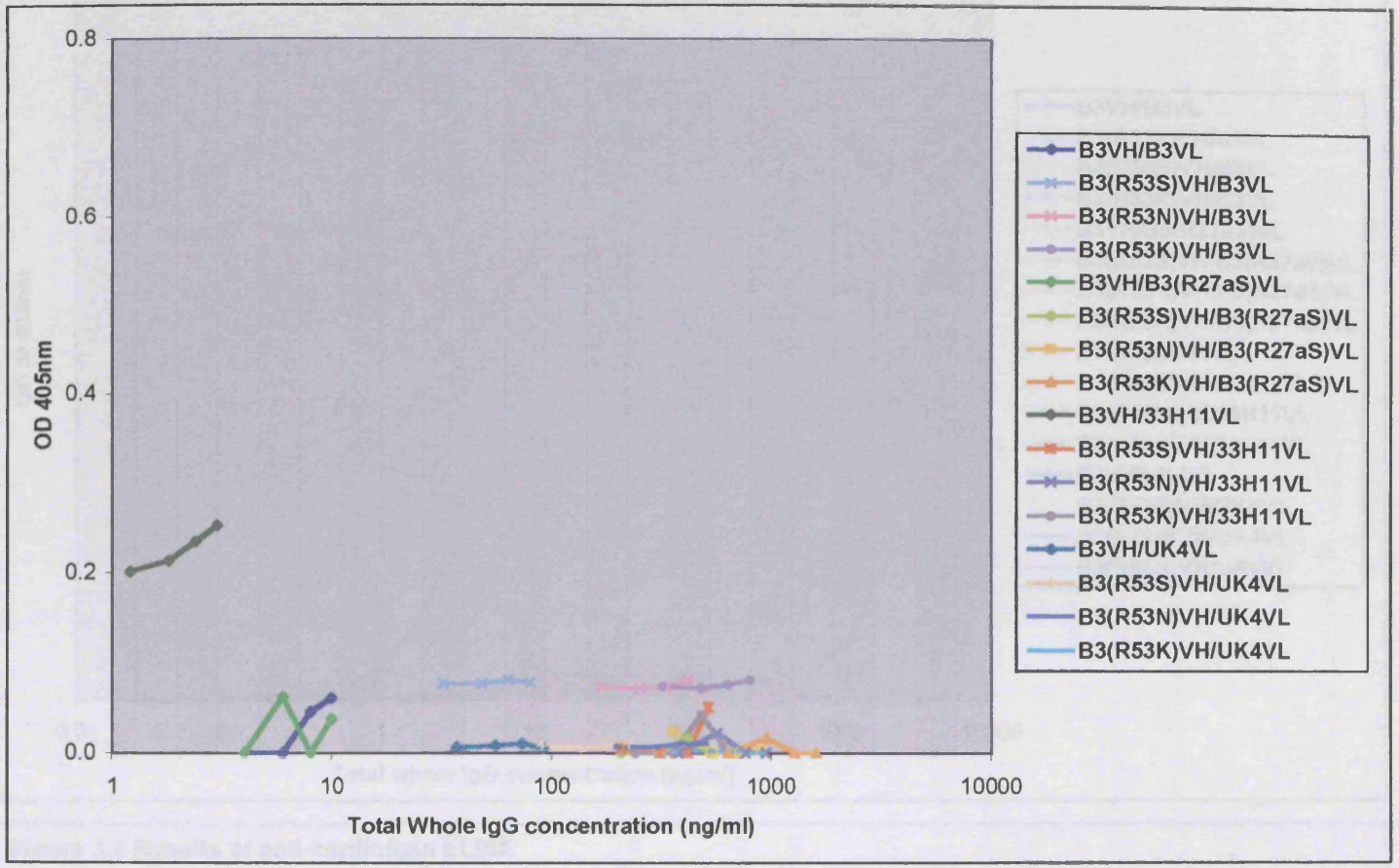


Figure 3.8 Results of anti-α -actinin ELISA

The graph shows binding of IgG in COS-7 cell supernatants containing each H/L chain combination to α-actinin (20μg/ml).

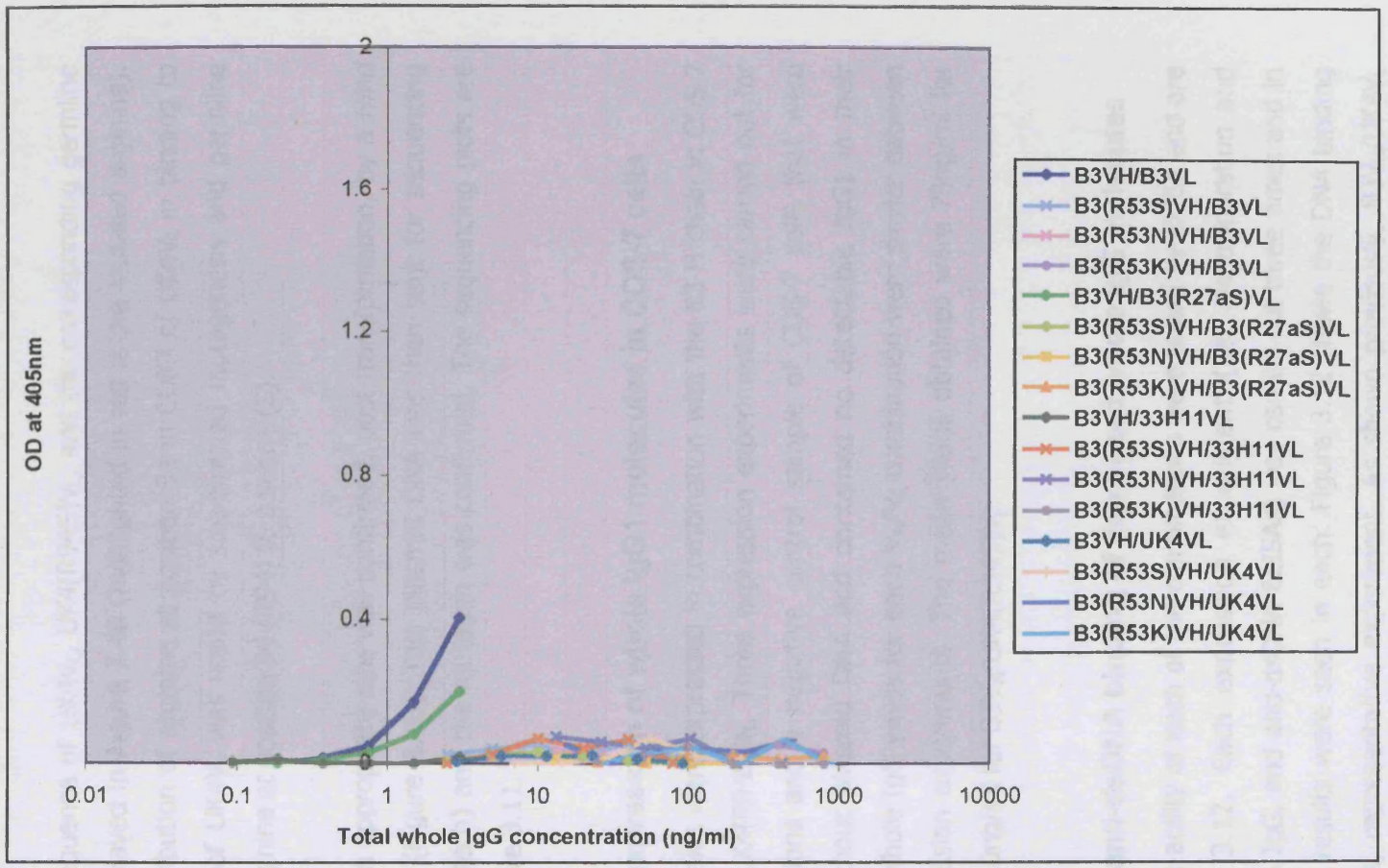


Figure 3.9 Results of anti-cardiolipin ELISA

The graph shows binding of IgG in COS-7 cell supernatants containing each H/L chain combination to CL (50μg/ml).

3.3 The importance of sequence features of UK4V$_L$ in binding to dsDNA

3.3.1 Results of site-directed mutagenesis for production of UK4V$_L$ variant

The amino acid sequence of UK4V$_L$, UK4(R94S)V$_L$, and the corresponding germline gene, 2a2 are displayed in Figure 3.4B (highlighted in red is the mutated arginine). To study the contribution of arginine at position 94 in CDR3 of UK4V$_L$ in binding to dsDNA, a variant of UK4V$_L$ was made by site-directed mutagenesis and germline reversion of the arginine at position 94 (R94) to a serine (S).

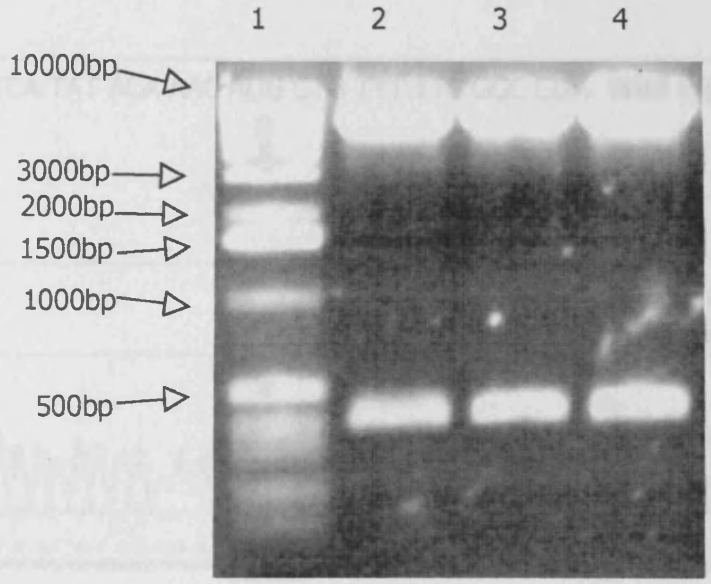
Plasmid DNA of the appropriate size was confirmed, post transformation, by a *Hind III/Bam HI* digest (Figure 3.10). This plasmid DNA was then sent for sequencing analysis (MWG-Biotech) and the mutation was confirmed. The sequencing plots are represented in Figure 3.11.

3.3.2 Transient expression of whole IgG1 molecules in COS-7 cells

The following L chains were expressed in combination with the B3 H chain in COS-7 cells; UK4V$_L$ and UK4(R94S)V$_L$. Three expression experiments were carried out for the two combinations and a negative control sample of COS-7 cells that were electroporated without plasmid DNA and contained no detectable IgG1 in their supernatant. The whole IgG yields for each V$_H$/V$_L$ combination were similar between each of the expression experiments. The mean yields obtained were 75ng/ml for B3V$_H$/UK4V$_L$ and 110ng/ml for B3V$_H$/UK4(R94S)V$_L$.

3.3.3 Results of anti-dsDNA binding of expressed whole IgG molecules

The dsDNA binding ability of each of the combinations were tested by ELISA and are shown in Figure 3.12. Each expression experiment (i.e. electroporation and subsequent human IgG, and anti-dsDNA ELISAs) was carried out three times and in each case, similar results were seen in each. Figure 3.12 shows the DNA binding results of a single, representative experiment. As shown previously, B3V$_H$/UK4V$_L$ showed no binding to dsDNA. In addition, the combination containing the UK4(R94S)V$_L$ CDR3 did not bind to dsDNA.



Lanes

1 = 1kb DNA ladder

2 = *Hind III/BamH I* digested UK4V_L/pLN10

3 - 4 = *Hind III/BamH I* digested UK4(R94S)V_L/pLN10

Figure 3.10 *Hind III/BamH I* restriction digest of pLN10 vector containing UK4V_L and UK4(R94S)V_L variant construct on 1% agarose gel

UK4V_L/pLN10 and UK4(R94S)V_L/pLN10 were digested with *Hind III* and *BamH I* and the fragments separated on a 1% agarose gel. The actual agarose gel is shown above, indicating the expected band sizes. It can be seen from the gel that DNA bands of 7091 and 355 bp are present in each.

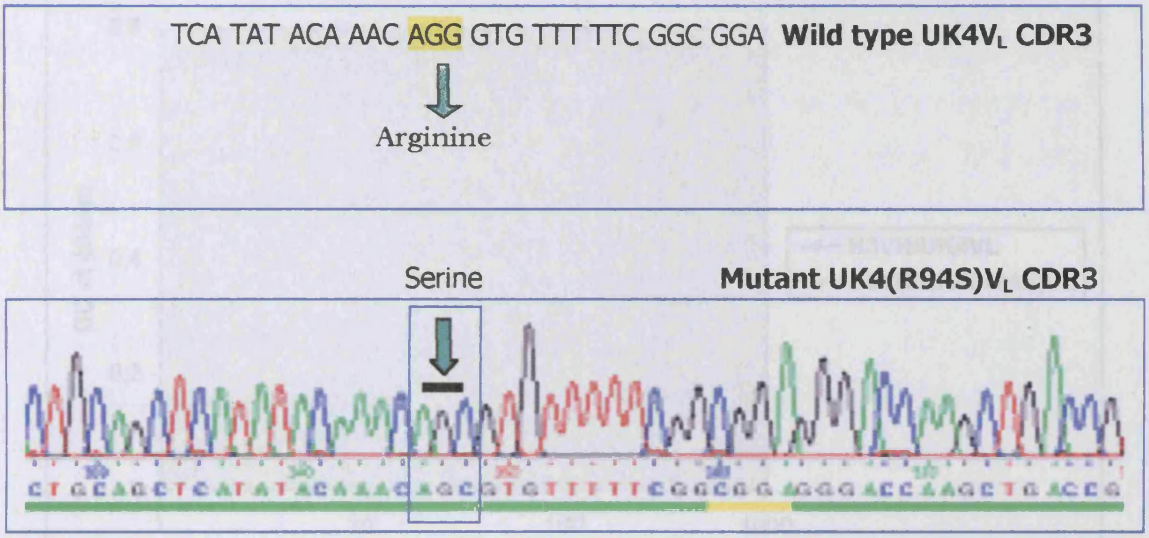


Figure 3.11 The sequence of UK4V$_L$ variant was confirmed by automated sequencing

The chromatograph trace represented in this figure shows the sequence of UK4(R94S)V$_L$ CDR3. The mutation introduced in the sequence is indicated by an arrow pointing to the single amino acid alteration.

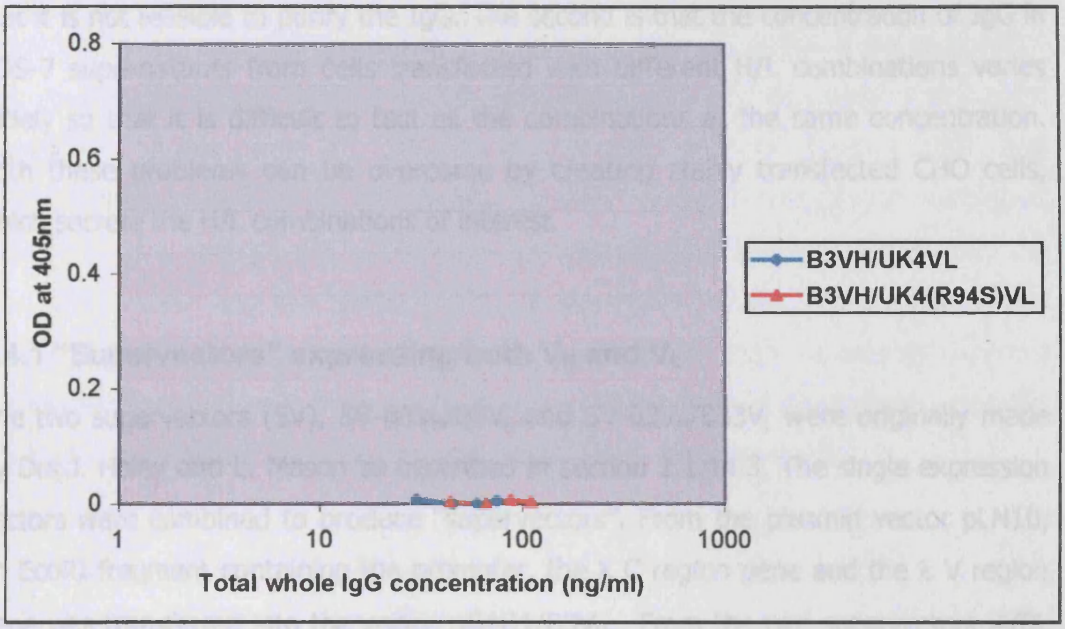


Figure 3.12 Effect of point mutation in UK4V_L

The graphs show binding of IgG in COS-7 cell supernatants containing B3V_H paired with UK4V_L and UK4V_L R94S to dsDNA. Diluted serum from a patient with SLE was run on every plate as a positive control. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained no IgG or anti-dsDNA activity.

3.4 Stable expression of whole IgG molecules in CHOdhfr⁻ cells

There are two main difficulties with the use of IgG in COS-7 cell supernatants. The first is that the total amount of IgG produced from transient expression is small so that it is not feasible to purify the IgG. The second is that the concentration of IgG in COS-7 supernatants from cells transfected with different H/L combinations varies widely so that it is difficult to test all the combinations at the same concentration. Both these problems can be overcome by creating stably transfected CHO cells, which secrete the H/L combinations of interest.

3.4.1 "Supervectors" expressing both V_H and V_L

The two supervectors (SV), SV-B3V_H/B3V_L and SV-B3V_H/B33V_L were originally made by Drs J. Haley and L. Mason as described in section 2.1.14.3. The single expression vectors were combined to produce "supervectors". From the plasmid vector pLN10, an EcoRI fragment containing the promoter, the λ C region gene and the λ V region gene was transferred into the vector pG1D1/B3V_H. From the two supervectors SVBL and SVB33, I produced four new variants by site-directed mutagenesis. Two of the variant supervectors had an arginine to serine mutation at position 53 in the B3 H chain and were paired with B3 L chain (SV-B3(R53S)V_H/B3V_L) and B33 L chain (SV-B3(R53S)V_H/B33V_L). The other two variant supervectors both had the same B3 L chain but a different H chain, one with an arginine to asparagine (SV-B3(R53N)V_H/B3V_L) and the other with an arginine to lysine (SV-B3(R53K)V_H/B3V_L) mutation at position 53 in B3 H chain. SV-B3(R53S)V_H/B33V_L was included because previous experiments had shown that the chimeric L chain B33V_L (i.e. B3V_L CDR1 and 33H11V_L CDR2 and CDR3) confers stronger anti-dsDNA and anti-nucleosome activity than the wild type L chain B3V_L, and we wanted to see if that effect would overcome the negative effect of the R53S change in the H chain.

Using an *EcoRI* restriction digest, plasmid DNA of the appropriate size was confirmed by the presence of a 6917bp band and a 2260bp band when run on an agarose gel. The incorporation of the mutation in the H chain of the supervectors was verified by sequencing analysis (MWG-Biotech).

3.4.2 Transfection of CHO*dhfr*⁻ cells with supervectors and methotrexate amplification of the transfected CHO cell lines

CHO*dhfr*⁻ cells were transfected with the four supervectors by electroporation. In addition, two aliquots of CHO*dhfr*⁻ cells were also electroporated (under the same conditions as the expression vectors), one with an empty pG1D210 expression vector (i.e. contains no H or L chain V region DNA sequences) and one without any DNA. Following 10-14 days of culture in growth medium lacking ribo and deoxyribonucleosides, all the cells in the "no DNA" negative control dish were dead and foci of cells were clearly visible in the dishes containing cells transfected with the four supervectors and the empty pG1D210 expression vector. The amount of IgG produced was measured from which the IgG production rate was determined for a number of clones of each supervector. The production rates of the highest producers are shown in Table 3.3. The two controls produced no detectable IgG1.

Supervector	IgG production rate ($ng/10^6$ cells/day)
SV-B3(R53S)V_H/B3V_L	413
SV-B3(R53S)$_H$/B33V_L	52
SV- B3(R53N)V_H/B3V_L	670
SV- B3(R53K)V_H/B3V_L	670

Table 3.3 Human IgG production rates of CHO cells transfected with the supervectors

Different amounts of IgG were obtained from each cell line after one round of MTX amplification. However, MTX amplification did not increase the yield of IgG in the case of these four stable cell lines. The variable expression from CHO cells is well documented in both our hands and others (Kim et al., 1998, Rahman et al., 2002). This phenomenon occurs during sub-cloning in successive amplification steps; when cells can increase the copy number of the *dhfr* gene without increasing their production rate. Thus, cells that do not produce an exogenous protein will usually have a higher growth rate and take over the culture thereby reducing the chance of selecting a high producer cell.

3.5 Discussion

The results described in this chapter show that whole human IgG1 molecules that bind dsDNA, nucleosomes alpha-actinin and CL can be produced through the transient expression of cloned autoantibody DNA sequences in eukaryotic cells. The total yield of IgG1 varied between 3ng/ml to 950ng/ml depending on the H/L chain combination. Zack et al. (1995) have reported low yields of expressed IgG1, through the use of a similar expression system in COS-7 cells. Kettleborough et al., (1991) have previously used this expression system to humanise a mouse mAb by CDR exchange and have seen that the yield of IgG varied between different constructs.

$B3V_H/B3V_L$ (wild type) bound dsDNA, nucleosomes and CL, which is consistent with the previously reported properties of this mAb, (Ehrenstein et al., 1995, Giles et al., 2003, Mason et al., 2005) as well as the computer model of the interaction between a dsDNA helix and B3, shown in Figure 1.5 (Kalsi et al., 1996). Although V_H CDR3 plays a major role in antigen binding of a number of other murine and human anti-dsDNA antibodies described in the past (Radic and Weigert, 1994, Li et al., 2000), I believe it is unlikely to play a major role in the interaction of B3 with antigen. The computer model of the B3/dsDNA complex shows no major sites of contact between dsDNA and V_H CDR3 and this CDR3 sequence is short and contains no arginine, asparagine or lysine residues (sequence shown in Figure 3.4) The results of the ELISA tests correlated with the predictions of the computer model in showing that the ability of B3 to bind dsDNA is dependent upon the arginine residues present at critical positions (position 53 in $B3V_H$ and positions 27a and 54 in $B3V_L$) that enable the antibody to form a specific electrostatic interaction with the phosphate backbone of dsDNA. This interaction is completely lost by mutation to serine (R53S), asparagine (R53N) or lysine (R53K), thus leading to a loss in DNA binding ability by the variant H chain when compared to the wild type. In contrast, in B3 L chain, this interaction is critically reduced but not lost by mutation to serine (R27aS), therefore leading to a reduction in DNA binding ability. The precise location of arginine residues has been shown to be important in the binding of both murine and human anti-dsDNA antibodies to DNA (Radic et al., 1993, Radic and Weigert, 1994). $33H11V_L$ contains a serine at position 27a rather than an arginine, however, despite this fact it is able to bind DNA when in combination with B3 H chain. The computer model of the $B3V_L/33H11V_L$ combination predicts that the arginine at position 92 in the CDR3 is able to interact with the dsDNA backbone. Therefore it is possible that the presence of the arginine residue in CDR3 of $33H11V_L$ could compensate for the

absence in CDR1. In conclusion, the results shown here suggest that in a 2a2 encoded L chain such as $B3V_L$ and $33H11V_L$, which has undergone extensive somatic mutation, DNA-binding ability is determined by the pattern of these somatic mutations. Also, the presence of the H chain of B3 plays a dominant role in binding dsDNA, nucleosomes and CL. $B3V_H$ binds nucleosomes and DNA with 3 of the 4 L chains tested (Figure 3.8). Single amino acid changes can affect DNA binding of these antibodies, especially where these changes involve arginine residues that have been predicted by the computer models to directly interact with the dsDNA helix. The arginine residues critical to DNA binding are those in $B3V_H$ CDR2, $B3V_L$ CDR1 and $33H11V_L$ CDR3 which may have an additive effect in enhancing binding to DNA whilst an arginine in $UK4V_L$ CDR3 blocks DNA binding (computer models shown in Figures 1.5, 1.6 and 1.7) (Haley et al., 2004). Replacement of this arginine residue with serine did not introduce DNA binding suggesting that the arginine is not solely responsible for blocking DNA binding.

To identify the features of $B3V_H$ that are responsible for conferring DNA and nucleosome binding ability, I focused on the germline V_H3-23. The position 53 is occupied by serine so I produced the opposite effect, which was addition of an arginine to investigate if this amino acid will re-introduce binding to nucleosomes and DNA. However, there was no binding observed with the variant form of V_H3-23 (Figure 3.7). Thus introducing the S53R mutation alone into this germline gene is not sufficient to engender binding to these antigens. This finding suggests that the arginine is not solely responsible for binding of $B3V_H$ to nucleosomes or DNA. Therefore, other somatically mutated amino acids present in the sequence of $B3V_H$ in combination with this arginine must also play a role in conferring the binding ability of this antibody.

$UK4V_L$ is very similar to $B3V_L$, however there is a serine rather than a glycine present at position 29 in CDR1 and an asparagine at position 27a rather than an arginine. As predicted by the computer model of $B3V_H/UK4V_L$ (Figure 1.7), the pattern of somatic mutations in $UK4V_L$ prevents any binding to DNA, even when paired with B3 heavy chain. From the computer model, it was hypothesized that the introduction of the arginine residue at position 94 in addition to the changes at positions 27a and 29 prevent DNA binding. This finding is in agreement with previous studies that have also shown that it is not the mere presence of arginine residues in the CDRs that confers the ability to bind DNA but their precise positions (Guth et al., 2003,

Stevenson et al., 1993). I had hypothesised that the blocking arginine at position 94 prevents the binding to DNA in the case of $B3V_H/UK4V_L$ combination. Mutation of this arginine to serine however, did not introduce binding to DNA. The reason for the lack of any DNA binding might involve other blocking amino acids in $UK4V_L$, and mutation of multiple residues might be required to produce an effect. Therefore, this hypothesis needs to be further investigated since the mutation introduced alone is not sufficient to free the binding cleft.

Various groups have previously used computer models to produce three-dimensional images of anti-DNA antibodies (mainly murine) interacting with DNA and to predict sites of contact between dsDNA and certain amino acid residues at the antigen-binding site (Radic and Weigert, 1994). They have followed a method similar to our group, where they used site-directed mutagenesis to test the validity of these predictions. In many cases the targets of mutagenesis have been arginine residues and as in our system the number of arginine residues did not always strictly correlate with the ability of the antibody to bind DNA (Radic et al., 1993). For example, Radic et al (1993) examined the effects of amino acid substitutions on DNA binding where they reverted three somatic mutations in the H chain of a murine anti-dsDNA antibody 3H9. Their results showed that arginine 53 eliminated virtually all dsDNA binding and this agreed with my findings shown in this chapter. Mutation of a leucine in $3H9V_H$ CDR2 to an arginine (L64 to R64), completely eliminated binding to ssDNA and dsDNA. The authors suggested that this may be due to the formation of a salt bridge of R64 with a neighbouring aspartic acid (D65) producing an Ig conformation incompatible with DNA binding (Radic et al., 1993). Wellmann et al., (2005) used human monoclonal anti-DNA antibodies that were systematically subjected to site-directed mutagenesis reverting amino acid substitutions to the germline sequence, and tested the antibody reactivity against dsDNA. They have shown that for the human antibody 33.F12, arginines enhance antibody affinity for DNA. In the case of another antibody however, asparagine and lysine residues in the L chain of 33.C9 are involved in the contact to DNA.

Katz et al., (1994) used site-directed mutagenesis to change amino acid residues in the H chain of the pathogenic R4A anti-dsDNA antibody to look for alterations in DNA binding and pathogenicity. Their results showed that single amino acid substitutions in both CDRs and FW regions reduced DNA binding. However, in contrast to the results of Radic and colleagues and my findings, this group showed that the V_H

sequence that resulted in the highest ability to bind DNA contained two fewer arginine residues than R4A, highlighting the fact that charge interaction is not the sole determinant of binding to DNA. These two arginines were both replaced by serine in R4AV$_H$ FR3, suggesting that FW regions not only perform a scaffolding function but also affect binding specificity. Furthermore, Radic et al., (1994) predicted from a computer model of a murine anti-dsDNA antibody that V_H FR3 is positioned so as to contribute contacts with the minor groove of the DNA.

Members of our group have used a bacterial expression system to produce Fab of three autoantibodies described in this chapter (B3/33H11, anti-DNA; UK4, aPL) and six related hybrids (Kumar et al., 2000, Kumar et al., 2001). The system described by Kumar et al., (2000) involved the transformation of *E. coli* with plasmid expression vectors containing the appropriate V_L-C_L and V_H-C_H DNA for each antibody. Following culture of the bacteria, Fab were harvested from the periplasm of the *E. coli* and then purified. SDS-PAGE and Western blot confirmed that the purified Fab was of the correct size and assembled form. Protein expression levels of 5-9mg per litre of culture were achieved. The comparative DNA/CL-binding analysis of the nine Fabs demonstrated that B3 and 33H11 L chains possess both anti-DNA and anti-CL activities (Kumar et al., 2001). These results are consistent with the binding results and computer generated models of these H and L chain combinations expressed in eukaryotic systems, as presented previously by our group (Haley et al., 2004) and in this chapter.

The amount of whole IgG produced by transient expression in COS-7 cells described in this chapter was too small to allow purification of the antibodies or for experiments on pathogenicity to be carried out. It was therefore necessary to establish a stable expression system for production of recombinant B3 and its variants in CHO cells to allow for more extensive *in vitro* and *in vivo* characterisation of the binding of the human IgG. The results described in this chapter also show that I have successfully developed a stable expression system to produce whole human recombinant anti-nucleosome IgG1 molecules from cloned PCR products encoding anti-nucleosome antibody sequences. This system has the potential to produce significant amounts of antibody whose sequence-related functional properties can be studied both in *in vitro* and *in vivo* assays. The total yield of IgG1 varied between 52 and 413 ng per 10^6 cells per day depending on the H/L chain combination expressed. The yield of these antibodies, however, was not increased by further selection of the

stable cell lines in selective growth media containing MTX. The process of MTX amplification selects the clones of cells with a higher *dhfr* copy number and consequently a higher copy number of IgG cDNA flanking the *dhfr* gene.

A number of groups have previously described stable expression of murine anti-DNA antibodies from cloned cDNA (Katz et al., 1994, Radic et al., 1993, Pewzner-Jung et al., 1996). In most cases, expression was achieved using HCLVs, which are hybridoma cells that have lost the ability to secrete H chains. By transfecting such variants with expression vectors encoding various different H chains, Radic et al., (1993), Katz et al., (1994) and Pewzner-Jung et al., (1996) were all able to demonstrate that altering the numbers of arginines in CDRs of the H chains altered the ability of murine mAbs to bind DNA. Of these groups, only Katz and colleagues went on to test the ability of the altered antibodies to cause pathogenic changes in mice. They produced antibodies based on the murine monoclonal anti-DNA antibody R4A. All these antibodies had the L chain of R4A, but the H chains were variants of the R4A V_H sequence. They found that the antibody with strongest binding to dsDNA did not have more CDR arginines than wild-type R4AV_H. This antibody actually showed less glomerular binding but more tubular binding to mouse kidneys *in vivo* than the wild-type R4A.

Only one group has previously reported stable expression of whole human anti-dsDNA molecules (as opposed to Fab or single chain Fv fragments) *in vitro*. Li and colleagues expressed the V region sequences of the human IgA anti-DNA antibody 412.67 in F3B6 human/mouse heteromyeloma cells (Li et al., 2000). The products were whole IgG molecules, since the expression vectors contained γ, rather than α, C regions. This group showed that reversion of two arginines in 412.67 V_H CDR3 totally removed the ability to bind ssDNA or dsDNA. All somatic mutations outside V_H CDR3 in either V_H or V_L of this antibody, however, could be reverted with no effect on DNA binding. No data were presented regarding the effect of these sequence changes on pathogenicity, and it is not known whether the original IgA antibody 412.67 is pathogenic in mice. The stable expression of antibody B3 described here, therefore, is only the second report of stable expression of a whole human anti-dsDNA antibody *in vitro*.

CHAPTER FOUR

Somatic mutations to arginine residues affect the binding of different human aPL derived heavy and light chains to cardiolipin, β_2-glycoprotein I and phosphatidylserine

CHAPTER FOUR.

SOMATIC MUTATIONS TO ARGININE RESIDUES AFFECT THE BINDING OF DIFFERENT HUMAN aPL DERIVED HEAVY AND LIGHT CHAINS TO CARDIOLIPIN, β_2-GLYCOPROTEIN I AND PHOSPHATIDYLSERINE

4.1 Introduction and aims of this chapter

Sequence analysis of human monoclonal aPL has shown that IgG aPL often contain large numbers of somatic mutations in their H and L chain V region sequences (Giles et al., 2003b). The distribution of these somatic mutations suggests they are antigen-driven (Shlomchik et al., 1987). Arginine, asparagine and lysine residues tend to accumulate in the CDRs of these monoclonal aPL and arginine residues have been shown to play an important role in the CDRs of some murine monoclonal aPL (Cocca et al., 2001, Kita et al., 1993).

Our group has previously described a system for *in vitro* expression of whole IgG molecules from cloned V_H and V_L sequences of human monoclonal aPL (Giles et al., 2003a). This system was used to test the binding properties of different H and L chain combinations derived from a range of antibodies. One of these is the human monoclonal IgG antibody IS4, which was derived from a patient with PAPS and binds to anionic (but not neutral) PL in the presence of the cofactor β_2GPI. This human mAb is pathogenic in a murine model, therefore it is likely to be relevant in the pathogenesis of APS (Pierangeli et al., 2000). Our group has shown that the sequence of IS4V_H was dominant in conferring the ability to bind CL whilst the V_L that was paired with was important in determining the strength of CL binding (Giles et al., 2003a).

Modelling studies have shown that multiple surface-exposed arginine residues are prominent features of the H chain of IS4 that conferred the highest ability to bind CL in earlier experiments (Giles et al., 2003a). The hypothesis is that in the arginine rich CDR3 of IS4V_H, four out of five arginine residues [at positions 96, 97, 100, 100g (Wu

and Kabat, 1970)] are surface exposed and therefore potentially important in binding to CL. Therefore, the aim of my experiments as described in this chapter was to define the contribution of individual arginine residues within CDR3, in binding to CL, β_2GPI and PS. To investigate this, patterns of CDR3 arginine residues in the V_H sequence were altered by site-directed mutagenesis. The altered sequences were expressed transiently in COS-7 cells and stably in CHO*dhfr*$^-$ cells. The binding of the different H and L combinations expressed was then assessed by direct ELISAs.

4.2 The importance of sequence features of IS4V_H in binding to CL

4.2.1 Sequences of heavy chains and light chains expressed

The V_L sequences of IS4V_L and B3V_L are both encoded by the same germline V_λ gene, 2a2, but differ in their patterns of somatic mutation. The amino acid sequences are displayed in Figure 4.1A. As described in the previous chapter, B3V_L contains two adjacent arginine residues in CDR1, both produced by somatic mutations. In CDR3 of IS4V_L a serine residue is replaced by asparagine. The amino acid sequences of IS4V_H and the corresponding germline gene are shown in Figure 4.1B. IS4V_H contains an asparagine residue in CDR2 that was created by somatic mutation and multiple arginine residues in CDR3. The four surface exposed arginine residues that were altered by site-directed mutagenesis are shown in bold.

4.2.2 Results of site-directed mutagenesis

Six mutant forms of IS4V_H were generated in which particular arginine residues were mutated to serine. Serine was chosen because it is a non-polar amino acid residue. Germline reversion could not be performed, as our group could not align IS4 D_H to the germline D_H gene using DNAplot, which has strict alignment criteria described in Corbett et al., (1997). Four mutants, named IS4V_Hi, IS4$V_H$ii, IS4$V_H$iii and IS4$V_H$iv contained single mutations of arginine residues at positions 96, 97, 100 and 100g respectively. The remaining mutants contained two arginine to serine mutations at positions 96 and 97 (IS4V_Hi&ii) and at all four positions in IS4V_Hx. Figure 4.2 shows the sites of site-directed mutagenesis in the V_H CDR3 of the wild type IS4V_H and its six variants IS4V_Hi, IS4$V_H$ii, IS4$V_H$iii, IS4$V_H$iv, IS4V_Hi&ii and IS4V_Hx. The mutations were confirmed by sequencing analysis.

(A) V_L

```
              FR1                           CDR1          FR2              CDR2   FR3                                  CDR3     Jλ
            1                    20  24 27              34 35  40            50     60      70        80         89        100
            |                    |   |  |abc             |  |   |            |      |       |         |          |         |
2a2         QSALTQPASVSG.SPGQSITISC  TGTSSDVGGYNYVS  WYQQHPGKAPKLMIY  EVSNRPS  GVSNRFSGSKSGNTASLTISGLQAEDEADYYC  SSYTSSST  VVFGGGTKLTVLG
IS4         ------------.----------  --A---I-S--S--  ---HL-------I--  D--S---  ------------F----------P--------  C---TIN-  W------------
B3          ------------.----------  ---RR------F--  ---------------  ---H---  ---T---A----S-------------------  ---S-TTR  -------------
```

(B) V_H

```
        FR1                                 CDR1   FR2              CDR2              FR3                                CDR3             JH
                                          30 31    36   40          50         60        70       82       90         100              101
                                           | |      |   |           |          |         |        |abc     |          |                |
1-03    QVQLVQSGAEVKKPGASVKVSCKASGYTFT  SYAMH  WVRQAPGQRLEWMG  WINSGNGNTKYSQKFQG  RVTITRDTSASTAYMELSSLRSEDTAVYYCAR                  YFDYWGQGTLVTVSS
IS4     ---F--------------------F--S--  --FL-  --------GP----  -------------N---  --S------------VV-----G---------  GRRDVRGVLWRGRHD  ---F-------I---
```

Figure 4.1 Sequence alignment of expressed V_L and V_H regions

(A) Sequences of expressed V_L regions compared to 2a2. (B) Sequence of expressed V_H region compared to 1-03. The amino acids altered by site-directed mutagenesis are shown in bold. The amino acids are numbered according to Kabat (Wu and Kabat, 1970). A dash indicates homology with the corresponding germline sequence.

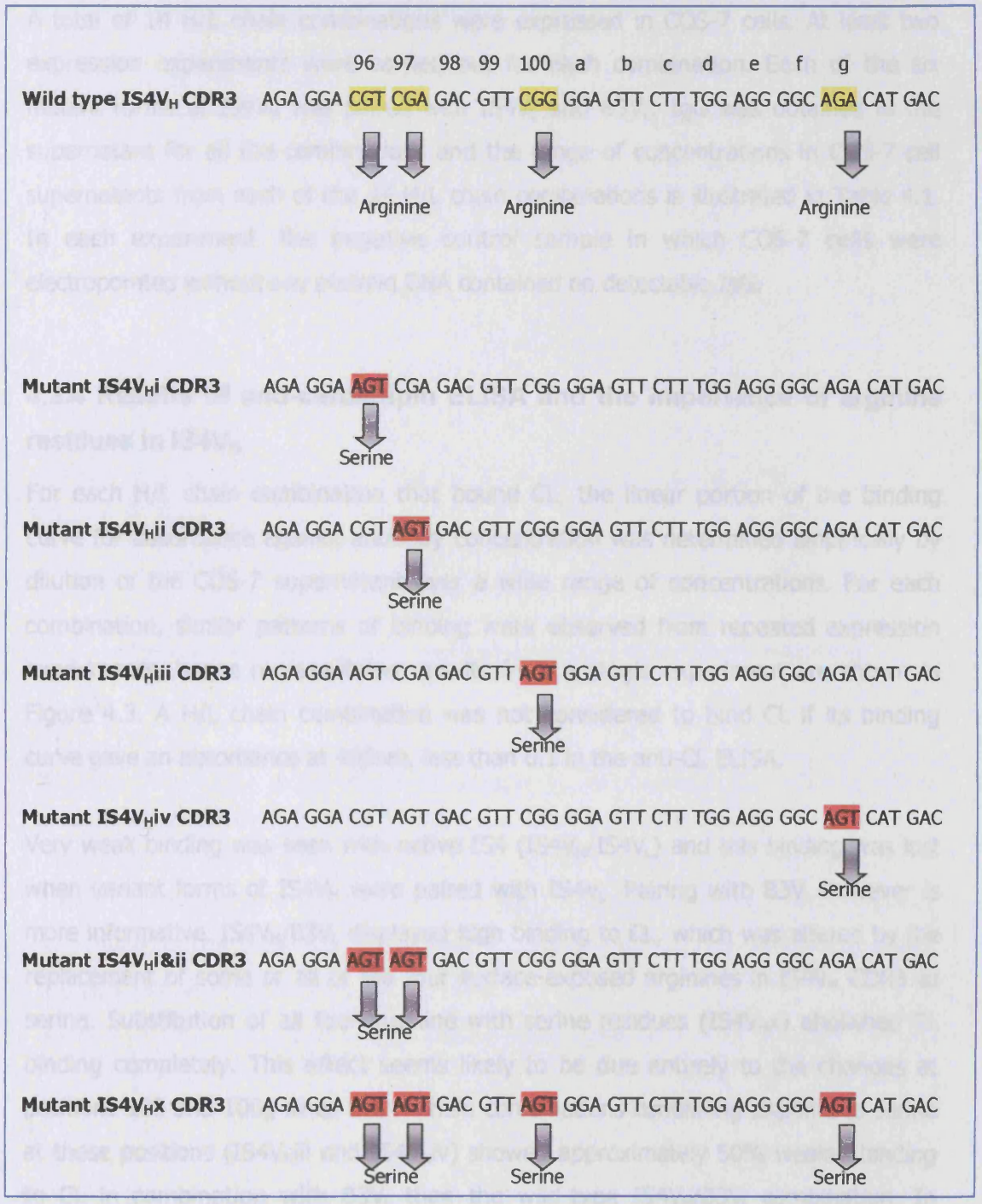


Figure 4.2 Production of IS4V$_H$ variants

This figure illustrates the results of the site-directed mutagenesis of IS4V$_H$ producing 6 new variant constructs. The amino acids mutated in the wild-type sequence are highlighted in yellow.

4.2.3 Transient expression of whole IgG in COS-7 cells

A total of 14 H/L chain combinations were expressed in COS-7 cells. At least two expression experiments were carried out for each combination. Each of the six mutant forms of $IS4V_H$ was paired with $IS4V_L$ and $B3V_L$. IgG was obtained in the supernatant for all the combinations and the range of concentrations in COS-7 cell supernatants from each of the 14 H/L chain combinations is illustrated in Table 4.1. In each experiment, the negative control sample in which COS-7 cells were electroporated without any plasmid DNA contained no detectable IgG.

4.2.4 Results of anti-cardiolipin ELISA and the importance of arginine residues in $IS4V_H$

For each H/L chain combination that bound CL, the linear portion of the binding curve for absorbance against antibody concentration was determined empirically by dilution of the COS-7 supernatant over a wide range of concentrations. For each combination, similar patterns of binding were observed from repeated expression experiments, hence representative results from a single experiment are shown in Figure 4.3. A H/L chain combination was not considered to bind CL if its binding curve gave an absorbance at 405nm, less than 0.1 in the anti-CL ELISA.

Very weak binding was seen with native IS4 ($IS4V_H/IS4V_L$) and this binding was lost when variant forms of $IS4V_H$ were paired with $IS4V_L$. Pairing with $B3V_L$ however is more informative. $IS4V_H/B3V_L$ displayed high binding to CL, which was altered by the replacement of some or all of the four surface-exposed arginines in $IS4V_H$ CDR3 to serine. Substitution of all four arginine with serine residues ($IS4V_HX$) abolished CL binding completely. This effect seems likely to be due entirely to the changes at positions 100 and 100g since the H chain combinations containing arginine to serine at these positions ($IS4V_Hiii$ and $IS4V_Hiv$) showed approximately 50% weaker binding to CL in combination with $B3V_L$ than the wild-type $IS4V_H/B3V_L$ combination. In contrast, the H chains containing arginine to serine mutations at position 96 ($IS4V_Hi$), position 97 ($IS4V_Hii$) or both ($IS4V_Hi\&ii$) dispayed no reduction in CL binding.

Heavy Chain	Light Chain	IgG concentration (ng/ml)
IS4	B3	5-14
IS4V_Hi	B3	24-54
IS4$V_H$ii	B3	30-34
IS4$V_H$iii	B3	30-34
IS4$V_H$iv	B3	28-30
IS4V_Hx	B3	32-34
IS4V_Hi&ii	B3	32-47
IS4	IS4	24-368
IS4V_Hi	IS4	50-56
IS4$V_H$ii	IS4	65-70
IS4$V_H$iii	IS4	48-90
IS4$V_H$iv	IS4	48-90
IS4V_Hx	IS4	78-94
IS4V_Hi&ii	IS4	74-80

Table 4.1 The range of IgG concentrations in ng/ml produced for each heavy/light chain combination during the COS-7 transient expression experiments

The IS4V_H mutants V_Hi, $V_H$ii, $V_H$iii and $V_H$iv contain single arginine to serine point mutations at positions 96, 97, 100 and 100g respectively; whilst V_Hi&ii contains arginine to serine mutations at positions 96 and 97; and V_Hx has arginine to serine mutations at all four positions.

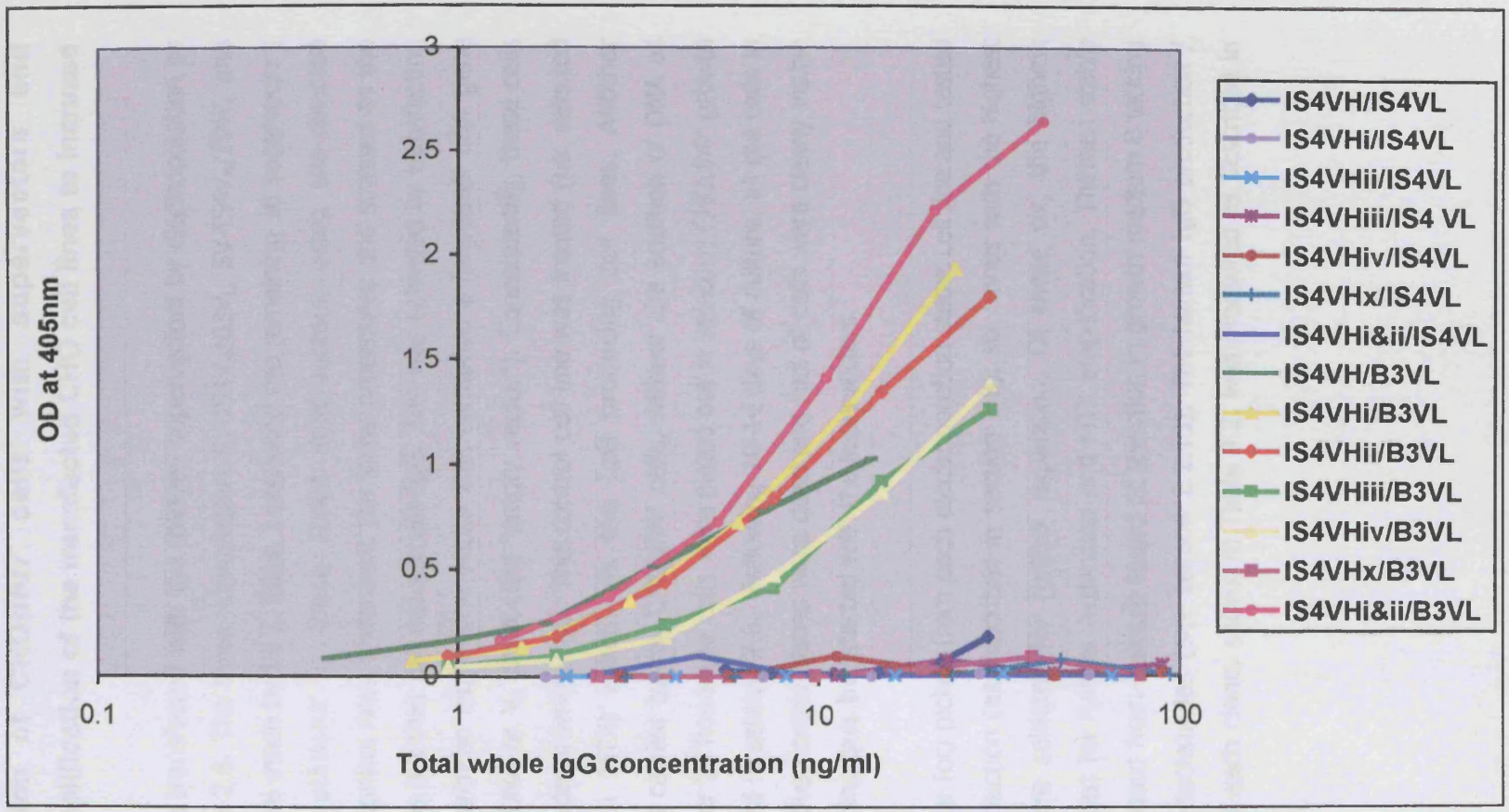


Figure 4.3 Effect of arginine to serine point mutations in IS4V_H CDR3

CL binding of IgG in COS-7 cell supernatants containing wild type or mutant forms of IS4 H chain with wild type IS4 or B3 L chains. The IS4V_H mutants V_Hi, $V_H$ii, $V_H$iii and $V_H$iv contain single Arg to Ser point mutations at positions 96, 97, 100 and 100g respectively; whilst V_Hi&ii contains Arg to Ser mutations at positions 96 and 97; and V_Hx has Arg to Ser mutations at all four positions.

4.3 Stable expression of whole IgG molecules in CHOdhfr⁻ cells

4.3.1 Transfection of CHO*dhfr⁻* cells with supervectors and methotrexate amplification of the transfected CHO cell lines to increase the yield of IgG

CHO*dhfr⁻* cells were transfected with the thirteen supervectors by electroporation as described in section 2.8. The three supervectors SV-IS4V_H/B3V_L, SV-IS4V_H/IS4V_L and SV-IS4V_H/UK4V_L were made by Dr I. Giles. I produced the remaining 10 supervectors by introducing mutations in these three supervectors, using site-directed mutagenesis. Two control lines underwent the same procedures and stresses as the supervector cell lines but they did not secrete IgG. This was achieved by transfecting the CHO*dhfr⁻* cells with an expression vector that contained a functional *dhfr* gene but no cloned V_H cDNA or V_λ cDNA (the "empty vector"). Consequently, these cells were not able to express whole IgG. This control cell line was treated (i.e. selected and amplified with MTX) exactly as the IgG producing cell lines. Another electroporation was carried out on CHO*dhfr⁻* cells, without the addition of DNA as another control. After 24 hours the cells were plated out in selective CHO*dhfr⁻* growth medium as described in section 2.8. Following 10-14 days of culture, all the cells in the "no DNA" negative control dishes were dead and foci of cells were clearly visible in the dishes containing cells transfected with the supervectors.

From the twenty-four foci picked from each electroporation stable cell line and tested for human IgG production (as described in section 2.8), six clones with the highest IgG production were selected for further expansion. Of these six, the highest producer was selected for further expansion and MTX amplification. Thirteen stable cell lines were produced from a single round of selection in growth medium B lacking ribo and deoxyribonucleosides (see section 2.1.13). The human IgG production / $1x10^6$ cells / day of each clone, shown in Table 4.2, was measured as described in section 2.8.5.

Supervector	IgG production rate ($ng/10^6$ cells/day)
SV-IS4V$_H$/IS4V$_L$	700
SV-IS4V$_H$i&ii/IS4V$_L$	1056
SV-IS4V$_H$iii/IS4V$_L$	5152
SV-IS4V$_H$iv/IS4V$_L$	3145
SV-IS4V$_H$iii&iv/IS4V$_L$	4103
SV-IS4V$_H$x/IS4V$_L$	1458
SV-IS4V$_H$/B3V$_L$	100
SV-IS4V$_H$i&ii/B3V$_L$	293
SV-IS4V$_H$iii&iv/B3V$_L$	2242
SV-IS4V$_H$x/B3V$_L$	4333
SV-IS4V$_H$/UK4V$_L$	100
SV-IS4V$_H$i,iii&iv/B3V$_L$	4103
SV-IS4V$_H$ii,iii&iv/B3V$_L$	293

Table 4.2 Human IgG production rates of CHO cells transfected with the supervectors
The IS4V$_H$ mutants V$_H$i, V$_H$ii, V$_H$iii and V$_H$iv contain single arginine to serine point mutations at positions 96, 97, 100 and 100g respectively; whilst V$_H$i&ii contains arginine to serine mutations at positions 96 and 97; and V$_H$x has arginine to serine mutations at all four positions.

4.3.2 Binding of IgG produced by CHO cells to CL

The supernatants from CHO cell cultures producing each of the human aPL were tested for binding to CL by ELISA. Figure 4.4 shows the results of a representative experiment (carried out three times). The negative controls used in the ELISAs were CHO*dhfr*$^-$ media and also supernatant from the CHO (empty pG1D210 vector) cell lines. Neither contained IgG nor anti-CL activity on testing by ELISA. The positive control in each case was serum from a patient with APS, containing anti-CL IgG. The same positive control was used for all the PL ELISAs and the SD of the positive control between the *OD* readings of the three experiments was always less than 0.1.

The IgG produced by the cell lines containing native IS4V$_H$ (SV-IS4V$_H$/B3V$_L$ and SV-IS4V$_H$/IS4V$_L$) were shown to bind CL with SV-IS4V$_H$/B3V$_L$ being the strongest binder. With the higher concentration of IgG obtained from the stable cell lines, there was binding to CL, which was not evident with my previous experiments with the IgG amounts obtained from the COS-7 expression system. From the results of the COS-7 expression system, IS4V$_H$/IS4V$_L$ appeared to be a weak binder whereas SV-IS4V$_H$/IS4V$_L$ binds well to CL. Mutation of the arginines at positions 96 and 97 to serine in IS4V$_H$, only has a slight effect on the binding of CL when paired with B3V$_L$ (SV-IS4V$_H$i&ii/B3V$_L$) and when paired with IS4V$_L$ the binding to CL is abolished. Mutations at positions 100 and 100g have a greater influence on the binding to CL with binding significantly reduced when paired with B3V$_L$ and completely lost when paired with IS4V$_L$. Mutation of three arginines in IS4V$_H$ (96, 100 and 100g / 97, 100 and 100g) reduces the binding even further. I observed weak binding to CL with SV-IS4V$_H$iv/IS4V$_L$, which I was unable to show in the COS-7 transient expression system.

4.3.3 Binding of IgG produced by CHO cells to β_2GPI and PS

The anti-β_2GPI and anti-PS binding activity of the IgG expressed by the stably transfected CHO*dhfr*$^-$ cells is shown in Figure 4.5 and 4.6 respectively, which are representative results of three experiments. The positive and negative controls used were the same as those used in the CL ELISA. A combination was not considered to bind β_2GPI if its binding curve gave an absorbance of less than 0.1 (at 405nm) for the concentration tested in the anti-β_2GPI ELISA. The strongest binder to both β_2GPI is SV-IS4V$_H$/B3V$_L$. Another four combinations show weak binding to β_2GPI. I successfully demonstrated weak binding of SV-IS4V$_H$/IS4V$_L$ to β_2GPI, something that I had been unable to show with the concentrations of antibody obtained previously

from the COS-7 expression system. This combination was described as a weak binder to β_2GPI by the original authors (Zhu et al., 1999). The combination containing the mutation at position 100g and IS4V$_L$ (i.e. SV-IS4V$_H$iv/IS4V$_L$) also bound β_2GPI and the binding was slightly stronger than the wild-type SV-IS4V$_H$/IS4V$_L$. Two other combinations gave similar weak binding to β_2GPI in combination with IS4V$_L$, one has the mutation at position 100 and the other has the mutation at positions 96 and 97 in IS4V$_H$. Any mutation in IS4V$_H$ in combination with B3V$_L$ abolishes the binding to β_2GPI.

The results obtained from the anti-PS ELISA are much clearer (Figure 4.6). Only two combinations bind PS. SV-IS4V$_H$/B3V$_L$ is a strong binder and mutation of arginines at positions 96 and 97 shows a marked reduction in the binding to PS. None of the other combinations bind PS at the concentrations tested.

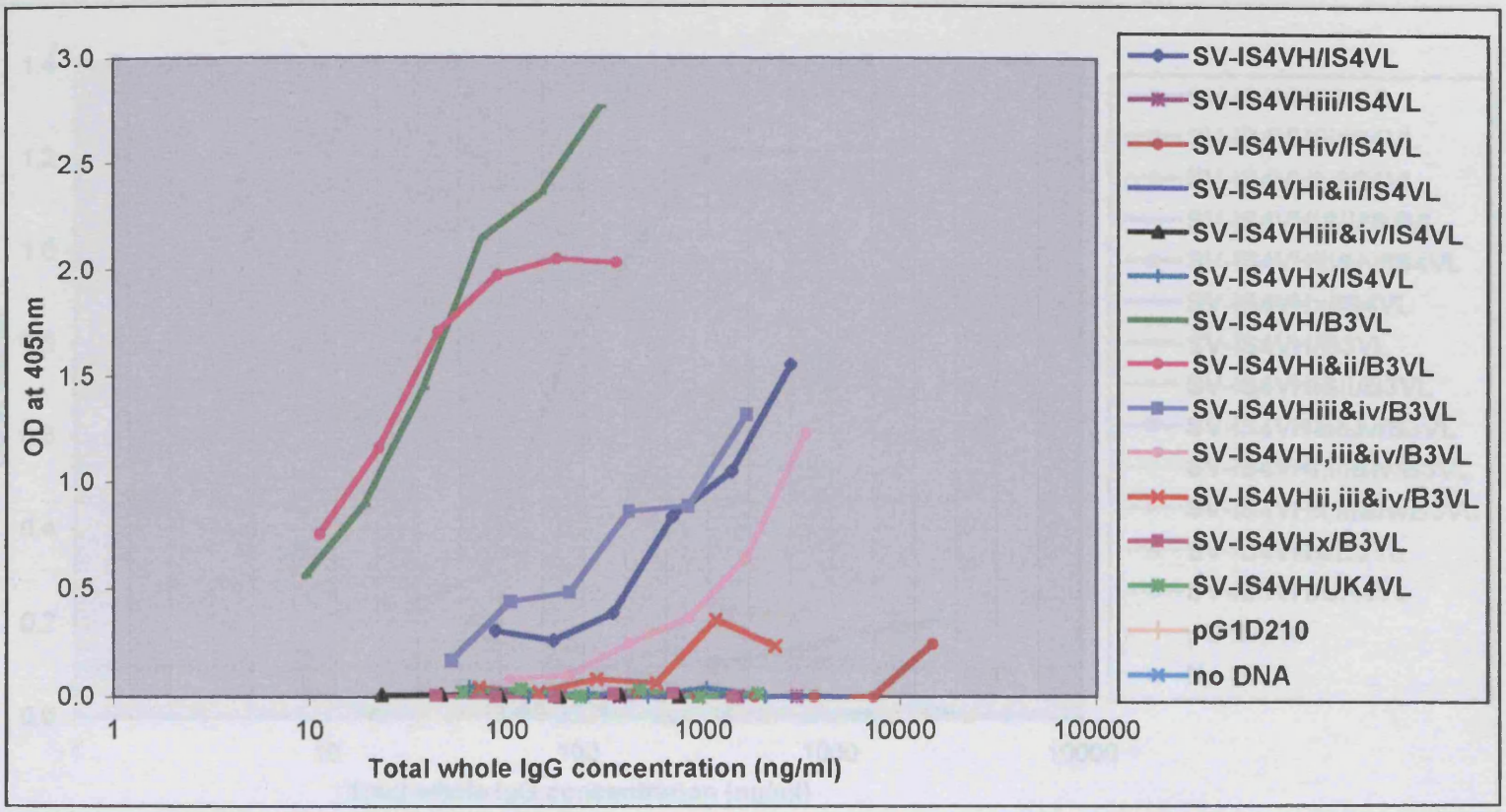


Figure 4.4 Results of aCL ELISA

CL binding of IgG in CHO cell supernatants containing supervectors with wild type or mutant forms of IS4 H chain with wild type IS4, B3 and UK4 L chains.

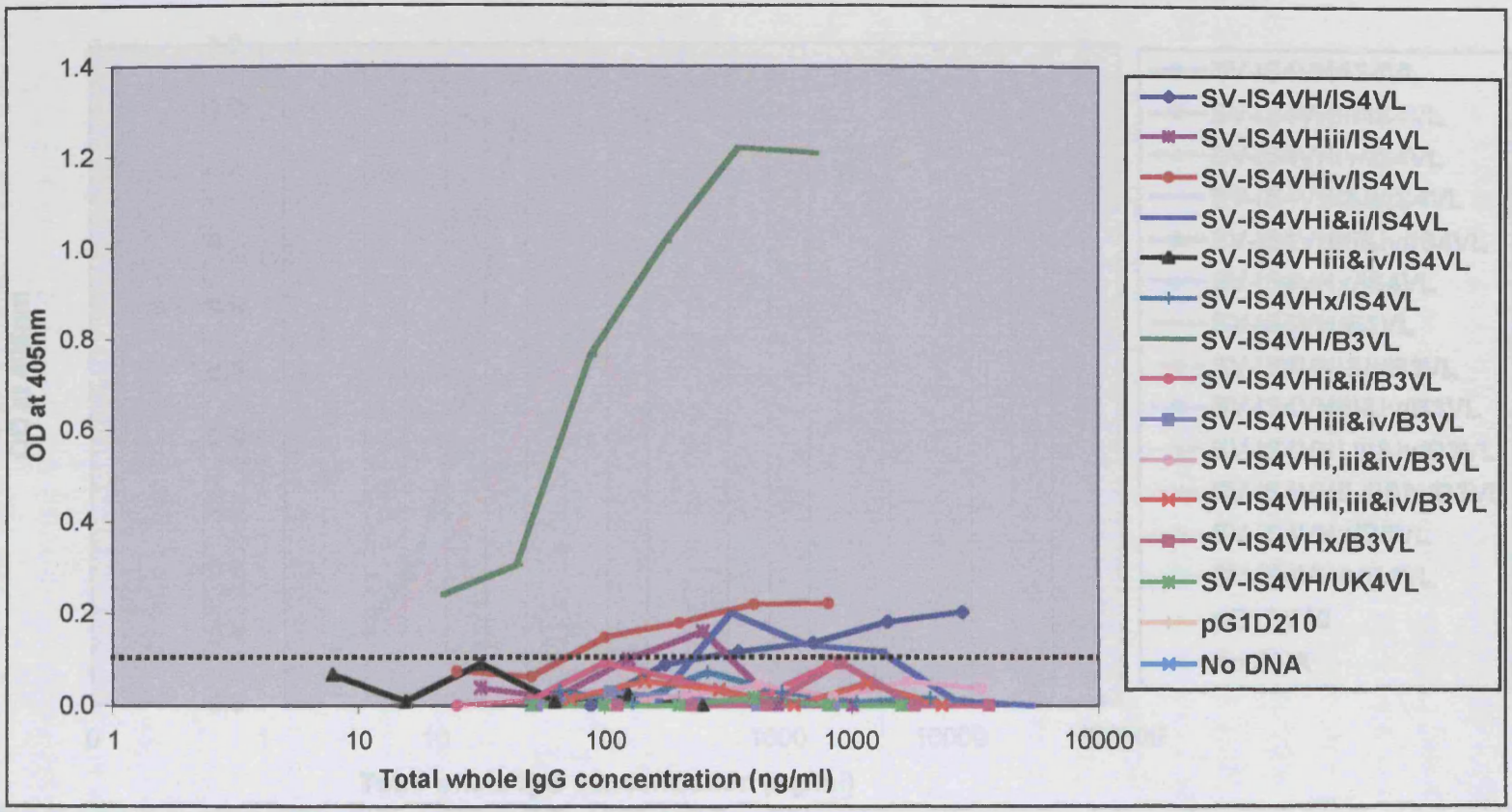


Figure 4.5 Results of anti-β_2GPI ELISA

β_2GPI binding of IgG in CHO cell supernatants containing supervectors with wild type or mutant forms of IS4 H chain with wild type IS4, B3 and UK4 L chains.

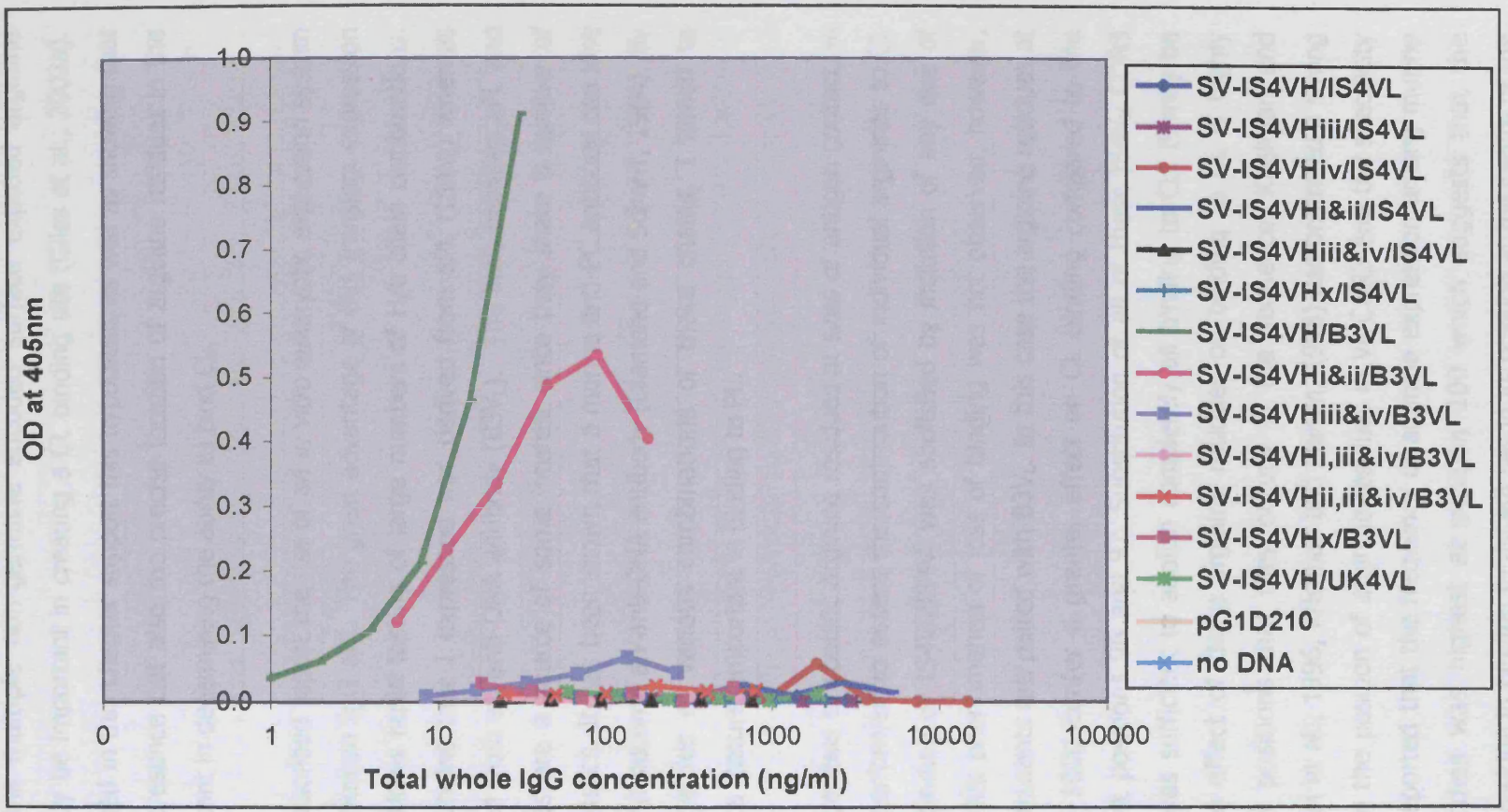


Figure 4.6 Results of anti-PS ELISA

PS binding of IgG in CHO cell supernatants containing supervectors with wild type or mutant forms of IS4 H chain with wild type IS4, B3 and UK4 L chains.

4.4 Discussion

Computer models and previous experiments performed by our group have shown that IS4V_H contains multiple non-germline encoded surface exposed arginine residues, which may be important in creating a CL binding site (Giles et al., 2003a). The results described in this chapter support this hypothesis as well as showing that it is not just the presence but also the precise location of arginine residues in the CDRs that is important in determining the ability to bind CL.

The experiments described report the use of an *in vitro* eukaryotic expression system to produce whole human IgG aPL. The great advantage of this transient expression system is that it allows rapid testing of large numbers of H/L chain combinations. One of the light chains that I expressed was derived from aPL (IS4V_L) whereas others were derived from an anti-DNA antibody (B3V_L). The links between aPL and anti-DNA antibodies are a source of some interest since they share a number of sequence characteristics. It has been shown that a murine anti-PC antibody can lose specificity for PL and become an anti-DNA antibody (Diamond and Scharff, 1984). By studying the properties of various combinations of these chains, I aimed to distinguish sequence features important in binding to PL.

Site-directed mutagenesis of specific arginine residues at sites of antigen contact, in IS4V_H CDR3, was performed to assess the contribution of individual arginines to CL binding. The low binding of IS4V_H/IS4V_L was abolished by inclusion of any one of these mutations. This phenomenon of loss of binding was not observed, however, when these IS4V_H mutants are paired with B3V_L. In this case the arginine residues at positions 100 and 100g confer a greater effect on CL binding compared to the arginine residues at positions 96 and 97. Substitution of all of these IS4V_H CDR3 arginine residues was sufficient to abolish completely all binding to CL. It may be concluded that the effect of these arginine residues on binding to CL is highly dependent on the positions that they occupy in the sequence. Krishnan and colleagues (Krishnan et al., 1996, Krishnan and Marion, 1998) demonstrated a strong correlation between the position of arginine residues in V_H CDR3 and the specificity for dsDNA. They reported that the frequency of arginine expression among murine anti-dsDNA antibodies was highest at position 100 which suggests that the importance of this residue in binding to dsDNA lies in its position at the centre of the V_H CDR3 loop in the structure of the antigen combining site (Krishnan et al., 1996).

In the previous chapter, I have shown the importance of an arginine residue at a specific position in the V_H of an anti-DNA antibody, B3. Many other groups have also expressed antibodies *in vitro*, altering the V_H or V_L sequence by mutagenesis and shown that altering the numbers of arginine residues in the CDRs of these antibodies can lead to significant changes in binding to DNA. Several groups have shown that arginines in V_HCDR3 often play a particularly important role in binding to DNA (Li et al., 2000, Radic et al., 1991, Mockridge et al., 1996). In the case of aPL, different groups have reported important contributions from the H and the L chain. Cocca et al., (2001) studied a murine antibody, 3H9 that has dual specificity for PLs and DNA and showed that reversion of an arginine in the H chain V region reduced binding to these antigens. In another study also by the same group (Cocca et al., 2002), arginine residues were introduced into the V_H region at positions known to mediate DNA binding and they observed enhanced binding to PS-β_2GPI complexes and to apoptotic cell debris.

The amount of whole IgG produced by transient expression in COS-7 cells was too small to allow purification or for experiments on pathogenicity to be carried out. It was therefore necessary to establish a stable expression system for production of recombinant IS4 and its variants in CHO cells to allow for more extensive *in vitro* and *in vivo* characterisation of the binding of the human IgG. In this chapter I also describe the successful development of a stable expression system to produce whole human recombinant aPL IgG molecules from cloned PCR products encoding aPL sequences. Furthermore, the stable cells lines were amplified using MTX.

The total yield of IgG1 varied between 6 and 5152 ng per 10^6 cells per day depending on the H and L chain expressed. With the larger amounts of IgG produced using MTX amplification, binding to CL as well as β_2GPI and PS was clearly demonstrated. The results demonstrating weak binding to β_2GPI in the absence of CL shown in Figure 4.5 confirm the observation of the original authors (Zhu et al., 1999) who found that IS4 secreted by hybridoma cells bound better to CL than to β_2GPI.

My results show that there are different patterns of binding to different antigens. It is possible that the effects of the CDR3 arginine residues on binding to anionic PL are due to charge interactions between different antigens and different antibodies. It is also likely that the conformation of the cofactor, β_2GPI, exposes different epitopes to different PL. The binding of human monoclonal aPL IS4 to β_2GPI is further evidence

that studying this antibody is relevant to increasing our understanding of the pathogenesis of APS given that β_2GPI is recognised as an important cofactor for pathogenic aPL. This aPL is likely to be typical of pathogenic aPL found in other patients with APS. Pewzner-Jung et al., (1996) is the only group that have described a stable expression system to produce a murine monoclonal anti-phosphorylcholine antibody (6G6) as whole IgM molecules. The stable expression of IS4 and variants that I have described in this thesis is the first report of stable *in vitro* expression of whole human IgG aPL.

In summary, I have demonstrated the relative importance of certain surface exposed arginine residues at critical positions within the heavy chain CDR3 of aPL (IS4) in conferring the ability to bind CL in a direct ELISA. After expression of the altered antibodies in larger quantities obtained from stably transfected cells, it is now important to test the effects of sequence changes involving these amino acids on pathogenic functions. More accurate information about changes in affinity of variant forms of IS4 for CL or β_2GPI could be obtained by testing larger quantities of purified antibodies.

CHAPTER FIVE

Investigation of the relationship between antibody sequence and binding properties of purified human monoclonal antibody B3 and its variants

CHAPTER FIVE.

INVESTIGATION OF THE RELATIONSHIP BETWEEN ANTIBODY SEQUENCE AND BINDING PROPERTIES OF PURIFIED HUMAN MONOCLONAL ANTIBODY B3 AND ITS VARIANTS

5.1 Introduction and aims of this chapter

When purified under rigorous conditions, Guth and colleagues have shown that some murine anti-dsDNA antibodies actually bind chromatin rather than dsDNA (Guth et al., 2003). This finding suggests that they may actually be anti-nucleosome antibodies, which only appear to bind dsDNA when they are incompletely dissociated from nucleosomes.

In order to obtain large quantities of purified human IgG secreted from the CHO cell lines produced and described in more detail in Chapter Three, they were grown in their selective amplification medium in a large volume commercial culture system. The human IgG was then purified from the supernatant using a protein A column and dialysed into PBS. The product was then analysed for purity by SDS-PAGE and quantified by spectrophotometry. This large-scale culture and purification was carried out by Chemicon Europe Ltd, Hampshire, UK. With the large amounts of purified antibody generated using this method, it was possible to obtain more accurate information regarding binding affinities to different clinically relevant antigens.

The aims of this chapter are to report the results of testing the purified antibodies to investigate whether the human mAb B3 is actually an anti-nucleosome antibody and also to investigate further the properties of the mutations introduced into B3, in binding to nucleosomes, ssDNA, α-actinin, CL, β_2GPI, Domain I of β_2GPI, PS and PS-PT.

5.2 Results of binding of purified human IgG molecules to clinically relevant antigens

5.2.1 Assembly of purified antibodies

Microgram quantities of recombinant human IgG were produced from each of the CHO cell lines 'wild-type' $B3V_H/B3V_L$, $B3V_H/B3(R27aS)V_L$, $B3V_H/B33V_L$ ($B3V_H$ co-expressed with $B3V_L$ CDR1 and $33H11V_L$ CDR2 and CDR3), $B3V_H/BUV_L$ ($B3V_H$ co-expressed with $B3V_L$ CDR1 and UK-$4V_L$ CDR2 and CDR3), $B3(R53S)V_H/B3V_L$, $B3(R53S)V_H/B33V_L$, $B3(R53N)V_H/B3V_L$, $B3(R53K)V_H/B3V_L$. The human IgG concentration of each antibody was verified using the whole human IgG ELISA, described in section 2.7.1.

5.2.1.1 Results of anti-dsDNA ELISA

Figure 5.1 shows binding of purified IgG from the four H/L combinations $B3V_H/B3V_L$, $B3V_H/B3(R27aS)V_L$, $B3V_H/B33V_L$ and $B3V_H/BUV_L$ to dsDNA under different conditions. Similar results were obtained in repeated ELISA assays. Figure 5.1A shows that when the samples are not treated with *DNase* after being simply diluted in SEC buffer, the combination $B3V_H/B33V_L$ binds dsDNA but the other three do not. Figure 5.1B, shows the effect of *DNase I* treatment of the purified antibodies, diluted in SEC buffer, prior to testing in the dsDNA ELISA. None of these combinations binds dsDNA and the *DNase I* treatment appears to have abolished the binding of the purified SVB33 to dsDNA. In order to investigate whether a 'cofactor' was necessary for DNA binding, which might be present in cell culture supernatant but which may have been removed by purification, the purified antibodies were also tested for binding to dsDNA after dilution in supernatant from COS-7 cells that had been electroporated in the absence of plasmid. The antibody/COS-7 supernatant solutions were pre-treated with *DNase I*, prior to testing in the dsDNA ELISA. The addition of the COS-7 supernatant allows the binding of the three combinations - $B3V_H/B3V_L$, $B3V_H/B3(R27aS)V_L$, $B3V_H/B33V_L$ – to dsDNA (Figure 5.1C), despite treatment with *DNase*. The strength of binding increased in the order $B3V_H/B3(R27aS)V_L$ < $B3V_H/B3V_L$< $B3V_H/B33V_L$. The same order was seen previously by expressing these combinations transiently in COS-7 cells (Haley et al., 2004). The combination $B3V_H/BUV_L$ does not bind dsDNA in ELISA under any conditions, which also corresponds to results obtained previously (Haley et al., 2004).

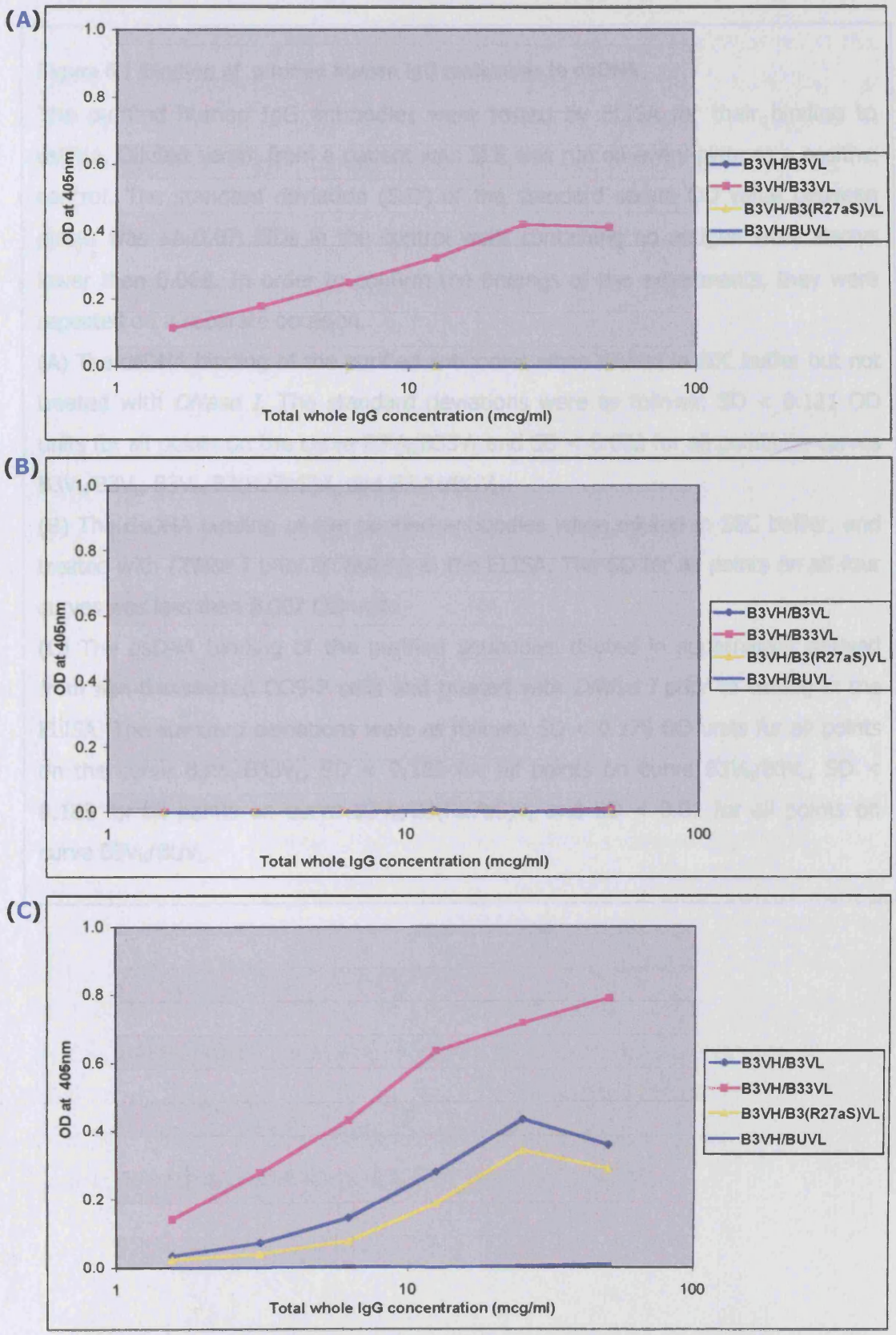


Figure 5.1 Binding of purified human IgG molecules to dsDNA

For legend see over

Figure 5.1 Binding of purified human IgG molecules to dsDNA

The purified human IgG antibodies were tested by ELISA for their binding to dsDNA. Diluted serum from a patient with SLE was run on every plate as a positive control. The standard deviation (S.D) of the standard serum OD value between plates was +/−0.07. ODs in the control wells containing no antigen were always lower than 0.068. In order to confirm the findings of the experiments, they were repeated on a separate occasion.

(A) The dsDNA binding of the purified antibodies when diluted in SEC buffer but not treated with *DNase I*. The standard deviations were as follows: SD < 0.121 OD units for all points on the curve $B3V_H/B33V_L$ and SD < 0.002 for all points on curves $B3V_H/B3V_L$, $B3V_H/B3(R27aS)V_L$ and $B3VH/BUV_L$.

(B) The dsDNA binding of the purified antibodies when diluted in SEC buffer, and treated with *DNase I* prior to testing in the ELISA. The SD for all points on all four curves was less than 0.002 OD units.

(C) The dsDNA binding of the purified antibodies diluted in supernatant derived from non-transfected COS-7 cells and treated with *DNase I* prior to testing in the ELISA. The standard deviations were as follows: SD < 0.176 OD units for all points on the curve $B3V_H/B33V_L$, SD < 0.185 for all points on curve $B3V_H/B3V_L$, SD < 0.185 for all points on curve $B3V_H/B3(R27aS)V_L$ and SD < 0.01 for all points on curve $B3V_H/BUV_L$.

5.2.1.2 Results of anti-nucleosome and anti-ssDNA ELISA

Figure 5.2 shows binding of the eight purified antibodies to nucleosomes, in the absence of COS-7 cell supernatant. The combinations $B3V_H/B3V_L$, $B3V_H/B33V_L$, and $B3V_H/B3(R27aS)V_L$) are good nucleosome binders. Strength of binding to nucleosomes was similar for the three combinations. There is a tendency to increased strength of binding in the order $B3V_H/B3(R27aS)V_L$ < $B3V_H/B3V_L$< $B3V_H/B33V_L$. $B3(R53S)V_H/B33V_L$ is a weak binder to nucleosomes. The other four combinations $B3(R53S)V_H/B3V_L$, $B3(R53N)V_H/B3V_L$, $B3(R53K)V_H/B3V_L$, and $B3V_H/BUV_L$ do not bind nucleosomes. Thus mutation of R53 to either S, N or K abolishes binding of B3 to nucleosomes (when paired with $B3V_L$) even when the antibodies are purified and tested at high concentrations. Similar patterns of binding were obtained with each antibody from repeated experiments hence representative results from a single experiment are shown in Figure 5.2. I also tested the antibodies for binding to ssDNA. There was no binding to ssDNA for any of the eight purified antibodies despite good binding of the positive control in the anti-ssDNA ELISA.

5.2.1.3 Addition of nucleosomes allows the reinstatement of dsDNA binding of the purified antibodies

Since the same three combinations which bound dsDNA on the addition of COS-7 supernatant (Figure 5.1C) also bound nucleosomes (Figure 5.2), I carried out experiments to test whether addition of purified nucleosomes (rather than cell supernatant) would have the same effect on binding of these antibodies to dsDNA. Figure 5.3 shows that binding of $B3V_H/B3(R27aS)V_L$ to dsDNA is reconstituted by adding purified nucleosomes at a concentration of 10μg dsDNA/ml. This effect was also seen for this H/L combination at a nucleosome concentration of 2.5μg dsDNA/ml (though the OD achieved was lower) but not at nucleosome concentrations of 1.5μg dsDNA/ml or 20μg dsDNA/ml. I was unable to demonstrate reconstitution of binding of purified $B3V_H/B3V_L$, $B3V_H/B33V_L$ or $B3V_H/BUV_L$ to dsDNA at any of these four concentrations of nucleosomes (1.5, 2.5, 10 or 20μg dsDNA/ml).

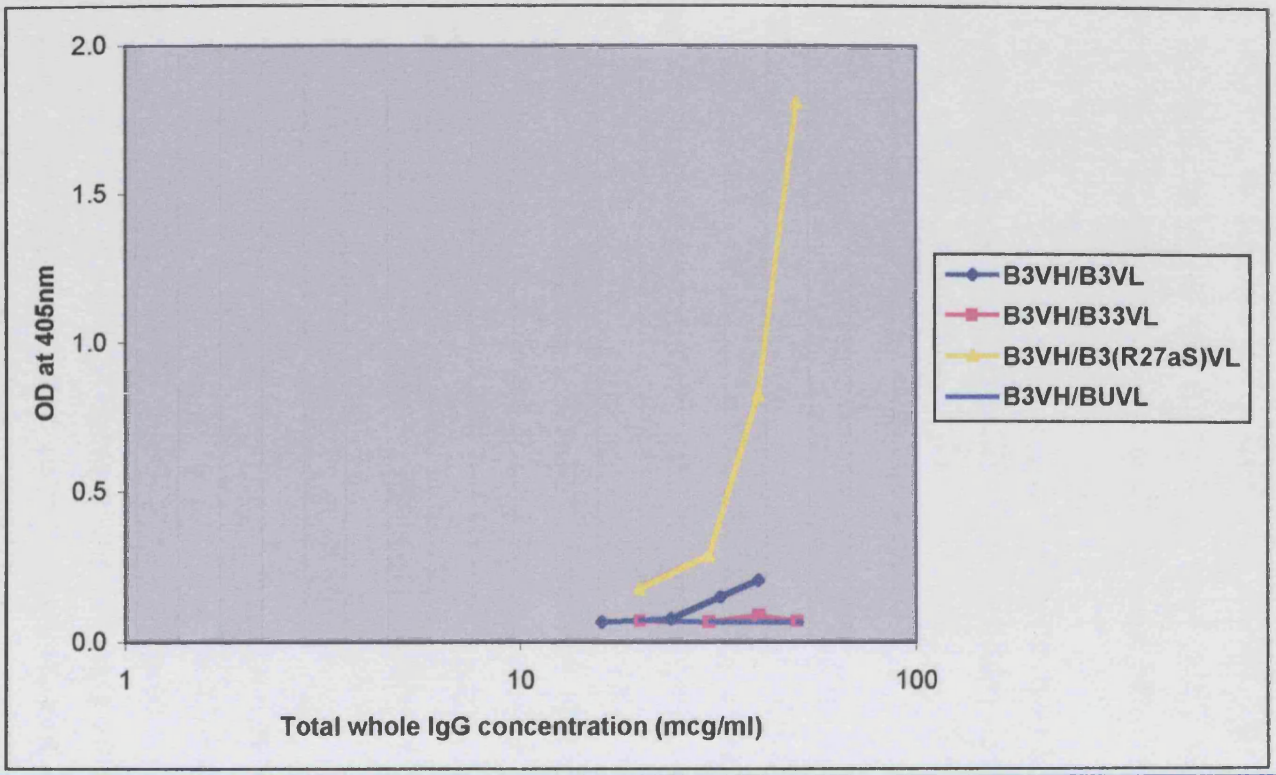


Figure 5.3 Binding to dsDNA of purified antibodies containing nucleosomes

Binding to dsDNA of purified *DNase I* treated antibodies diluted in SEC containing nucleosomes at a concentration of 10μg dsDNA/ml. At this concentration of nucleosomes only $B3V_H/B3(R27aS)V_L$ binds to dsDNA. The same results were obtained when the experiment was repeated at a concentration of 2.5μg dsDNA/ml except that the peak OD for the $B3V_H/B3(R27aS)V_L$ curve was lower (OD values was 0.2. At nucleosome concentrations of 1.5μg dsDNA/ml or 20μg dsDNA/ml, none of these H/L combinations bound dsDNA.

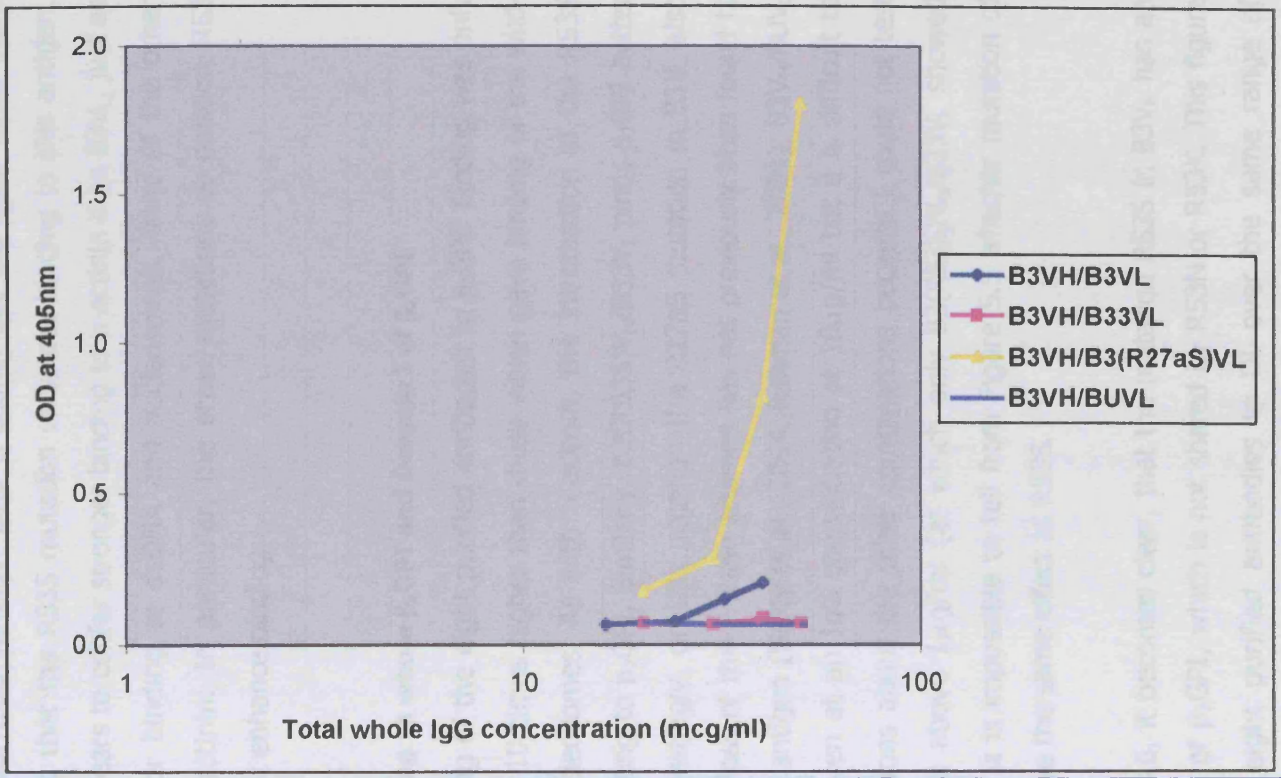


Figure 5.3 Binding to dsDNA of purified antibodies containing nucleosomes

Binding to dsDNA of purified *DNase I* treated antibodies diluted in SEC containing nucleosomes at a concentration of 10μg dsDNA/ml. At this concentration of nucleosomes only $B3V_H/B3(R27aS)V_L$ binds to dsDNA. The same results were obtained when the experiment was repeated at a concentration of 2.5μg dsDNA/ml except that the peak OD for the $B3V_H/B3(R27aS)V_L$ curve was lower (OD values was 0.2. At nucleosome concentrations of 1.5μg dsDNA/ml or 20μg dsDNA/ml, none of these H/L combinations bound dsDNA.

5.2.1.4 Results of α-actinin ELISA

Figure 5.4 shows binding of the eight purified antibodies to α-actinin. In complete contrast to the results of the anti-nucleosome ELISA, the presence of the R53S mutation enhances binding to α-actinin. The wild-type combination $B3V_H/B3V_L$ did not bind α-actinin in this experiment. However, $B3(R53S)V_H/B3V_L$ did bind α-actinin. Both $B3V_H/B33V_L$ and $B3(R53S)V_H/B33V_L$ bind α-actinin but the latter binds more strongly, again showing that the R53S mutation enhances binding to this antigen. The B33 light chain appears to confer stronger binding to α-actinin than $B3V_L$, just as was previously seen for binding to dsDNA and nucleosomes. None of the other combinations binds α-actinin. In particular, the other mutations at position R53 (R53N and R53K) did not enhance binding.

5.2.1.5 Results of binding to whole β_2GPI and Domain I of β_2GPI

Figure 5.5 shows binding of the eight purified antibodies to β_2GPI. Binding was only seen at concentrations 10 times higher than those which gave binding of the wild-type $B3V_H/B3V_L$ to nucleosomes. As with α-actinin, the introduction of the R53S mutation enhances binding to β_2GPI. Similarly, $B3(R53S)V_H/B33V_L$ binds β_2GPI better than $B3V_H/B33V_L$. Interestingly, on this occasion the R27aS mutation in $B3V_L$ also enhances binding. The loss of this L chain arginine has not previously been found to enhance binding to any antigen (Haley et al., 2004, Rahman et al., 2001). $B3V_H/BUV_L$ does not bind β_2GPI even at an IgG concentration of 10μg/ml but it is difficult to reach any firm conclusions about the other combinations because I could not test them at concentrations above 1μg/ml (at which only $B3(R53S)V_H/B33V_L$ showed binding). In particular, it is impossible to tell from Figure 5.5 whether mutation of R53 to N or K would have the same effect as R53S.

If we consider Figure 5.6, it becomes clear, that the mutation R53S in $B3V_H$ has an effect on binding to DI of β_2GPI, which is not shared by R53N or R53K. This figure shows binding of all eight purified antibodies to DI over the same range of concentrations up to a peak of 1μg/ml. Only $B3(R53S)V_H/B3V_L$ shows any significant binding to DI at these concentrations. Here, $B3(R53S)V_H/B33V_L$ binds much less strongly than $B3(R53S)V_H/B3V_L$ showing that the B33 L chain does not enhance binding to DI, contrary to its effects on binding to nucleosomes and α-actinin.

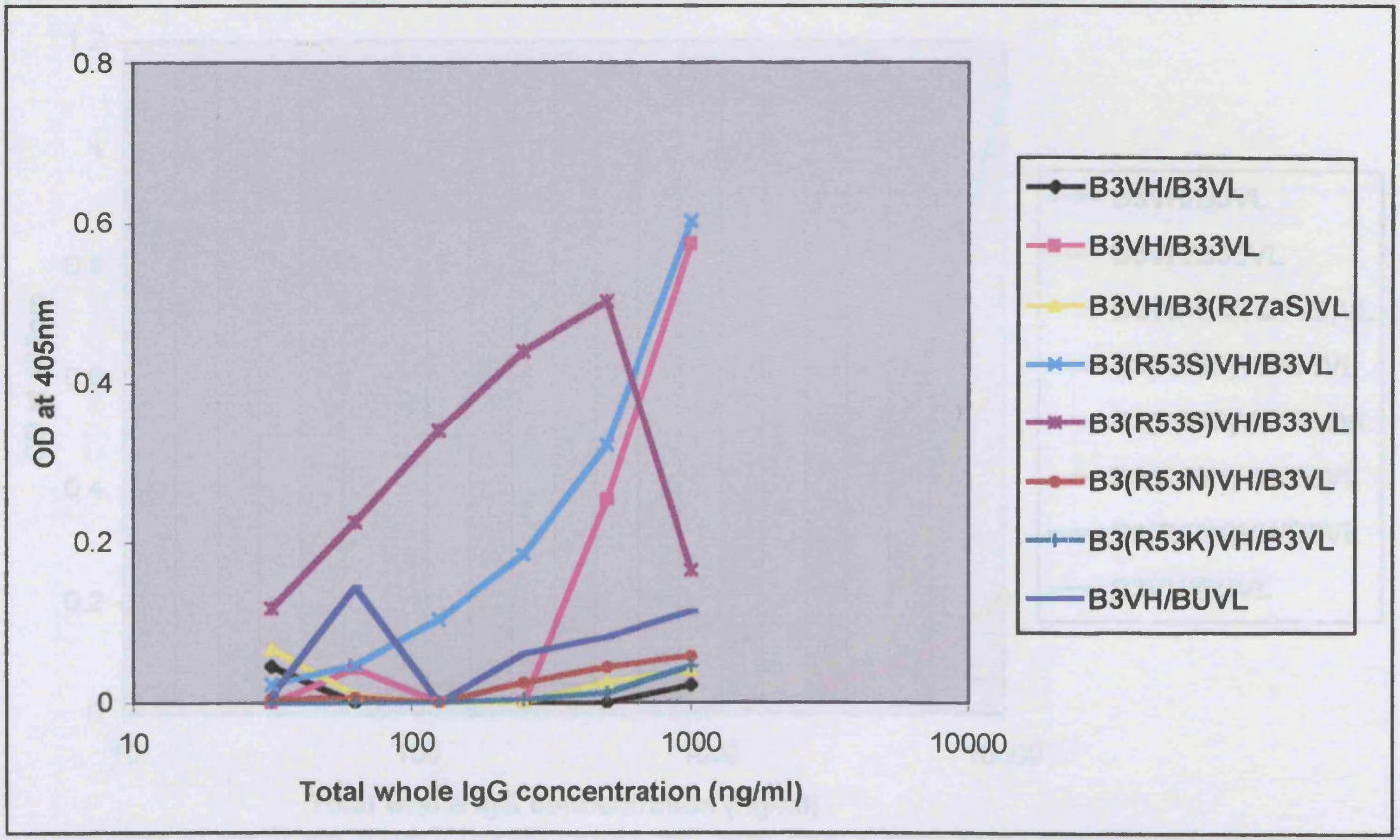


Figure 5.4 Results of anti-α-actinin ELISA

This figure shows the binding of the eight purified antibodies in direct ELISA assay to α-actinin. Diluted serum from a patient with SLE was run as a positive control.

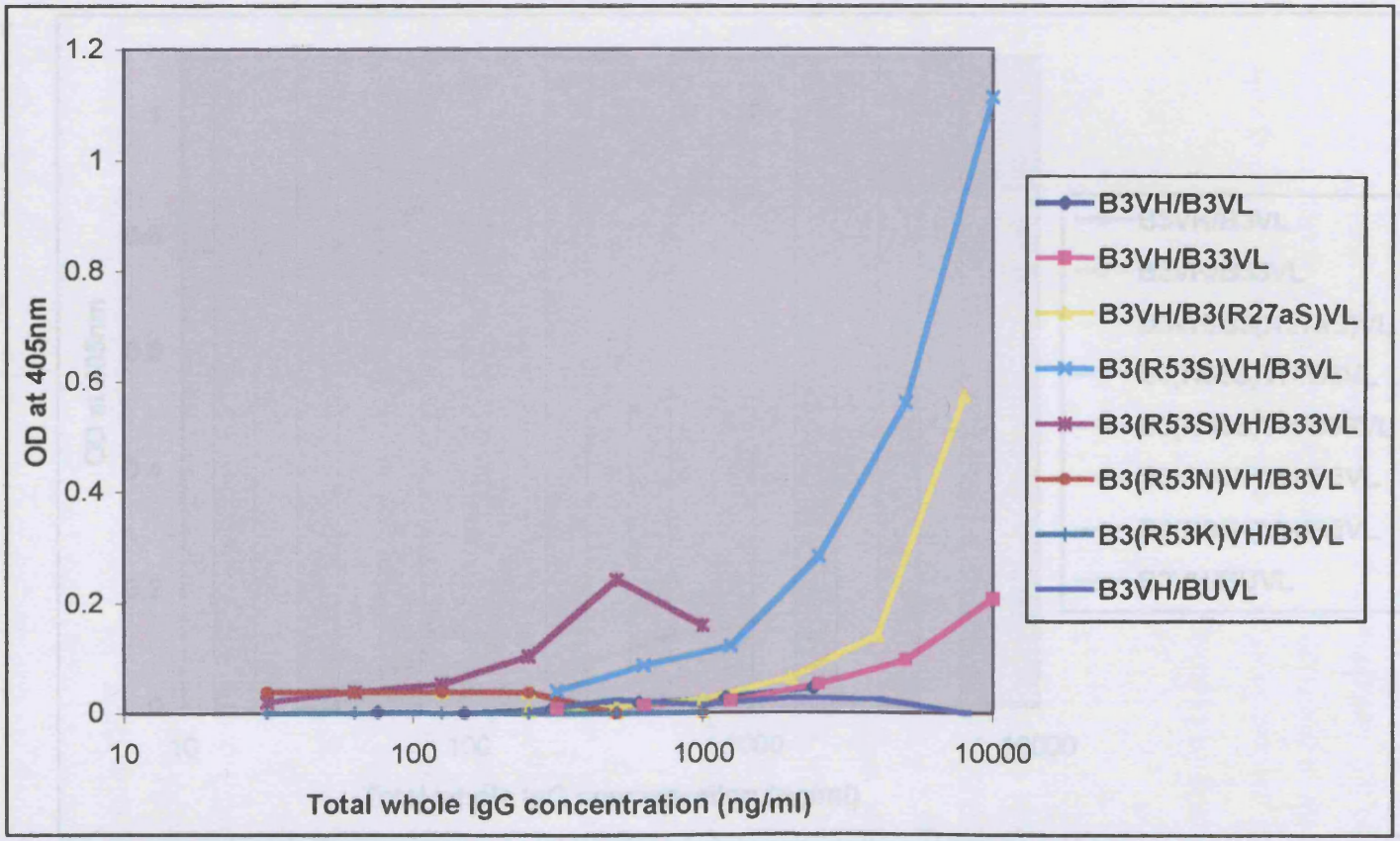


Figure 5.5 Results of anti-β_2GPI ELISA

This figure shows the binding of the eight purified antibodies in direct ELISA assay to β_2GPI. Diluted serum from a patient with APS was run as a positive control.

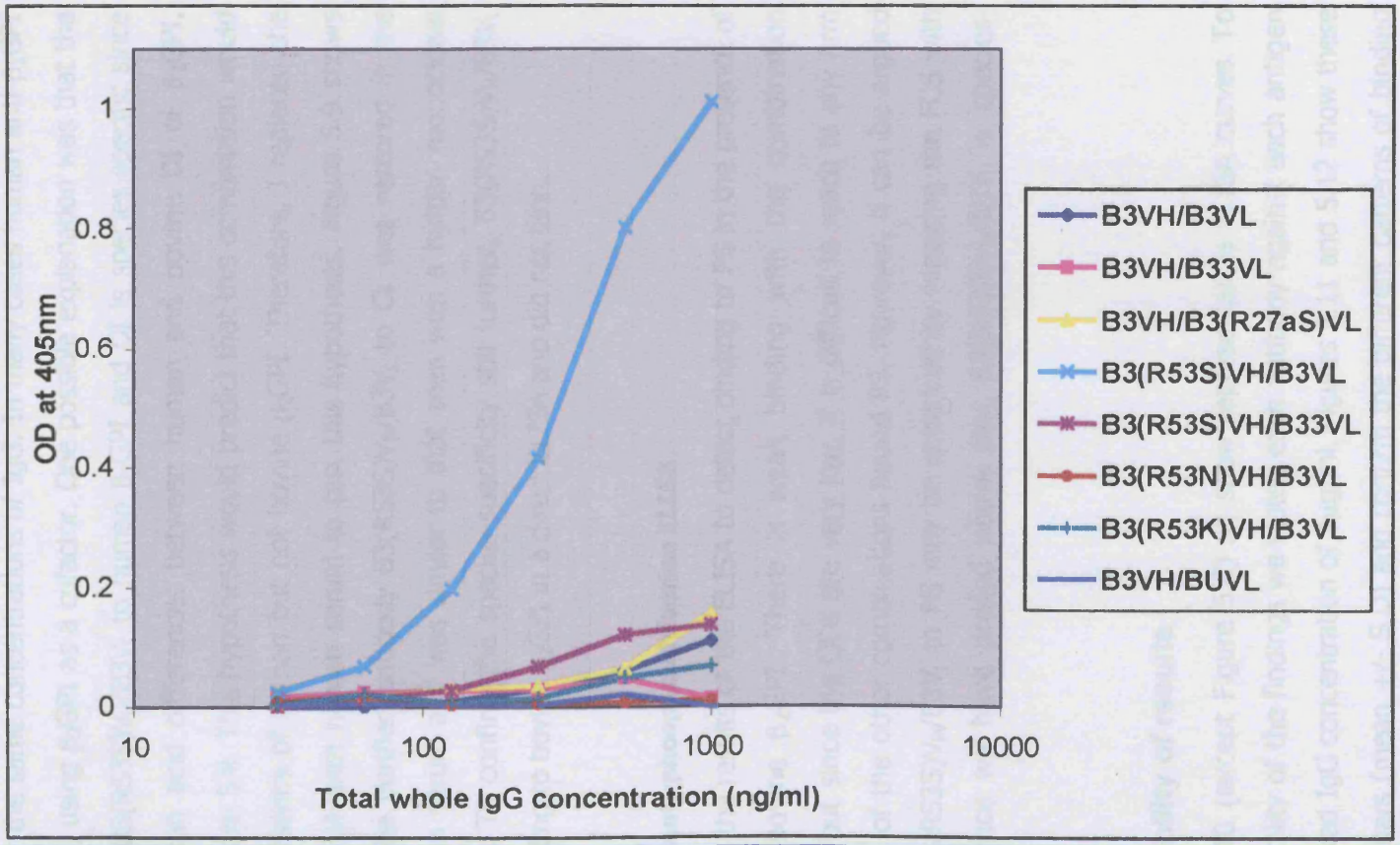


Figure 5.6 Results of anti-Domain I ELISA

This figure shows the binding of the eight purified antibodies in direct ELISA assay to DI of β_2GPI. Diluted serum from a patient with APS was run as a positive control.

5.2.1.6 Results of anti-cardiolipin ELISA

Figure 5.7 shows results of the ELISA to detect binding to CL in the presence of FCS, containing bovine β_2GPI. With the exceptions of combinations $B3V_H/B3V_L$ and $B3(R53S)V_H/B33V_L$, binding is either absent or weak and only present above 1μg/ml. Surprisingly, the combination $B3(R53S)V_H/B3V_L$ did not bind CL despite binding well to β_2GPI and DI at the same concentrations of IgG. In many cases human anti-β_2GPI antibodies bind CL, using β_2GPI as a cofactor. One possible explanation was that the strong binding of $B3(R53S)V_H/B3V_L$ to human β_2GPI and DI is species-specific since there are 11 amino acid differences between human and bovine DI of β_2GPI, represented in Figure 5.8. This hypothesis would predict that this combination would bind CL in the presence of human but not bovine β_2GPI. Therefore, I replaced the FCS in the CL ELISA with human serum to test this hypothesis. Figure 5.9 shows weak binding of the purified antibody $B3(R53S)V_H/B3V_L$ to CL was restored in the presence of human serum and was similar to that seen with a human monoclonal aPL ($IS4V_H/IS4V_L$). To confirm the species-specificity still further, $B3(R53S)V_H/B3V_L$ was tested for binding to bovine β_2GPI in a direct ELISA and did not bind.

5.2.1.7 Results of anti-phosphatidylserine ELISA

Figure 5.10 shows the results of the ELISA to detect binding to PS in the presence of FCS, containing bovine β_2GPI. There is weak binding with the combination $B3(R53S)V_H/B3V_L$, but since the ODs are very low, it is difficult to reach to any firm conclusions. None of the other combinations bound PS. However, it can be argued that binding of $B3(R53S)V_H/B3V_L$ to PS may be enhanced by replacing the FCS with human serum (since we have proved above that $B3(R53S)V_H/B3V_L$ is species-specific).

5.2.2 Reproducibility of results

Figures 5.1 to 5.10 (except Figure 5.8) all show representative ELISA curves. To confirm reproducibility of the findings we tested each antibody against each antigen in triplicate at a fixed IgG concentration of 1μg/ml. Figures 5.11 and 5.12 show these results with error bars (mean +/- S.D) and confirm the different patterns of binding seen against different antigens. These results show that the presence or absence of R53 in the V_H sequences of these eight H/L combinations has profound effects on binding to multiple clinically relevant antigens but has different effects on different antigens.

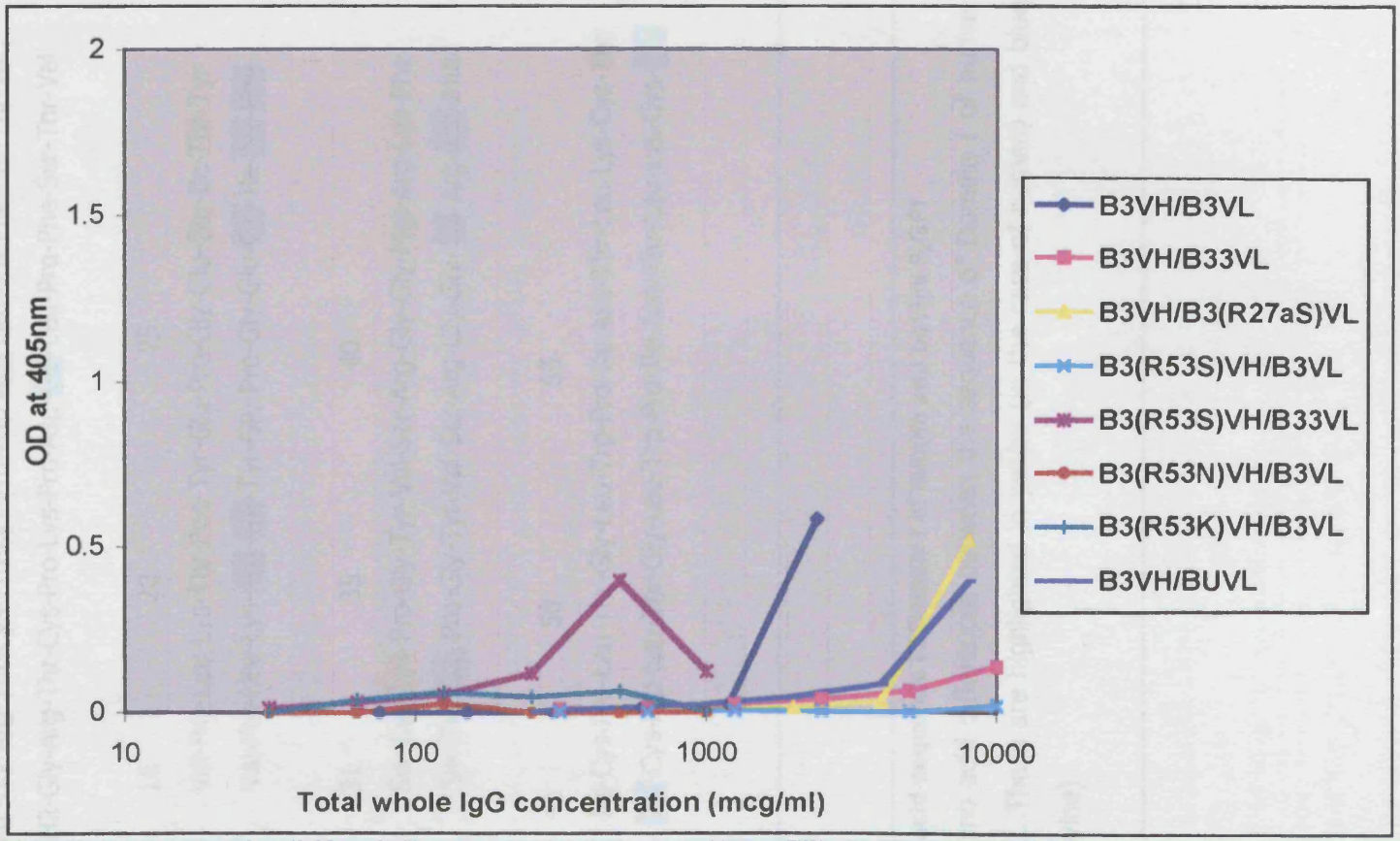


Figure 5.7 Results of anti-cardiolipin ELISA

This figure shows the binding of the eight purified antibodies in direct ELISA assay to CL. Diluted serum from a patient with the Antiphospholipid syndrome was run as a positive control.

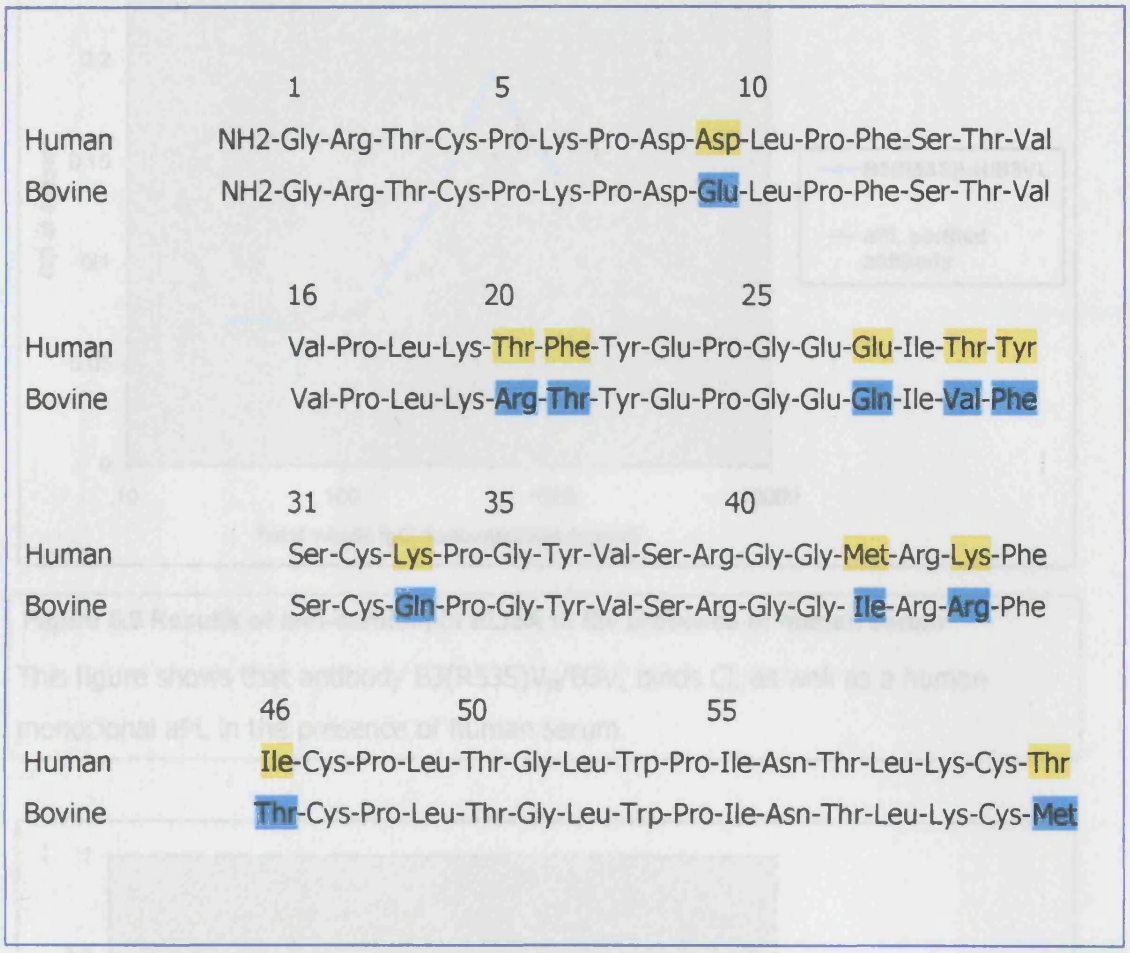


Figure 5.8 Amino acid sequence of Domain I of human and bovine β_2GPI

There are 11 amino acid differences between the sequence of Domain I of human and bovine β_2GPI. These are highlighted in yellow (in the case of human) and blue (in the case of bovine).

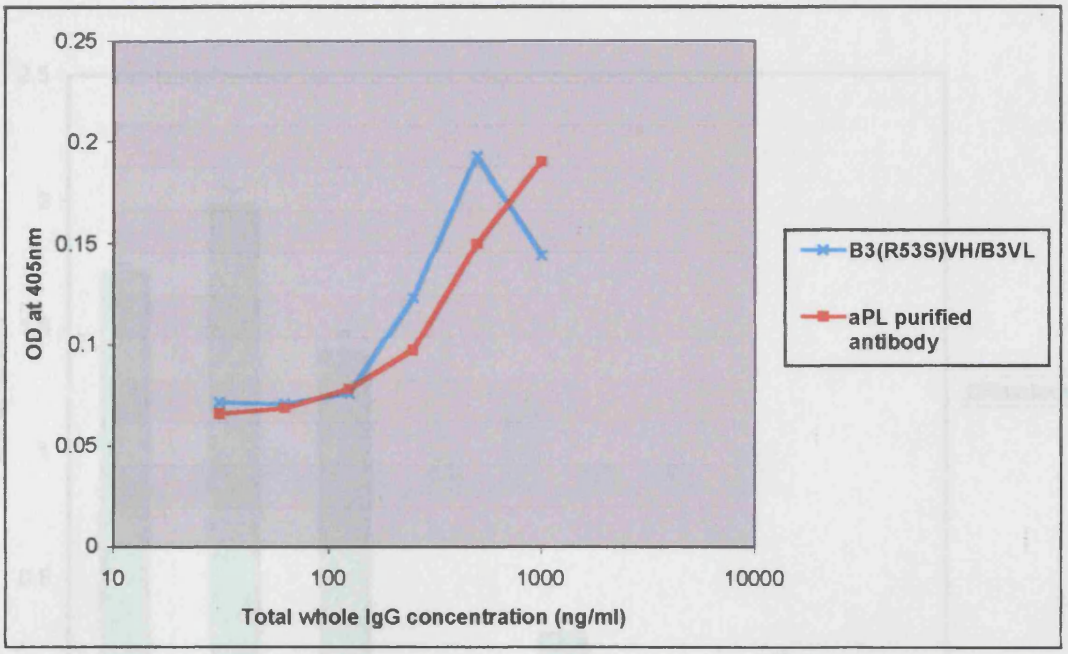


Figure 5.9 Results of anti-cardiolipin ELISA in the presence of human serum

This figure shows that antibody B3(R53S)V_H/B3V_L binds CL as well as a human monoclonal aPL in the presence of human serum.

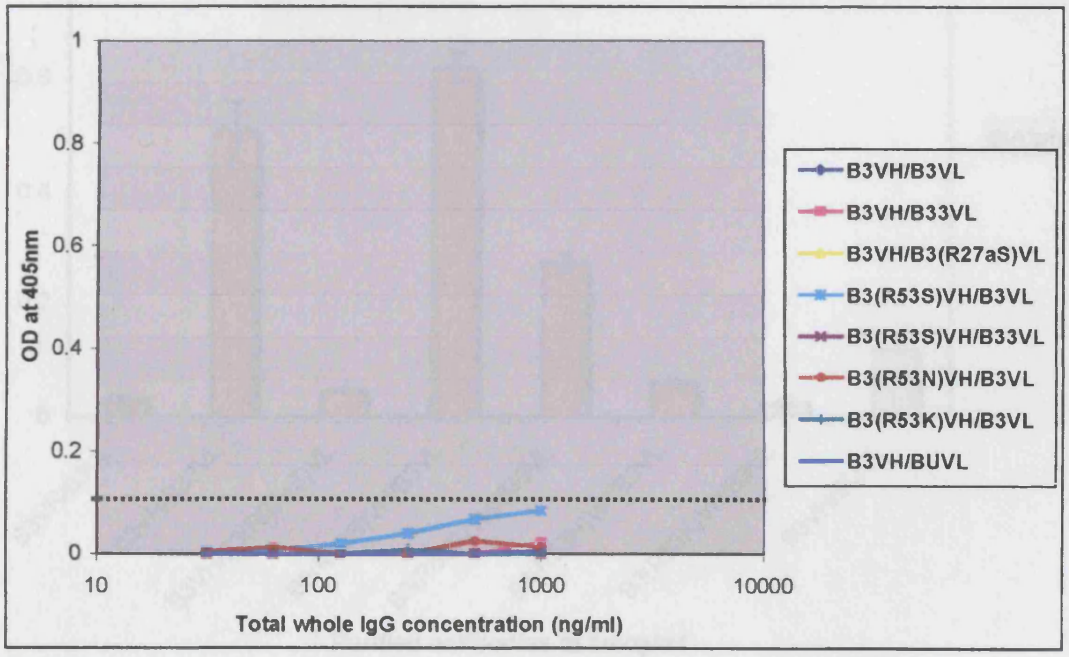


Figure 5.10 Results of anti-phosphatidylserine ELISA

This figure shows the binding of the eight purified antibodies in direct ELISA assay to PS. Diluted serum from a patient with APS was run as a positive control. A combination was not considered to bind PS if its binding curve gave an absorbance of less than 0.1 (at 405nm) (represented by the dotted line) for the concentration tested in the anti-PS ELISA.

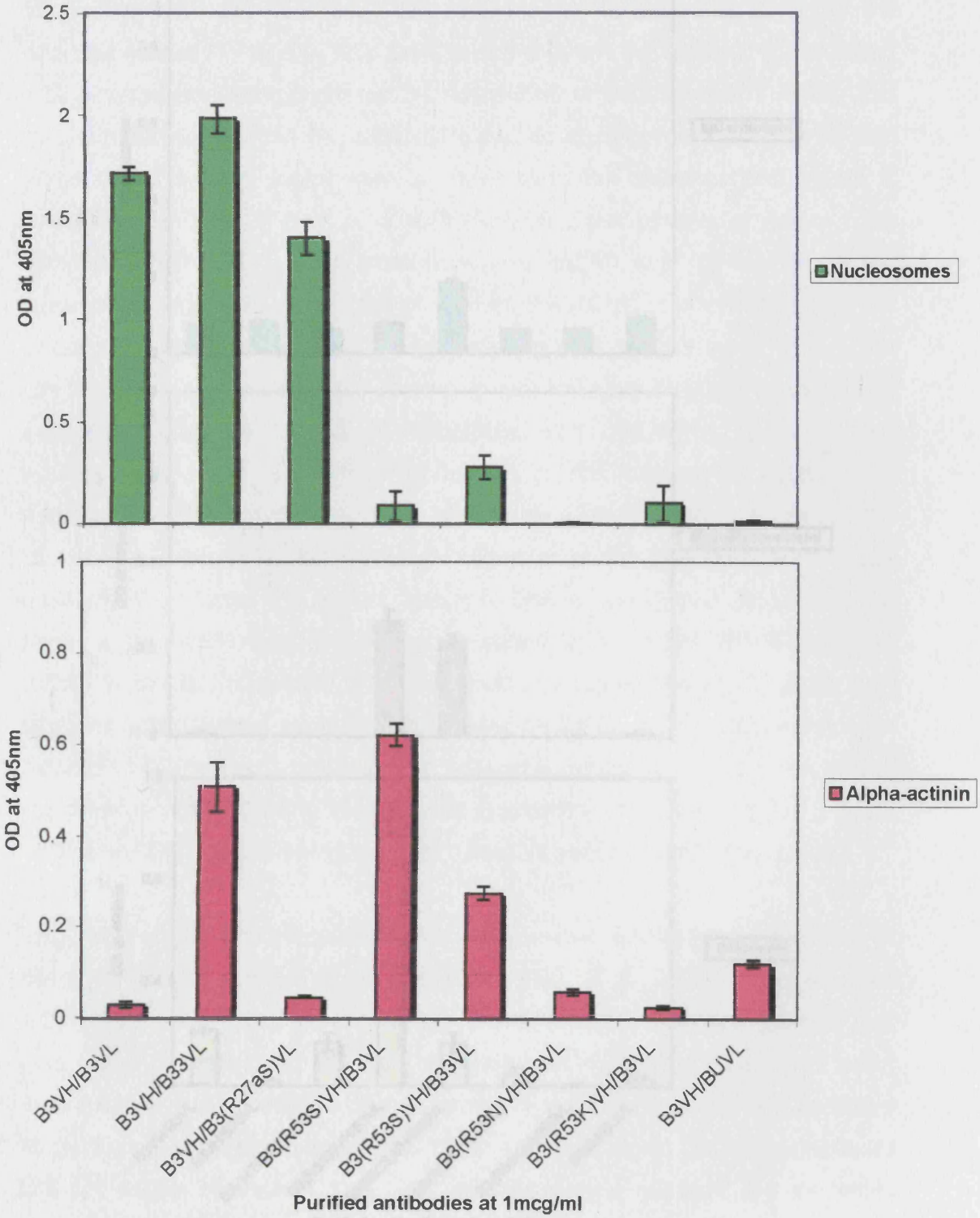


Figure 5.11 Summary of binding to nucleosomes and α-actinin

The chart represents the binding of the eight purified antibodies to nucleosomes (equivalent to a concentration of 10μg/ml dsDNA) and α-actinin (20μg/ml). All antibodies were tested at a concentration of 1μg/ml in triplicate and the error bars represent mean +/– S.D.

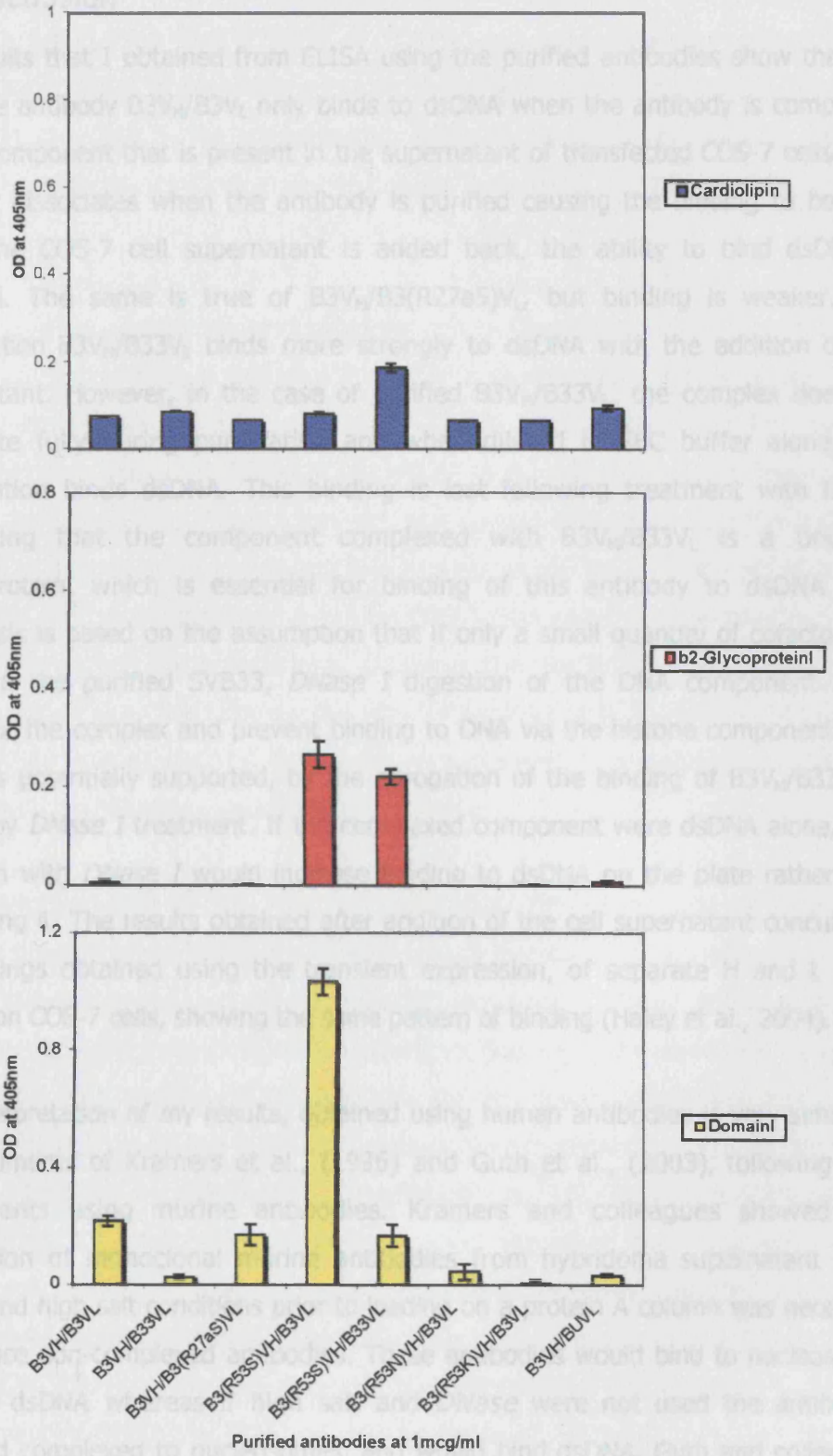


Figure 5.12 Summary of binding to cardiolipin, β_2GPI and Domain I

The chart represents the binding of the eight purified antibodies to CL (50μg/ml), β_2GPI (10μg/ml) and DI (5μg/ml). All antibodies were tested at a concentration of 1μg/ml in triplicate and the bars represent mean +/– S.D.

5.3 Discussion

The results that I obtained from ELISA using the purified antibodies show that the wild-type antibody $B3V_H/B3V_L$ only binds to dsDNA when the antibody is complexed with a component that is present in the supernatant of transfected COS-7 cells. This complex dissociates when the antibody is purified causing the binding to be lost. When the COS-7 cell supernatant is added back, the ability to bind dsDNA is restored. The same is true of $B3V_H/B3(R27aS)V_L$, but binding is weaker. The combination $B3V_H/B33V_L$ binds more strongly to dsDNA with the addition of the supernatant. However, in the case of purified $B3V_H/B33V_L$, the complex does not dissociate fully during purification and when diluted in SEC buffer alone, this combination binds dsDNA. This binding is lost following treatment with DNase suggesting that the component complexed with $B3V_H/B33V_L$ is a bridging nucleoprotein, which is essential for binding of this antibody to dsDNA. This hypothesis is based on the assumption that if only a small quantity of cofactor was bound to the purified SVB33, *DNase I* digestion of the DNA component might dissociate the complex and prevent binding to DNA via the histone component. This theory is potentially supported, by the abrogation of the binding of $B3V_H/B33V_L$ to dsDNA by *DNase I* treatment. If the complexed component were dsDNA alone, then digestion with *DNase I* would increase binding to dsDNA on the plate rather than decreasing it. The results obtained after addition of the cell supernatant concur with the findings obtained using the transient expression, of separate H and L chain vectors on COS-7 cells, showing the same pattern of binding (Haley et al., 2004).

This interpretation of my results, obtained using human antibodies is very similar to the arguments of Kramers et al., (1996) and Guth et al., (2003), following their experiments using murine antibodies. Kramers and colleagues showed that purification of monoclonal murine antibodies from hybridoma supernatant using *DNase* and high salt conditions prior to loading on a protein A column was necessary to produce non-complexed antibodies. These antibodies would bind to nucleosomes but not dsDNA whereas if high salt and *DNase* were not used the antibodies remained complexed to nucleosomes, and would bind dsDNA. Guth and colleagues studied antibodies 3H9 and SN5-18 derived from two different autoimmune mouse strains and obtained similar results. Both antibodies bound chromatin, but not histones or dsDNA, when highly purified whereas unpurified hybridoma supernatant or standard Protein G preparation of 3H9 did bind dsDNA and SN5-18 supernatant or Protein G preparation bound histones. When cell culture supernatant from SP2/0

cells was added to highly purified 3H9, its ability to bind dsDNA was restored and is similar to our finding that addition of COS-7 supernatant restores binding of the purified antibody B3 to DNA. The conclusion was that incomplete purification of such antibodies can lead to the interpretation that they bind dsDNA whereas the true antigen is nucleosomes (a complex of dsDNA, histone 2A and histone 2B) (Guth et al., 2003).

There is another possible explanation of my results. Treatment with *DNase* may alter DNA in solution in such a way that it acts as a competitive inhibitor of binding of antibody to dsDNA on the plate. When *DNase I* is added to an incompletely dissociated complex of dsDNA with $B3V_H/B33V_L$ it may generate small fragments of DNA or perhaps ssDNA. These fragments may remain associated with the antibody, or may gain access to the combining site, and act as efficient competitors of binding to dsDNA. The supernatants of the dying COS-7 cells could release other nucleases cleaving the phosphodiester bonds between the nucleotide subunits of DNA. However, this theory does not explain why $B3V_H/B3V_L$ and $B3V_H/B3(R27aS)V_L$ do not bind dsDNA in the absence of COS-7 supernatants even in the absence of *DNase I*, but will bind it when these supernatants are added.

During electroporation of COS-7 cells, approximately 70% of the cells are believed to die. The supernatant collected from these cells is therefore rich in debris from dying cells. Nucleosomes are part of this material and contain DNA. Nucleosomes might well be the cofactor from the COS-7 supernatant that binds the expressed antibodies and enables them to bind dsDNA in ELISA. For this assumption to be correct, then the purified *DNase* treated antibodies should bind nucleosomes. Results shown in Figure 5.2 confirm this assumption. The combinations $B3V_H/B3V_L$, $B3V_H/B3(R27aS)V_L$, and $B3V_H/B33V_L$ bind nucleosomes, without requiring the addition of cell supernatant. Guth et al., (2003) showed that arginine to serine mutations in V_H CDR3 of SN5-18 eliminates binding to chromatin. The single arginine to serine mutation in $B3(R27aS)V_L$ did not remove binding to nucleosomes but reduced it slightly. Replacement of CDR2 and CDR3 of $B3V_L$ by those of 33H11 or UK4 gave different effects on binding to nucleosomes, even though these three L chains are derived from the same germline gene and differ only at positions of somatic mutations (see Figure 5.2). In particular, the combination $B3V_H/BUV_L$ does not bind nucleosomes at all.

It was confusing that addition of purified nucleosomes could reconstitute the binding of $B3V_H/B3(R27aS)V_L$ to dsDNA but not that of combinations $B3V_H/B3V_L$, or $B3V_H/B33V_L$, which bind nucleosomes at least as well as $B3V_H/B3(R27aS)V_L$ in direct ELISA. However, it is clear from the experiments with $B3V_H/B3(R27aS)V_L$ that the concentration of nucleosomes is critical to their ability to act as a cofactor in the binding of this antibody to dsDNA. This finding may well also be the case for the other two combinations. Perhaps some non-nucleosome component of the supernatant, such as a nuclease, which removes a competitive inhibitor from the binding site, is playing a role in promoting the binding of $B3V_H/B3V_L$ and $B3V_H/B33V_L$ to dsDNA. Alternatively, these two H/L combinations may require the presence of a nucleoprotein cofactor, which is not found in our nucleosome preparation, in order to bind dsDNA.

My results from the binding studies described in this chapter show that a single change from arginine to serine in V_H CDR2 causes a dramatic shift in the binding properties of the human monoclonal IgG antibody B3. Arginine at position 53 is critical for binding to dsDNA and nucleosomes. Changing this arginine to serine, in the absence of any other change in sequence in either the H or L chain, abolishes binding to those two antigens but creates binding to α-actinin, whole β_2GPI and DI. Other groups have previously shown simultaneous decreases in binding of human anti-dsDNA antbodies to dsDNA and nucleosomes (Wellmann et al., 2005) or to dsDNA and PLs (Cocca et al., 2001) after loss of particular arginine residues by mutagenesis. In this thesis I have reported dramatic shifts of binding in opposite directions to different clinically relevant antigens caused by a single arginine to serine mutation and to my knowledge this finding has not been reported in the literature thus far.

Mostoslavsky and colleagues studied a panel of 15 monoclonal murine anti-DNA, anti-histone and anti-nucleosome antibodies and have shown that their ability to cause nephritis was closely associated with their ability to bind α-actinin (Mostoslavsky et al., 2001). α-actinin is an important structural protein in the renal podocyte and the theory that anti-α-actinin antibodies are important in lupus nephritis has been supported by experiments in another murine model (Deocharan et al., 2002, Zhao et al., 2005) and in a clinical study (Mason et al., 2004). This thesis describes an analysis of the effects of somatic mutations in anti-dsDNA antibodies on

ability to bind α-actinin and to my knowledge, this has not been reported in the literature thus far.

Anti-nucleosome antibodies are thought to cause glomerulonephritis by the formation of nucleosome/anti-nucleosome complexes, which interact with HS in the GBM (Kramers et al., 1994, van Bruggen et al., 1997). Wellmann et al., (2005) recently showed that two somatic mutations in the L chain and one in the H chain of the human monoclonal anti-dsDNA antibody 33.C9 were essential for binding to either dsDNA or nucleosomes. In summary, there is convincing evidence that anti-nucleosome antibodies are important in the pathogenesis of lupus nephritis and that somatic mutations are important in controlling affinity for nucleosomes.

Cocca et al., (2001) showed that reversion of an arginine at position 53 in the H chain of the murine anti-dsDNA antibody 3H9 to serine reduces binding to DNA, PL, β_2GPI and apoptotic cells. They suggested that some autoantibodies are produced in response to autoantigens derived from apoptotic cell debris and that some somatic mutations could therefore enhance binding to several such antigens. Accumulation of further mutations might then be expected to increase the affinity of the antibody for one autoantigen while reducing its affinity for others. My results could suggest that the wild-type B3 antibody which contains some somatic mutations (94% homology to the germline gene V3-23) had high affinity for nucleosomes, but the introduction of the H chain germline reversion R53S created an antibody with specificity and high affinity for α-actinin and β_2GPI. My experiments showed that $B3V_H/B3V_L$ binds nucleosomes at much lower antibody concentrations than the concentrations of $B3(R53S)V_H/B3V_L$ required to bind α-actinin and β_2GPI. The computer model of the B3/dsDNA complex (Figure 1.5) showing an interaction between dsDNA and R53 supports the idea that R53 is important in affinity maturation of the antibody (Kalsi et al., 1996). Although some previous authors had shown that an increase of arginine residues in CDRs led antibodies to develop first binding to ssDNA then binding to dsDNA (Li et al., 2000, Wellmann et al., 2005), in my experiments none of the H/L chain combinations bound ssDNA.

One strength of my work compared to previous studies is that I compared the effects of mutations to differently charged amino acids (R53S, R53N and R53K). I did this comparison because several authors have noted a prevalence of R, N and K residues at the antigen-binding sites of human and murine anti-dsDNA antibodies (Radic and

Weigert, 1994, Winkler et al., 1992, Rahman et al., 2002). Using a different approach (Collis et al., 2003) reviewed the sequences of 1875 antibodies in the Kabat database and showed that both R and N, but not K, are significantly over-represented in the combining sites of antibodies that bind nucleotide antigens, in comparison to antibodies binding other types of antigen. Lysine, like arginine, is positively charged and could potentially interact with negatively charged antigens. Arginine, asparagine and lysine can all form hydrogen bonds with dsDNA. Therefore, this might lead to the hypothesis that the R53N and R53K mutations would have less adverse effect on binding to dsDNA and nucleosomes than R53S but I did not find this to be the case in my study. It seems likely that a property of arginine apart from charge or hydrogen bond formation is crucial to its interaction with dsDNA. Perhaps the length and shape of the arginine side chain are important. In contrast R53S, R53N and R53K had very different effects on interaction with α-actinin, and DI.

The production of an antibody, B3(R53S)V_H/B3V_L which was found to distinguish human from bovine β_2GPI was unexpected and has not previously been described according to my literature search. As shown in Figure 5.8, there are 11 amino acid differences between the sequence of DI of human and bovine β_2GPI, therefore it is likely one of these differences plays a role in the specificity of the antibody for human β_2GPI.

CHAPTER SIX.

Arginine residues are important in determining the binding of purified hnman monecienal antibody IS4 to clinically relevant antigens

CHAPTER SIX.

ARGININE RESIDUES ARE IMPORTANT IN DETERMINING THE BINDING OF PURIFIED HUMAN MONOCLONAL ANTIBODY IS4 TO CLINICALLY RELEVANT ANTIGENS

6.1 Introduction and aims of this chapter

In Chapter Four, I have shown that the sequence of IS4V_H conferred the ability to bind PL whilst the identity of the paired V_L was important in determining the strength of PL binding. Subsequently, I reported that specific arginine residues in IS4V_H CDR3 were especially important in conferring the ability to bind CL, β_2GPI and PS and described the production of stable cell lines that provided an increase in the yield of IgG compared to the transient expression system. Thus, it was feasible to purify IgG and remove apoptotic/necrotic debris from the supernatant. This large-scale culture and purification was carried out by Chemicon Europe Ltd, Hampshire, UK.

The aims of the work described in this Chapter are to report the experiments performed with each purified H/L combination in ELISA assays for binding to anionic PL, such as CL and PS, as well as β2GPI and PS-PT that have been proposed as important targets in APS. I have also tested the purified antibodies for binding to a neutral PL (PC) and dsDNA, which shares some structural similarities with anionic PL but is not involved in the pathogenesis of APS. PC and dsDNA were included to determine whether the relationships between antibody sequence and binding that I observed were specific for clinically important antigen/antibody interactions.

6.2 Assembly of aPL antibodies

6.2.1 Sequences of expressed antibody variable regions

The sequences of IS4, B3 and UK4 have all been described in the previous chapters. All three antibodies contain L chains encoded by the germline V_λ gene 2a2. These L chains differ in nucleotide sequence only by 7% or less, as a result of their different patterns of somatic mutation. There are five Arg residues in IS4V_H CDR3, four of

which are surface exposed and were mutated to serine to create the mutant forms of $IS4V_H$ (discussed in more detail in Chapter Four). The nomenclature of the mutant H chains is summarised in Table 6.1.

Heavy chain	Position 96	Position 97	Position 100	Position 100g
IS4VH	R	R	R	R
IS4VHiii	R	R	R100S	R
IS4VHiv	R	R	R	R100gS
IS4VHi&ii	R96S	R97S	R	R
IS4VHiii&iv	R	R	R100S	R100gS
IS4VHi,iii&iv	R96S	R	R100S	R100gS
IS4VHii,iii&iv	R	R97S	R100S	R100gS
IS4VHx	R96S	R97S	R100S	R100gS

Table 6.1 Patterns of Arginine (R) to serine (S) replacements in $IS4V_H$CDR3

6.2.2 Stable expression of whole IgG in CHO cells and purification of antibody

A total of 13 H/L chain combinations were expressed in CHO cells, which contained wild type or mutant $IS4V_H$. Different amounts of IgG were obtained from each cell line after successive rounds of methotrexate amplification (detailed in Chapter Four). Human IgG was purified and quantified (as described in Chapter Five). Western blot analyses of the purified antibodies confirmed the presence of a strong band consistent with whole IgG on non-reducing gels (Figure 6.1) with no free H chains.

6.3 Characterisation of the binding properties of monoclonal aPL by ELISA

6.3.1 Results of anti-cardiolipin ELISA

Figure 6.2 shows binding the degree of the thirteen purified antibodies to CL. Similar patterns of binding were obtained with each antibody from repeated experiments hence representative results from a single experiment with dilutions of antibody over a wide range of concentrations are shown in Figure 6.2. These results are also confirmed in Figure 6.3, which represents binding of each combination in triplicate using a fixed antibody concentration of 1μg/ml to prove reproducibility. The wild-type combination $IS4V_H/IS4V_L$ bound CL, but any reduction in the number of CDR3 arginines abolished binding. In contrast, replacement of the L chain of IS4 by $B3V_L$ ($IS4V_H/B3V_L$) increased binding to CL. Since the binding of the combination $IS4V_H/IS4V_L$ was abolished by substitution of any one of the four V_H CDR3 arginine residues, the effects of V_H CDR3 arginine to serine substitutions in combination with $IS4V_H/B3V_L$ on binding to CL are more informative. The combination containing mutations at positions 96 and 97 ($IS4V_Hi\&ii/B3V_L$) has actually increased the binding to CL. With all the other combinations, binding was reduced, but not always abolished by different patterns of arginine to serine substitutions, therefore useful comparisons between the degrees of reduction caused by substitutions at different positions can be made. Substitution of all four arginine with serine residues ($IS4V_HX/B3V_L$) abolished binding to CL completely. Substitution of the arginines at positions 100 and 100g ($IS4V_Hiii\&iv/B3V_L$) reduced binding to CL. However, in the case of the triple mutants, either 96, 100 and 100g ($IS4V_Hi,iii\&iv/B3V_L$) or 97, 100 and 100g ($IS4V_Hiii\&iv/B3V_L$), binding was abolished. The combination $IS4V_H/UK4V_L$ conferred weak binding to CL (Figure 6.2).

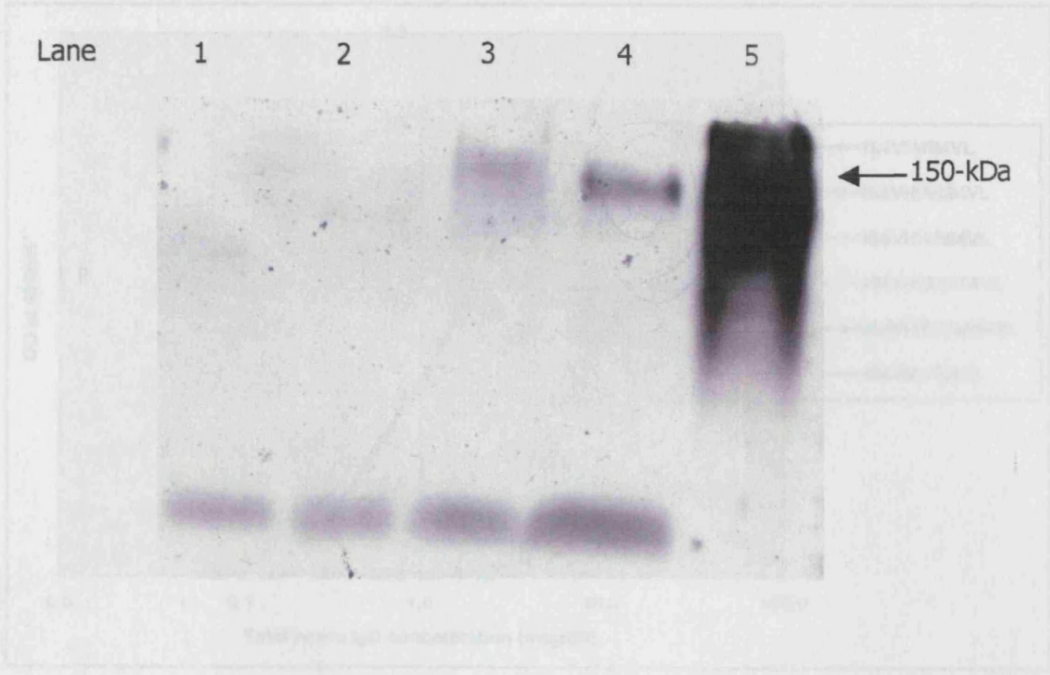


Figure 6.1 Western blot showing the presence of whole IgG

Lane 1: COS-7 cell supernatant from electroporation without any plasmid DNA

Lane 2: CHO cell supernatant from electroporation without any plasimd DNA

Lane 3: Shows the presence of whole IgG in the COS-7 supernatant produced from the $IS4V_H/IS4V_L$ combination

Lane 4: Shows the presence of whole IgG in the CHO supernatant produced from the $IS4V_H/IS4V_L$ combination

Lane 5: This band represents the positive control, whole IgG in the purified $IS4V_H/IS4V_L$ (150-kDa)

The second band obtained in lanes 1-4 is likely to be some form of impurity present in the cell supernatant since this band is not visible in the purified sample. This band was visible in the negative control lanes 1 and 2 ("no DNA" control supernatants), therefore it rules out the possibility that this band represents any free H or L chain. A marker was not available for a non-denaturing gel therefore the size of the band is not known.

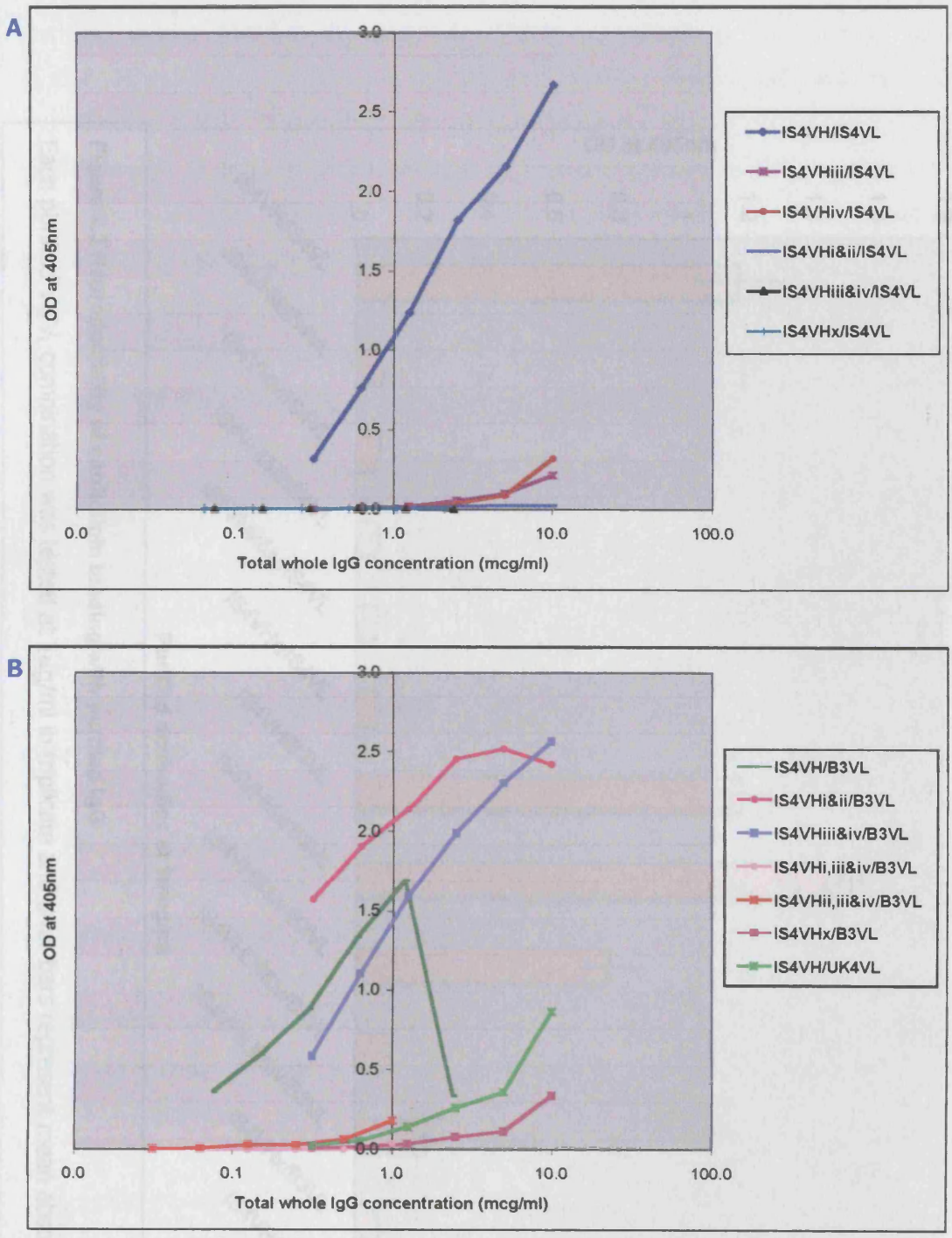


Figure 6.2 Results of cardiolipin ELISA

The graph shows binding of purified IgG antibodies to CL in a direct ELISA when $IS4V_H$ and variants are paired with A) native $IS4V_L$ and B) $B3V_L$ and $UK4V_L$.

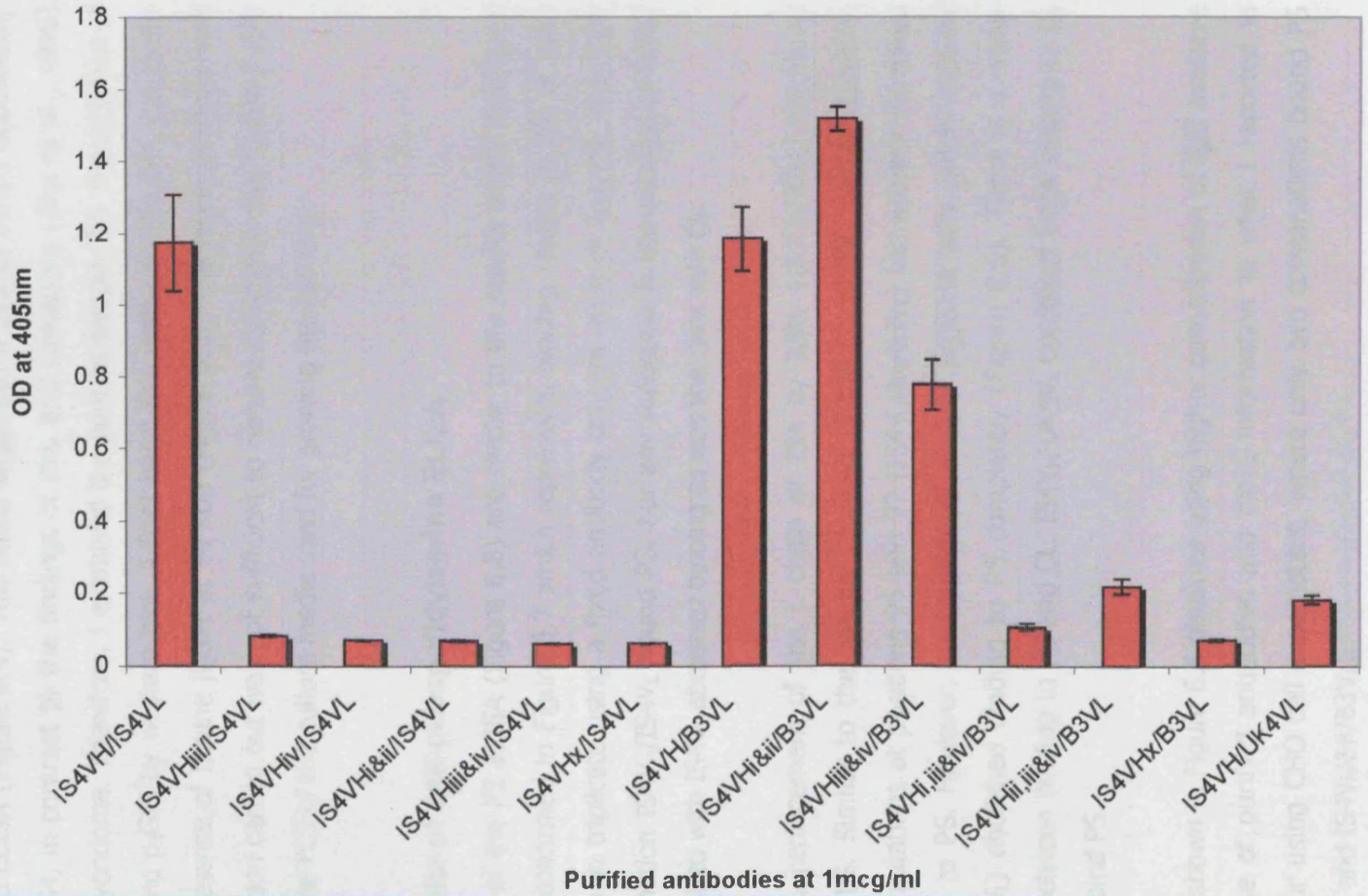


Figure 6.3 Reproducibility of cardiolipin binding with purified IgG

Each purified V_H/V_L combination was tested at 1μg/ml in triplicate and error bars represent mean absorbance +/- SD.

6.3.2 Results of modified anti-CL ELISA

Purified native IS4V_H/IS4V_L and two other combinations (IS4V_H/B3V_L and IS4$V_H$iii&iv/B3V_L) that bound CL in the standard ELISA, bound equally well to CL in the modified ELISA (Figure 6.4). This result suggested a lack of serum dependency of native IS4, in contrast to the findings of Zhu and colleagues (Zhu et al., 1999) using IS4 hybridoma. Therefore, I examined the purified samples for the presence of contaminating β_2GPI by western blot. A faint band was visible on the blot consistent with the presence of bovine β_2GPI at 54-kDa (Figure 6.5). This result indicates that the purification carried out was not sufficient to remove completely the bovine β_2GPI present in the FCS of the culture media used for growing the cell lines.

6.3.3 Results of anti-phospatidylserine ELISA

The results of the PS ELISA (Figure 6.6) are similar to the results of the binding to CL. This is confirmed in Figure 6.7 which represents binding results to PS, of each combination in triplicate using a fixed antibody concentration of 1μg/ml. The wild-type combination IS4V_H/IS4V_L bound PS, but any reduction in the number of CDR3 arginines paired with IS4V_L abolished binding as was the case with CL.

In contrast, replacement of the L chain of IS4 by B3V_L (IS4V_H/B3V_L) increased binding to PS. Similar to the results obtained in the CL ELISA, the combination containing mutations at positions 96 and 97 (IS4V_Hi&ii/B3V_L) has actually increased the binding to PS. However, substitution of all four arginine with serine residues (IS4V_Hx/B3V_L) abolished binding to PS completely (Figure 6.6). There is a minor difference between binding to PS and CL. IS4V_H/UK4V_L conferred weak binding to CL but did not bind PS.

The results shown in Figure 6.6 obtained using higher concentration of IgG available with the use of purified antibodies add more information to what I reported in Chapter Four using CHO cell supernatant, where only two combinations bound PS (IS4V_H/B3V_L and IS4V_Hi&ii/B3V_L).

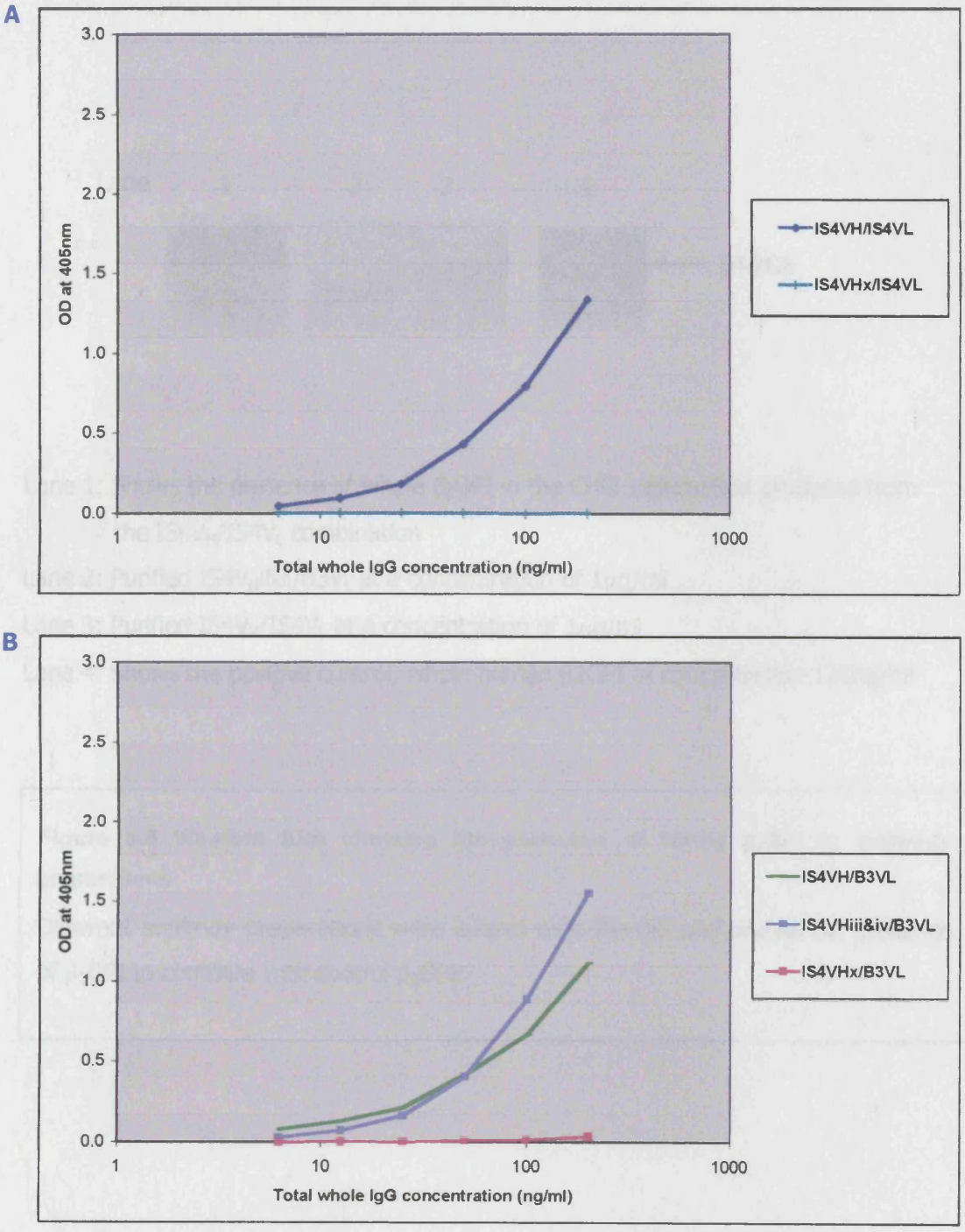
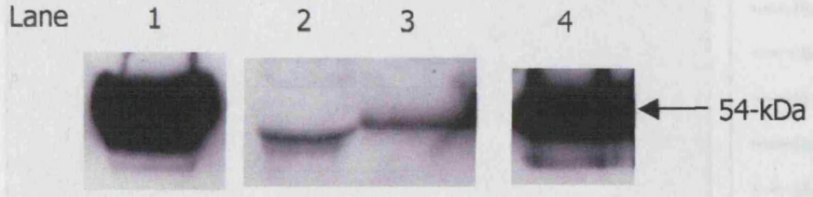


Figure 6.4 Results of modified anti-cardiolipin ELISA

The graph shows binding of purified IgG antibodies to CL detected by ELISA in the absence of FCS, when IS4V$_H$ and variants are paired with A) native IS4V$_L$ and B) B3V$_L$.



Lane 1: Shows the presence of whole β_2GPI in the CHO supernatant produced from the IS4V_H/IS4V_L combination

Lane 2: Purified IS4V_Hi&ii/B3V_L at a concentration of 1μg/ml

Lane 3: Purified IS4V_H/IS4V_L at a concentration of 1μg/ml

Lane 4: Shows the positive control, whole human β2GPI at concentration 125ng/ml

Figure 6.5 Western blot showing the presence of whole β_2GPI in antibody preparations

Different antibody preparations were loaded onto the gel and probed for presence of β_2GPI to compare with control β_2GPI.

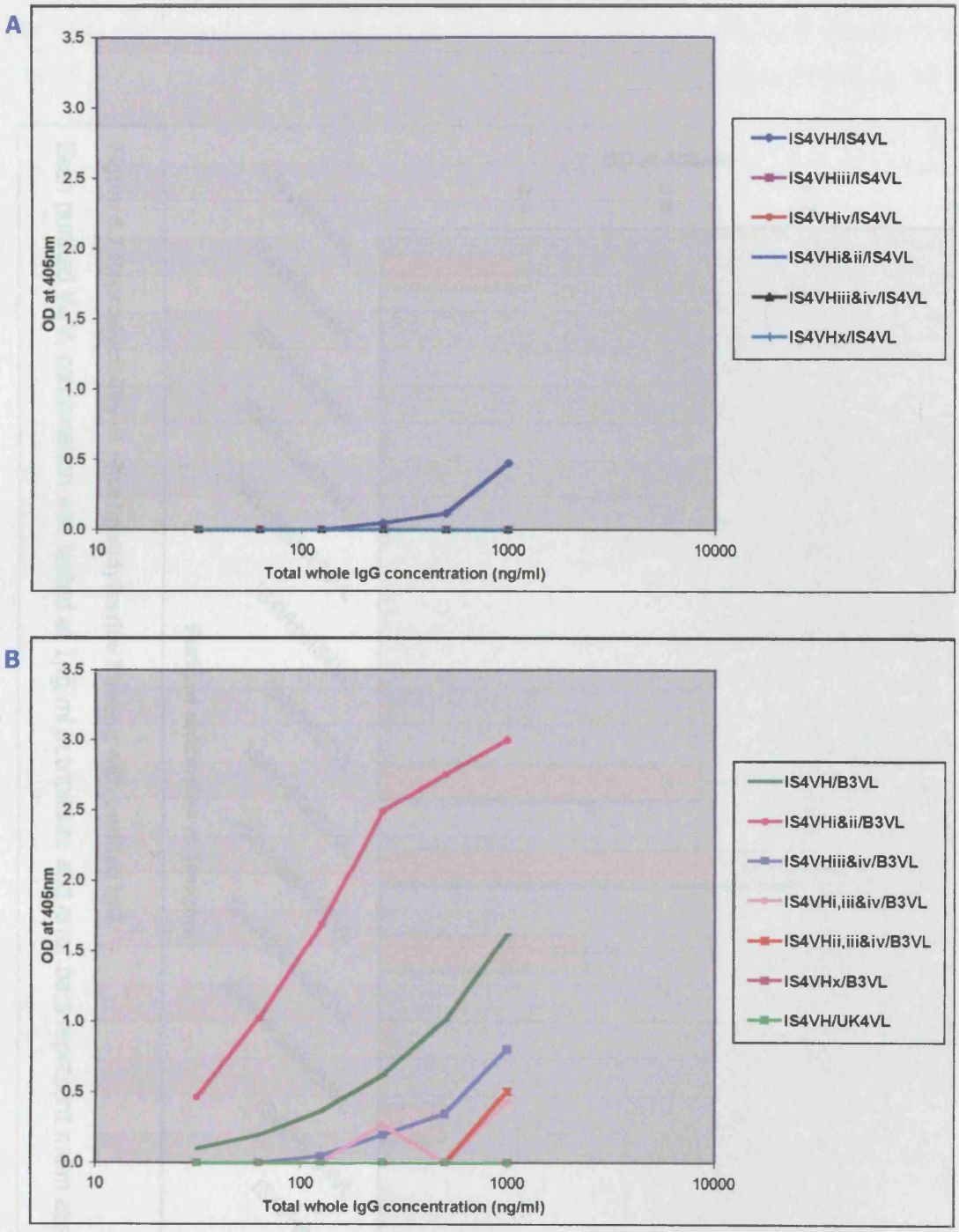


Figure 6.6 Results of phosphatidylserine ELISA

The graph shows binding of purified IgG antibodies to PS in a direct ELISA when $IS4V_H$ and variants are paired with A) native $IS4V_L$ and B) $B3V_L$ and $UK4V_L$.

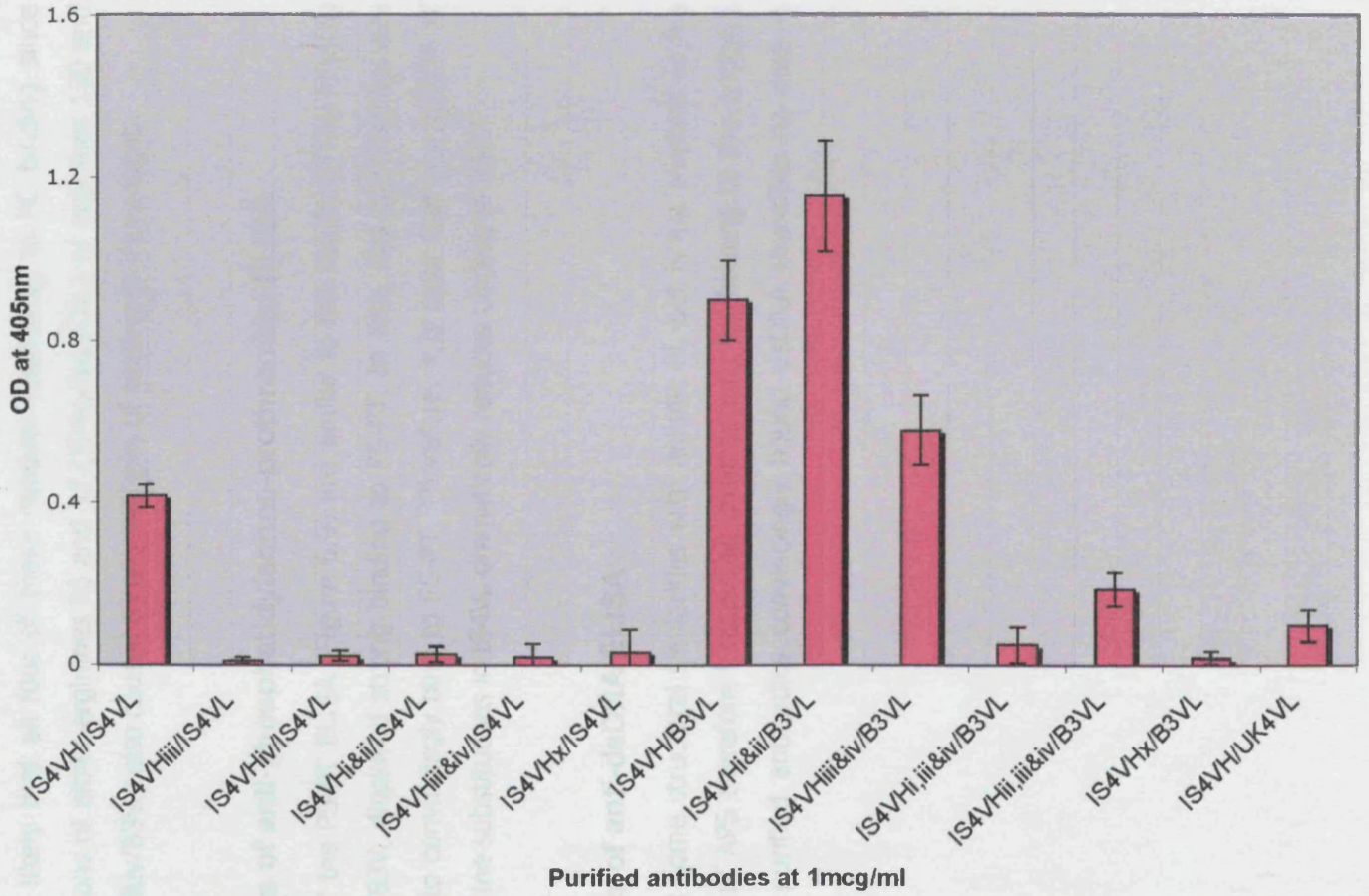


Figure 6.7 Reproducibility of phoshpatidylserine binding with purified IgG

Each purified V_H/V_L combination was tested at 1μg/ml in triplicate and error bars represent mean absorbance +/- S.D.

6.3.4 Results of anti-phosphatidylcholine ELISA

Figure 6.8 shows the binding results to PC where the wild-type combination $IS4V_H/IS4V_L$ did not bind PC. However, replacement of the L chain of IS4 by $B3V_L$ ($IS4V_H/B3V_L$) created the ability to bind PC. None of the other combinations bound PC. It seems likely that all four of these residues contribute to PC binding since double mutations of either arginines 96 and 97 ($IS4V_Hi\&ii/B3V_L$) or arginines 100 and 100g ($IS4V_Hiii\&iv/B3V_L$) also caused a complete loss of binding to this antigen.

6.3.5 Results of anti-phosphatidylserine-prothrombin ELISA

The results of the PS-PT ELISA (Figure 6.9) are similar to the results of the binding to PC. $IS4V_H/B3V_L$ displayed strong binding to PS-PT. In fact, this combination was the only one to convincingly bind to PS-PT. Therefore, it is clear that any pattern of arginine to serine substitutions in $IS4V_H$ dramatically reduces binding to PS-PT.

6.3.6 Results of anti-dsDNA ELISA

dsDNA shares some structural similarities with anionic PL but is not involved in the pathogenesis of APS therefore I tested all combinations for binding to this antigen. None of the purified antibodies convincingly bound dsDNA therefore no data is shown.

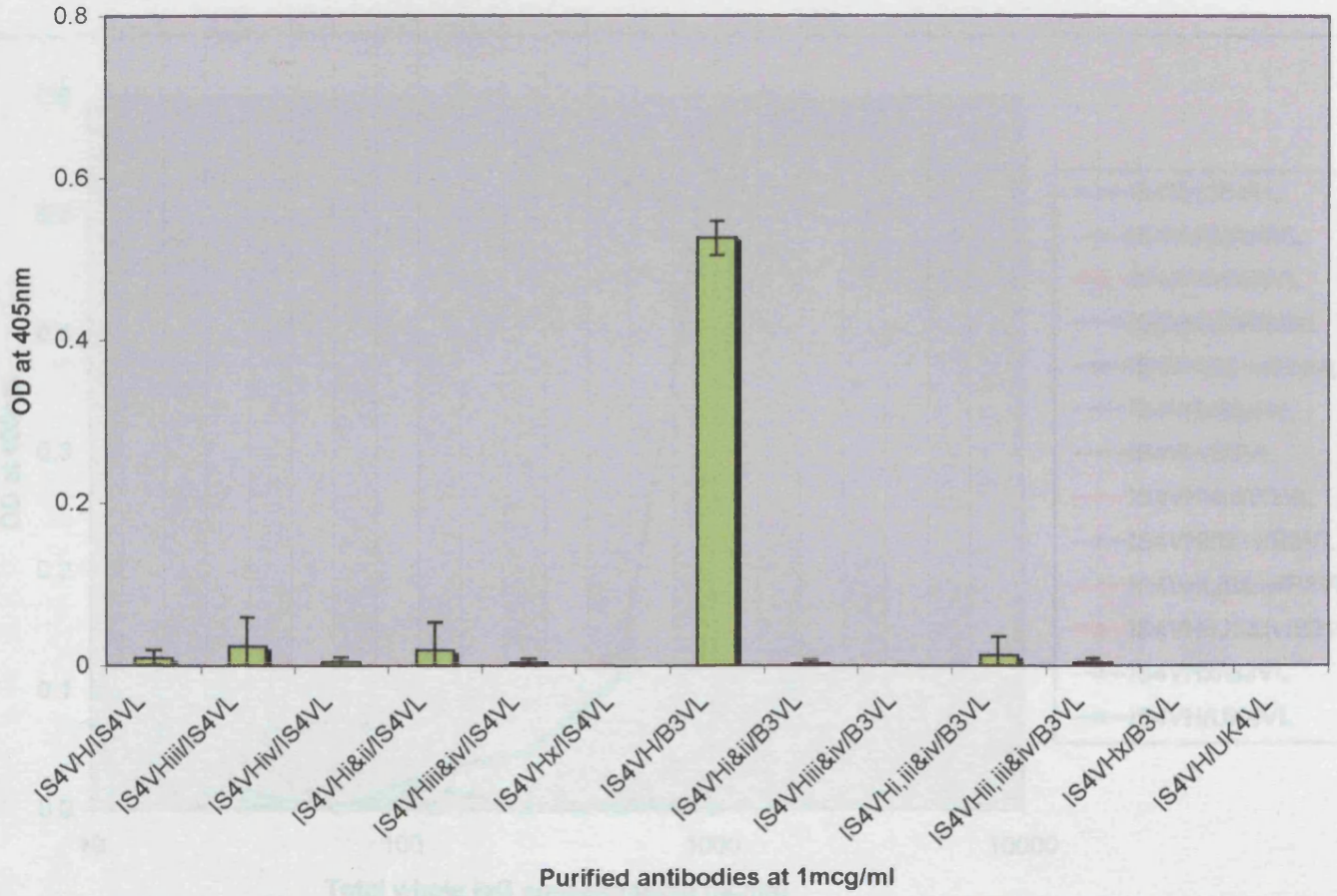


Figure 6.8 Phosphatidylcholine binding with purified IgG

Each purified V_H/V_L combination was tested at 1μg/ml in triplicate and error bars represent mean absorbance +/- S.D.

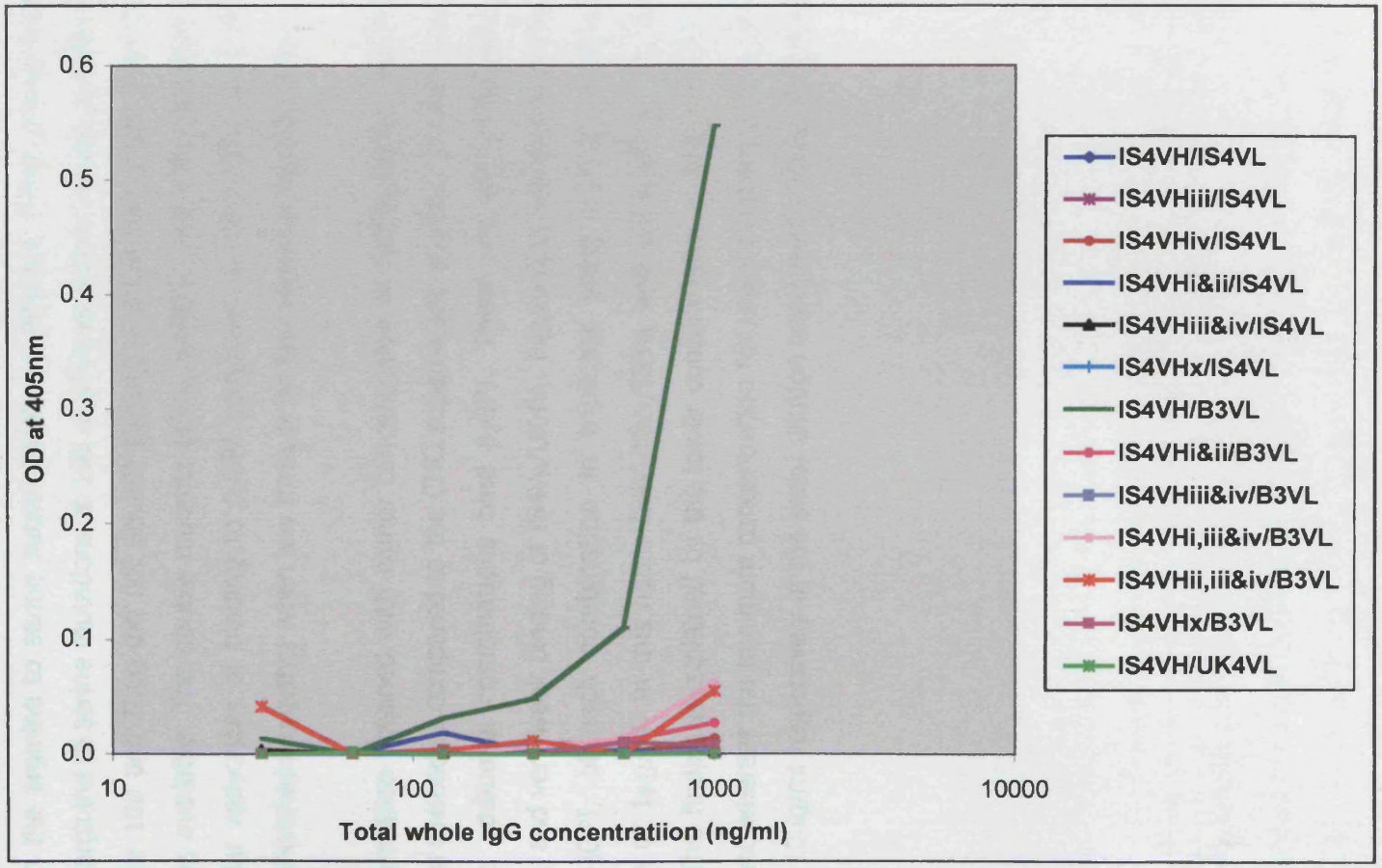


Figure 6.9 Results of phosphatidylserine-prothrombin ELISA

The graph shows binding of purified IgG antibodies to PS-PT in an ELISA.

6.3.7 Results of anti-β_2GPI ELISA

Figure 6.10 shows the binding of the purified antibodies to β_2GPI. I found that IS4V$_H$/IS4V$_L$ and IS4V$_H$/B3V$_L$ bound β_2GPI but IS4V$_H$/UK4V$_L$ did not. Although this order of binding was similar to the results obtained in the PL ELISAs, the results obtained with the arginine to serine substitutions are different. When paired with IS4V$_L$, single arginine to serine mutations at 100 or 100g as well as double mutations at 96 or 97 or 100 and 100g did not abolish binding when tested at the maximal concentrations available. The double mutants (IS4V$_H$i&ii/B3V$_L$ and IS4V$_H$iii&iv/B3V$_L$) showed similar reductions in binding to β_2GPI compared to IS4V$_H$/B3V$_L$ and this binding was abolished altogether when any three or all four arginines were mutated.

The new information obtained with higher concentration of IgG available with the use of purified antibodies compared to the CHO supernatant results in Chapter Four, is that three additional combinations bind β_2GPI. These are IS4V$_H$iii&iv/IS4V$_L$, IS4V$_H$i&ii/B3V$_L$ and very weak binding of IS4V$_H$/UK4V$_L$. Figure 6.11 represents binding results to β_2GPI, of each combination in triplicate using a fixed antibody concentration of 1μg/ml. In this figure IS4V$_H$iii&iv/IS4V$_L$ and IS4V$_H$/UK4V$_L$, do not bind β_2GPI. This finding is explained by the lower concentration of 1μg/ml used in this experiment whereas the minimum concentration for these combinations to bind was 2.4 and 10μg/ml respectively in the serial dilution experiments shown in Figure 6.10.

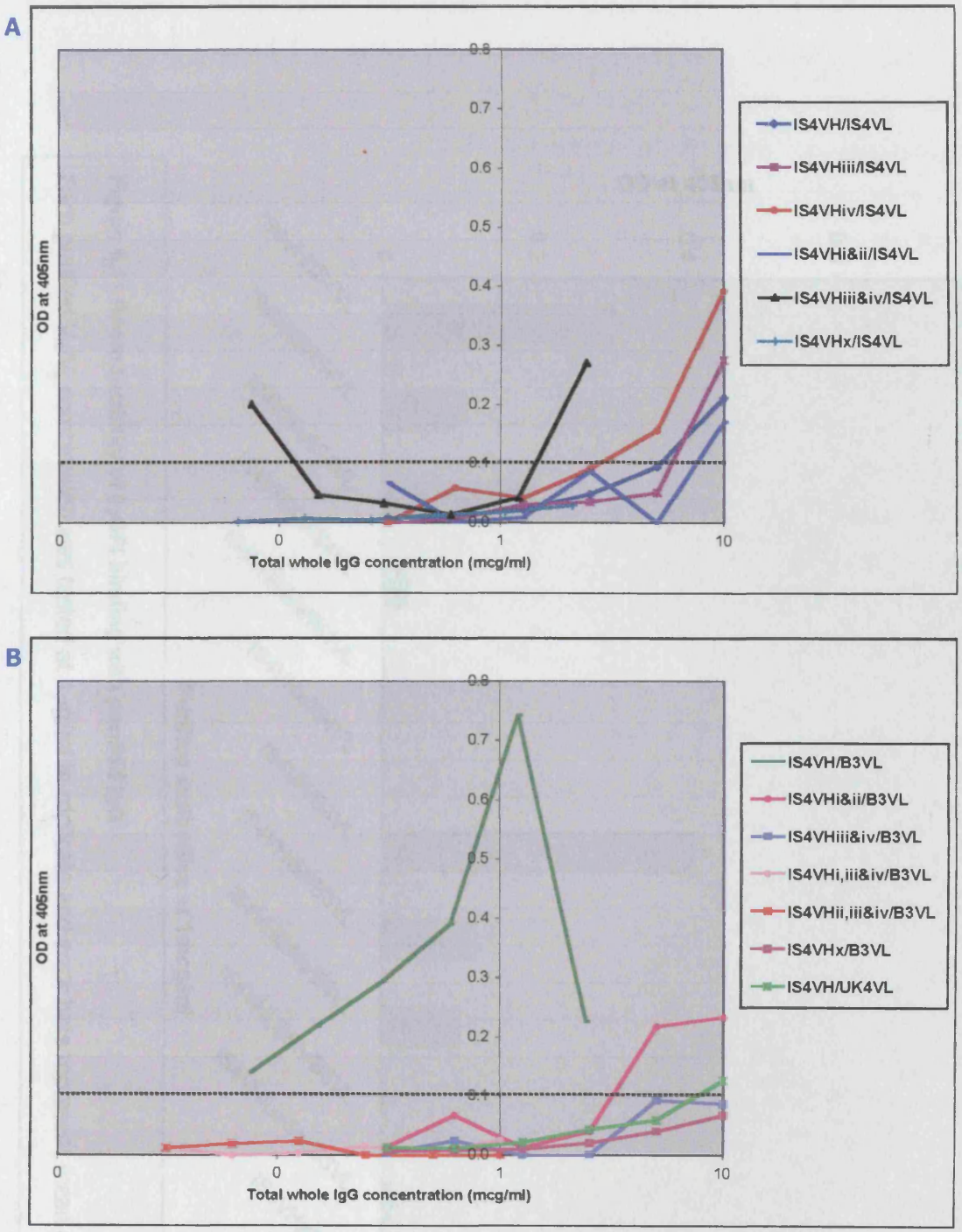


Figure 6.10 Results of β_2GPI ELISA

The graph shows binding of purified IgG antibodies to β_2GPI in a direct ELISA when IS4V$_H$ and variants are paired with A) native IS4V$_L$ and B) B3V$_L$ and UK4V$_L$. A combination was not considered to bind β_2GPI if its binding curve gave an absorbance of less than 0.1 (at 405nm) (represented by the dotted line) for the concentration tested in the anti-β_2GPI ELISA.

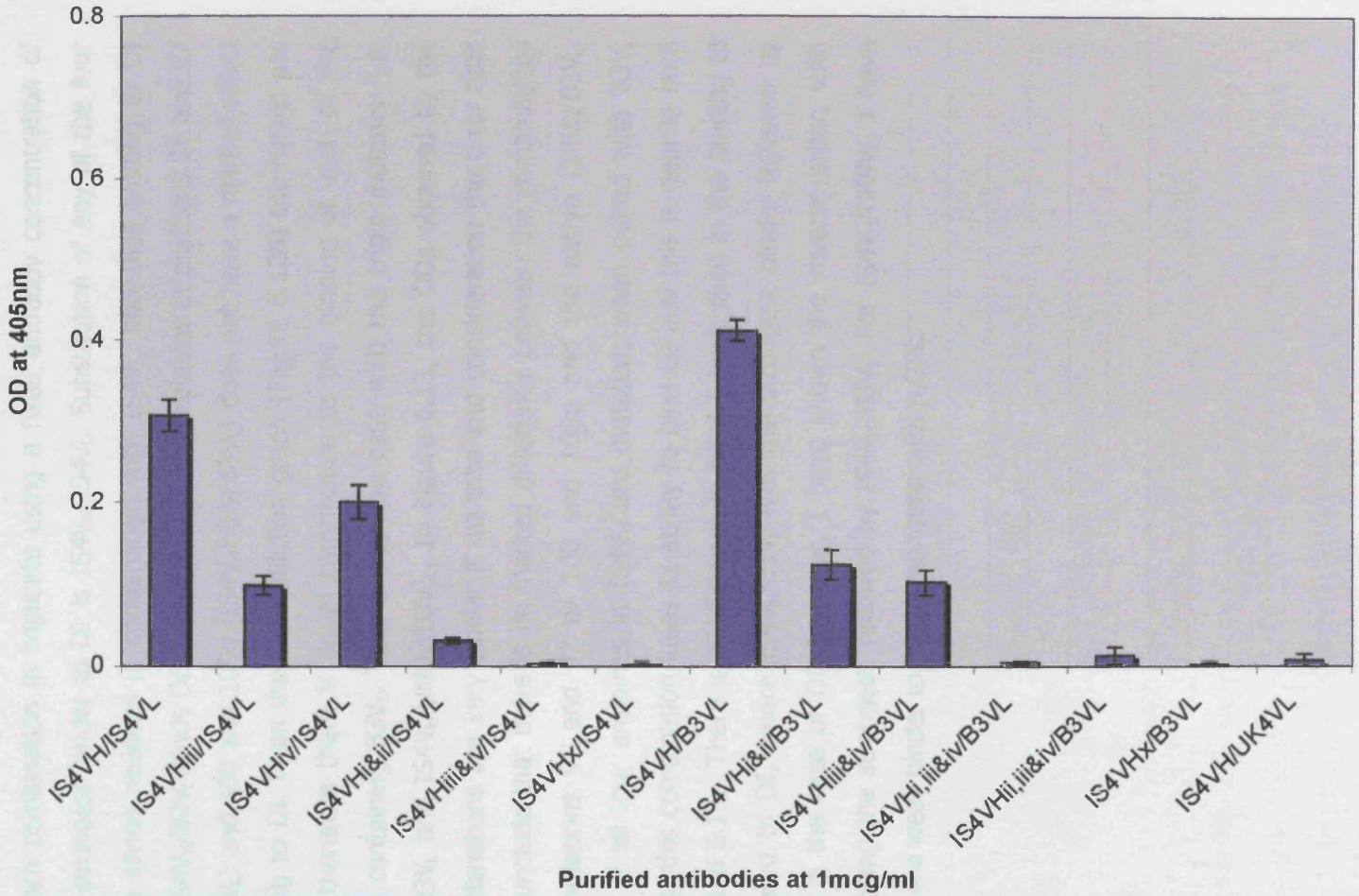


Figure 6.11 Reproducibility of β_2GPI binding with purified IgG

Each purified V_H/V_L combination was tested at 1μg/ml in triplicate and error bars represent mean absorbance +/- S.D.

6.3.8 Results of anti-Domain I ELISA

The results obtained in the anti-DI ELISA, shown in Figure 6.12, differ from the results obtained in the anti-β_2GPI ELISA in both the V_H/V_L combinations that bind and their pattern of binding. Figure 6.13 also confirms that these results are reproducible by testing each combination in triplicate using a fixed antibody concentration of 1μg/ml. The strongest binder to DI is $IS4V_H/IS4V_L$. Substitution of any of the four arginine with serine residues in combination with $IS4V_L$, abolishes binding to DI completely. $IS4V_H/B3V_L$ binds DI but removal of the arginines at positions 96 and 97 ($IS4V_Hi\&ii/B3V_L$) and 100 and 100g ($IS4V_Hiii\&iv/B3V_L$) does not have a marked effect on the binding to DI. From the serial dilution graph (Figure 6.12B) the curves are very similar therefore there is equal contribution to the binding of each of the combinations containing $B3V_L$. This is also the case with the triple mutants (i.e. $IS4V_Hi,iii\&iv/B3V_L$ and $IS4V_Hii,iii\&iv/B3V)_L$. In Figure 6.13, the ODs obtained by the different combinations are very similar if we take into consideration the error bars therefore I conclude that there is no marked difference between the combinations containing mutations 96 and 97 or 100 and 100g and the native $IS4V_H/B3V_L$. Furthermore, if all four arginines in $IS4V_H$ are mutated, when paired with $B3V_L$ ($IS4V_HX/B3V_L$), this combination loses its ability to bind DI and this is clear in both Figure 6.12 and 6.13. There is a difference in what I have seen in the binding to β_2GPI compared to DI, where $IS4V_H/B3V_L$ was the strongest binder followed by $IS4V_H/IS4V_L$. In the case of DI, however, I have shown the reverse order, with $IS4V_H/IS4V_L$ being the strongest, followed by $IS4V_H/B3V_L$. For $IS4V_H/UK4V_L$, I have shown that it is a weak binder to DI as was found with β_2GPI.

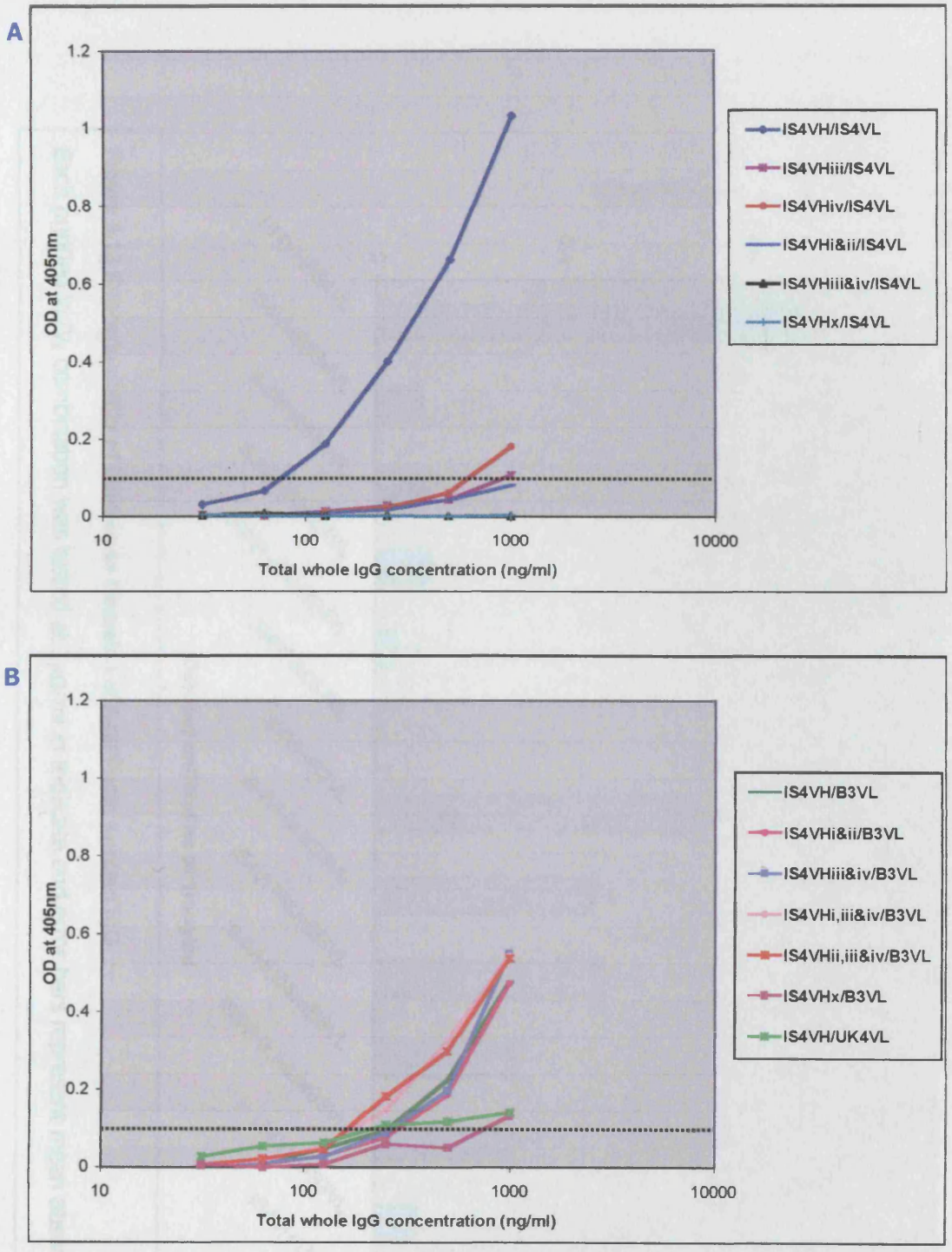


Figure 6.12 Results of Domain I of β_2GPI ELISA

The graph shows binding of purified IgG antibodies to DI in a direct ELISA when $IS4V_H$ and variants are paired with A) native $IS4V_L$ and B) $B3V_L$ and $UK4V_L$. A combination was not considered to bind DI if its binding curve gave an absorbance of less than 0.1 (at 405nm) (represented by the dotted line) for the concentration tested in the anti-DI ELISA.

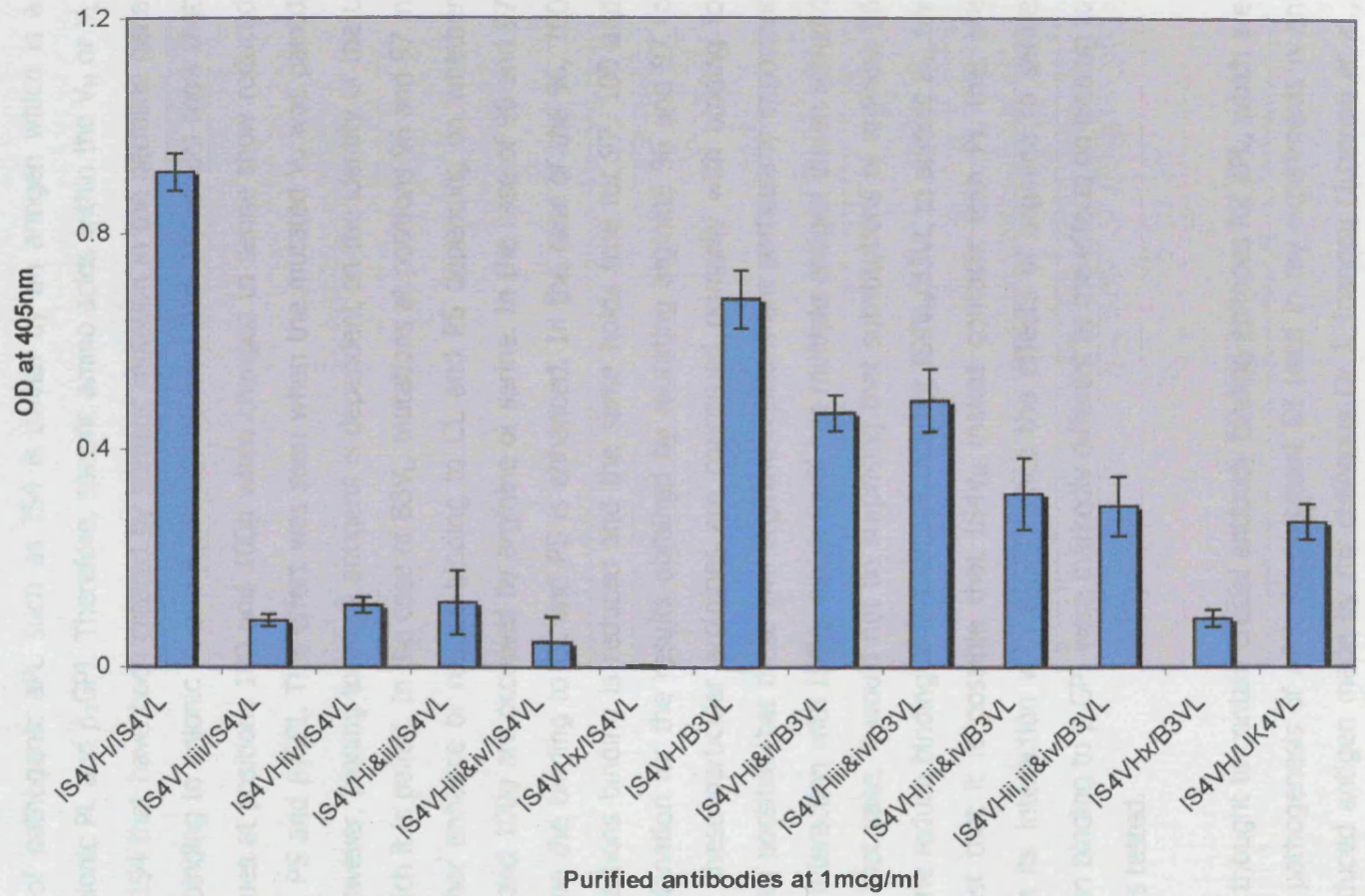


Figure 6.13 Reproducibility of binding to Domain I of β_2GPI with purified IgG

Each purified V_H/V_L combination was tested at 1μg/ml in triplicate and error bars represent mean absorbance +/- S.D.

6.4 Discussion

In this Chapter, I have examined the binding of human monoclonal purified aPL to a variety of antigens. I found that the wild-type IS4V_H/IS4V_L antibody binds to CL, PS, β_2GPI and DI of β_2GPI. This finding is consistent with the hypothesis that development of pathogenic aPL such as IS4 is driven by an antigen which is a complex of anionic PL and β_2GPI. Therefore, specific amino acids within the V_H or V_L sequences of IS4 that have been created by somatic mutation in the germline gene and promote binding to anionic PL and β_2GPI can be identified. My results show that arginine residues at positions 100 and 100g when changed to serine show reduced binding to CL, PS and β_2GPI. This effect was seen when the mutated V_H was paired with IS4V_L. However, binding to these antigens is dependent on the identity of the L chain with which it is paired. In the case of B3V_L, mutations at positions 96 and 97 in IS4V_H can either enhance or reduce binding to CL and PS depending on whether positions 100 and 100g are occupied by arginine or serine. In the case of 96 and 97 mutations alone the binding to CL and PS is enhanced. In the case of the 96, 100 and 100g mutations binding is reduced and the same holds true for 97, 100 and 100g. My explanation for the results obtained by mutating arginine 96 and 97 to serine is that these particular arginines are concerned primarily with binding to β_2GPI. It is also possible that these two arginine residues are particularly important in forming an interaction with IS4V_L, which itself has multiple antigen driven somatic mutations. In that case, it would not be surprising that substitutions of arginine 96 and 97 to serine reduce binding of IS4V_H/IS4V_L but not IS4V_H/B3VL to anionic PL. My results suggest that it is possible that IS4V_H makes contacts with PL that are separate from its interaction with β_2GPI, since the effects of arginine to serine substitutions on binding to β_2GPI were markedly different to the effects on binding to the PL antigens tested.

DI of β_2GPI is thought to contain crucial antibody binding epitopes for aPL, which are critical to the pathogenesis of APS. Recombinant DI used in my experiments is an artificially produced antigen made by my colleague Dr J. Ioannou (Ioannou et al., 2006). My explanation for the fact that some antibodies bind DI better than whole β_2GPI could be that the isolated domain has greater exposure of aPL binding epitopes that are shielded when attached to the rest of β_2GPI when bound to an irradiated plate. It is likely that aPL bind weakly to β_2GPI in solution than in solid

phase because of the conformation of the antigen. Dr J. Ioannou has also shown that DI inhibits the binding of $IS4V_H/IS4V_L$ to DI.

There were two limitations with my experiments. Firstly, the antibody preparations did not remove β_2GPI completely, as I have shown by Western blotting (see Figure 6.1). Therefore, I was unable to demonstrate a loss of CL binding when purified $IS4V_H/IS4V_L$ was tested in the modified CL ELISA in the absence of FCS (see Figure 6.4). By carrying out more rigorous purification of IS4 from hybridoma supernatant, Zhu et al., (1999) were able to remove β_2GPI and hence, demonstrate that IS4 does not bind CL in the absence of β_2GPI. Secondly, the concentration of purified antibodies available was limited and not all antibodies were available at 10μg/ml.

It is important that I have been able to show reactivity to PS with IS4, since it is now recognised as another clinically relevant antigen important in the pathogenesis of the APS, in addition to CL (Gharavi et al., 1987, Nash et al., 2004) and β_2GPI (McNally et al., 1995). Anti-PS antibodies have been shown to have a significant association with arterial thrombosis (Lopez et al., 2004) and studies have also found a relationship between anti-PT antibodies and clinical manifestations of the APS (Amengual et al., 2003). Cocca et al., (2002) have shown that by introducing arginine residues into the V_H of a murine antibody, 3H9, which has dual specificity for PL and DNA, the binding to PS-β_2GPI complexes and to apoptotic cells was enhanced. dsDNA is negatively charged and similar to anionic PL, however, none of the 13 H/L chain combinations that I tested bind to dsDNA. This observation shows that the effects of the CDR3 arginine residues on binding to anionic PL are not simply due to non-specific charge interactions between antigen and antibody and suggests that dsDNA is not part of the immunogenic antigen that has driven the accumulation of these arginine residues.

$IS4V_H/B3V_L$ is a strong binder to a number of clinically relevant antigens such as β_2GPI and PT, and my prediction would be that it is likely to be pathogenic *in vivo*. However, this combination also binds PC. I was not expecting $IS4V_H/B3V_L$ to bind to the neutral PC because this characteristic is generally a feature of non-pathogenic aPL (Loizou et al., 1990). The real test of my prediction, will however come from experiments testing this combination in biological assays, which are currently in progress by my colleague Dr I. Giles. The aim is to study the variants of IS4 for their thrombogenic properties in an *in vivo* pinch-induced thrombosis model developed by

Pierangeli et al., (1999) at the Morehouse School of Medicine in Antlanta, USA. Native IS4 isolated from hybridoma cells has previously been shown to be thrombogenic (Pierangeli et al., 2000). Pierangeli and colleagues injected groups of mice either with monoclonal Igs isolated from patients with the APS or with monoclonal Igs of the same isotype from healthy controls. A non-occlusive thrombus was induced in the femoral veins of experimental mice by a pinch injury (Pierangeli et al., 1999). Igs from patients with APS caused a significant increase in mean thrombus area and a significant delay in mean thrombus disappearance time as compared with normal controls.

Two groups have shown that germline reversion of certain somatic mutations in both murine (Cocca et al., 2001) and human (Wellmann et al., 2005) anti-dsDNA antibodies reduced the ability of the antibodies to bind DNA and nucleosomes but enhanced their PS binding. I have also shown this phenomenon in Chapter Five where I mutated an arginine residue into the germline serine residue in a human mAb, B3 and abolished binding to dsDNA and nucleosomes but introduced binding to PL in the presence of human β2GPI. This abolition of binding was not demonstrated with any of the mutated IS4 antibodies however since none of the antibodies bound dsDNA regardless of reactivity to PS. Given that I have shown that purified native B3, an anti-nucleosome antibody, did not bind dsDNA unless supernatant from electroporated cells was added, it would be interesting to examine these purified recombinant aPL for binding to nucleosomes. It is possible that IS4V_Hi&ii/B3V_L (with arginine 96 and 97 substituted to Ser), which binds well to anionic CL and PS, may also bind nucleosomes.

There have been very few reports investigating the relationship between sequence, structure and pathogenicity of aPL. Pierangeli and colleagues have tested a range of aPL in *in vivo* models of microcirculation and have shown that there is no simple relationship between pathogenicity and strength of binding to CL (Pierangeli et al, 2000). Two of the antibodies tested, IS1 and IS2, contained identical V_H and their L chains differed from each other by only five amino acids (Chukwuocha et al., 2002). IS1V_L contained a few more mutations than IS2V_L which improved its reactivity for antigen but removed its thrombogenic activity (Zhu et al., 1999, Pierangeli et al., 2000). Lieby et al., (2004) studied 5 monoclonal aPL, all originating from a single patient suffering from this autoimmune disease. They have found that for only one antibody (CIC15) its antiphospholipid activity was dependent on annexin A5, and its

V region contained mainly 3 replacement mutations, which were *in vitro* reverted to the germline configuration (asparagines to serine in V_LCDR1), to demonstrate the role of these mutations in the pathogenicity of the antibody. The resulting "germline" antibody reacted with multiple self-antigens and only partially lost its reactivity against PLs, but it was no more dependent on annexin A5 and, more importantly was no more pathogenic. Germline reversion caused the antibody to lose its ability to induce fetal loss in BALB/c mice. In conclusion, somatic mutations affect the binding of antibodies and the effect on function requires further studies.

CHAPTER SEVEN

Overview and ideas for future work

CHAPTER SEVEN.

OVERVIEW AND IDEAS FOR FUTURE WORK

The work described in this thesis increases our understanding of the importance of somatic mutations and the critical effect that the precise position of arginine residues in the antigen-binding site may have on the binding properties of anti-DNA antibodies and aPL. Computer models for the human mAbs, B3 and IS4 have provided more insight about the likely positions of key arginine residues leading to the hypothesis that these residues are likely to contribute to the binding affinity of the antibodies. To investigate this hypothesis I have produced variant forms of $B3V_H$ and $IS4V_H$ using site-directed mutagenesis, and have shown that arginines in the CDRs play a major role in the binding properties of anti-dsDNA antibodies and aPL.

Both transient and stable expression of cloned cDNA from human monoclonal anti-DNA antibodies and aPL in the form of whole IgG molecules has been accomplished and allowed me to test the properties of $B3V_H$ and $IS4V_H$ variant forms. In total I have produced 17 stable cell lines. The antibody production of the CHO cells transfected with the supervectors was higher than that of the COS-7 cells co-transfected with separate H and L chain vectors. The main advantage of the transient expression system however, was that it allowed testing of a range of H/L combinations and provided evidence as to which chains are essential in binding to nucleosomes, dsDNA, CL, and β_2GPI.

It was vital to express antibodies stably in CHO cells to increase the volume and concentration of IgG expressed, in order to test these antibodies against a wider variety of antigens at a higher dose than was possible with COS-7 cells. The larger amounts of antibodies produced from CHO cells were crucial for purification. This purification could not be carried out using the much smaller amounts of IgG produced by the transient system because during purification much Ig protein is lost, therefore requiring larger initial quantities of Ig. I have summarised and represented the binding results of all purified antibodies against all antigens in Table 7.1 and 7.2.

The abolition of dsDNA binding by the purification and *DNase I* treatment of antibodies, which had previously bound dsDNA when suspended in supernatant, and

the subsequent reinstatement of dsDNA binding by reconstitution with supernatant, suggested that B3 might in fact recognise an epitope comprising both dsDNA and histone, such as nucleosomes. To determine the nature of the factor in the supernatant that was required for dsDNA binding, pure nucleosomes were added to the purified and *DNase I* treated antibodies prior to assaying for dsDNA binding. In the case of purified $B3V_H/B3(R27aS)V_L$, addition of nucleosomes allowed the reconstitution of dsDNA binding. In light of the lack of DNA binding by the highly purified antibodies, I also assessed the binding of these antibodies to nucleosomes on the solid-phase and confirmed anti-nucleosome activity. The additional binding of purified $B3V_H/B33V_L$ to α-actinin suggests that it actually recognises a conformational epitope that allows limited cross-reactive binding. Thus it is possible that anti-nucleosome and anti-dsDNA antibodies may bind more than one antigen.

The major achievement from my work however, is that I have shown dramatic shifts of binding in opposite directions to different clinically relevant antigens caused by a single arginine to serine mutation (refer to summary table 7.1). In the combination $B3V_H/B3V_L$, an arginine in V_H CDR2 at position 53 is critical for binding to dsDNA and nucleosomes. Changing this arginine to serine, in the absence of any other change in sequence in either the H or L chain, abolished binding to those two antigens but created binding to α-actinin, whole β_2GPI and DI of whole β_2GPI. In addition, the production of an antibody [$B3(R53S)V_H/B3V_L$] that distinguishes human from bovine β_2GPI has not previously been described. There are 11 amino acid differences between the sequence of DI of human and bovine β_2GPI, therefore it is likely one of these differences plays a role in the specificity of the antibody for human β_2GPI. Our group has expressed two variant forms of DI that specifically target these residues of interest, therefore it will be possible in future experiments to determine the binding affinity of $B3(R53S)V_H/B3V_L$ to these variant forms of DI. Only the germline reversion R53S (not R53N or R53K) enhanced binding to α-actinin, whole β_2GPI and DI, supporting the idea that the germline sequence of the V3-23 gene in that region promoted low affinity binding to these antigens. Since I did not produce a stably transfected CHO line producing the germline V3-23 sequence, I was not able to produce sufficient quantities of purified germline-encoded antibody to test its binding to α-actinin, whole β_2GPI or DI. However, there are many descriptions in the literature of polyreactive antibodies with H chains encoded by the unmutated V3-23 gene [reviewed in (Giles et al., 2003)].

Antibody	dsDNA (Figure 5.1)	Nucleosomes (Figure 5.2)	α-actinin (Figure 5.4)	CL (Figure 5.7)	PS (Figure 5.10)	β_2GPI (Figure 5.5)	DI (Figure 5.6)
$B3V_H/B3V_L$	+++	+++	-	+	-	-	-
$B3V_H/B33V_L$	+++	+++	+++	-	-	+	-
$B3V_H/B3(R27aS)V_L$	++	++	-	+	-	+	-
$B3(R53S)V_H/B3V_L$	-	-	+++	+++	-	+++	+++
$B3(R53S)V_H/B33V_L$	-	-	+++	+	-	+	+
$B3(R53N)V_H/B3V_L$	-	-	-	-	-	-	-
$B3(R53K)V_H/B3V_L$	-	-	-	-	-	-	-
$B3V_H/BUV_L$	-	-	-	-	-	-	-

Table 7.1 Summary of the binding properties of the B3 variants

The presence of arginine at position 53 in $B3V_H$ results in binding to dsDNA and nucleosomes. On the contrary, the presence of this arginine reduced binding to alpha-actinin, β_2GPI and Domain I of β_2GPI. Binding to these antigens was shown when the arginine was substituted to serine. (+++: indicates strong binding, ++: medium, +: weak and –: denotes no binding)

Antibody	CL (Figure 6.2)	PS (Figure 6.6)	PC (Figure 6.8)	PS-PT (Figure 6.9)	β_2GPI (Figure 6.10)	DI (Figure 6.12)
$IS4V_H/IS4V_L$	+++	++	-	-	+++	+++
$IS4V_Hiii/IS4V_L$	-	-	-	-	+	+
$IS4V_Hiv/IS4V_L$	-	-	-	-	+	+
$IS4V_Hi\&ii/IS4V_L$	-	-	-	-	-	-
$IS4V_Hiii\&iv/IS4V_L$	-	-	-	-	-	-
$IS4V_HX/IS4V_L$	-	-	-	-	-	-
$IS4V_H/B3V_L$	+++	+++	+++	+++	+++	+++
$IS4V_Hi\&ii/B3V_L$	+++	+++	-	-	+	++
$IS4V_Hiii\&iv/B3V_L$	++	++	-	-	+	++
$IS4V_Hi,iii\&iv/B3V_L$	-	-	-	-	-	++
$IS4V_Hii,$ iii &iv/$B3V_L$	+	+	-	-	-	++
$IS4V_HX/B3V_L$	-	-	-	-	-	-
$IS4V_H/UK4V_L$	+	-	-	-	-	+

Table 7.2 Summary of the binding properties of the IS4 variants

Arginine residues in $IS4V_H$ substituted to serine, at positions 100 and 100g reduced binding to all antigens, while at positions 96 and 97 reduced binding to β_2GPI but increased or decreased binding to CL and PS (in combination with $B3V_L$). When paired with $IS4V_L$, arginine residues substituted to serine at positions 100 and 100g abolish binding to CL and PS but confer weak binding to β_2GPI and DI. Only the $IS4V_H/B3V_L$ combination bound PC and PS-PT. (+++: indicates strong binding, ++: medium, +: weak and –: denotes no binding)

The direct binding ELISAs used in this thesis were sufficient to determine the binding ability of the purified antibodies tested however, may be possible in future to obtain more accurate information using assays such as surface plasmon resonance (SPR) or inhibition ELISAs. SPR can be applied in the case of the two antibodies that did not show any binding to the any of the antigens tested [B3(R53K)V_H/B3V_L and B3(R53N)V_H/B3V_L]. In inhibition ELISAs, the affinity of Igs in a test sample is determined according to the amount of antigen that has to be added to the test sample in order to inhibit binding to the same or another antigen coated to the plate. Alternatively the dissociation constant (Kd), as a measure of antibody affinity, can be determined by incubation of the antibodies with varying concentrations of antigen prior to assaying in an ELISA with antigen on the solid-phase (Friguet et al. 1985). In this system the concentration of antigen must be in great excess of the concentration of antibody. Functional affinity of the antibodies can also be measured by addition of the chaotropic agent diethylamine (DEA) to inhibit the binding of low affinity antibodies to the antigen coated on the solid-phase (Devey et al 1988). The higher the affinity of the antibodies tested, the smaller the shift in binding caused by the presence of DEA in the assay. The results are expressed in terms of the inhibition index, which is the reciprocal $\log_{10}$ of the shift, thus a small shift in binding gives a high inhibition index for a high affinity antibody. In SPR the antibody affinity for an antigen is measured through the calculation of kinetic rate constants detected for the binding and dissociation of an antibody when it is passed over a chip coated with antigen.

In future, it is vital to assess the pathogenicity of these antibodies *in vivo* to correlate binding with functional effects. For example, our group and others have studied the effects of whole IgG secreted from hybridoma or CHO cells administered intra-peritoneally to immunodeficient mice (Mason et al., 2005, Mostoslavsky et al., 2001, Zhao et al., 2005, Ehrenstein et al., 1995, Ravirajan et al., 1998). It would be interesting to compare the effects of the nucleosome-binding antibody B3V_H/B3V_L and the α-actinin-binding antibody B3(R53S)V_H/B3V_L on the kidneys of such mice. The use of murine models to study human diseases may be criticised especially since studying the role of human mAbs in normal, immunodeficient or autoimmune mice is clearly complex. Given their increased availability, it would seem reasonable to consider using simpler *in vitro* systems, such as human kidney cells. Using a conditionally immortalised human podocyte line, Coward and colleagues recently showed that exposure to plasma from patients with nephrotic syndrome (including

one with SLE) altered the distribution of intracellular proteins essential to podocyte function (Coward et al., 2005). The podocyte is highly specialised and is especially interesting since several hereditary human nephrotic syndromes have been shown to be attributable to defects in podocyte specific genes including those for nephrin and podocin (Pavenstadt et al., 2003). It would be fascinating to see if the anti-nucleosome and/or anti-α-actinin IgG antibodies expressed in this experiment have similar effects on those podocytes and if they can bind to other podocyte molecules such as nephrin and podocin, which have been implicated in other human nephrotic diseases. As well as the binding to the individual cells, antibody binding could also be assessed in the human glomerular basement membrane assay (Lefkowith and Gilkeson, 1996) and the rat perfused kidney system (Raz et al., 1989). The perfused rat kidney has previously been used to measure the effect of both murine and human anti-DNA antibodies on induction of proteinuria (Raz et al., 1989).

Assays of the effects of monoclonal aPL on thrombosis (Pierangeli et al., 1994) and pregnancy loss (Blank et al., 1991) in mice have been developed and it would be possible to compare the effects of anti-β_2GPI antibodies such as B3(R53S)V_H/B3V_L with antibodies that do not bind β_2GPI (e.g. B3V_H/BUV_L) in these assays. IS4 has been shown to be highly thrombogenic and to enhance endothelial activation in the *in vivo* model of microcirculation (Pierangeli et al., 2000). As described in section 6.4, my colleague Dr I. Giles is currently looking at the effects of the IS4 variants upon thrombus formation and leucocyte adhesion in this model. In addition, it would be interesting to study the effects of the IS4 variants upon vascular EC activation *in vitro* by examining the expression of adhesion molecules (E-Selectin, ICAM-1 and VCAM-1) which have all been shown to be up-regulated by native IS4 (Pierangeli et al., 2000)

TF is a major initiator of coagulation *in vivo* produced from EC and monocytes. Several studies have shown both *in vivo* and *in vitro* up-regulation of monocyte TF expression in patients with APS. aPL from APS patients, have been shown to enhance TF expression and procoagulant activity on normal monocytes (Kornberg et al., 1994, Reverter et al., 1998). Additionally, TF expression on monocytes from patients with PAPS (compared to monocytes from normal controls) is directly involved in the pathogenesis of thrombotic complications (Cuadrado et al., 1997). *In vitro* studies have also shown that IgG purified from patients with APS increases surface expression of TF in normal human EC (Ferrara et al., 2004). Recent studies have

shown that aPL activate monocytes and EC through several signalling pathways such as p38MAPK and NF-κB. Having extensively characterised the binding properties of IS4 (both wild-type and mutant forms), it would be very interesting to study its effects upon monocyte and EC expression and function of TF and characterise the signalling pathways involved.

It is therefore urgent to carry out this future work since it would help to elucidate the precise role of arginine residues in the binding of autoantibodies to their target antigen and provide better understanding of how manipulation of antibody binding sites may affect their function. The long-term goal of this future research would be to design a more effective and targeted therapy to block the effects of these autoantibodies in patients with SLE and APS.

An attempt has made to re-educate the immune system with toleragenic therapies, by administration of a structure which contains oligovalent B cell epitopes on a nonimmunogenic molecular framework, lacking T cell epitopes. This has been developed as a means to inactivate pathological B cells in an antigen-specific manner. These types of agents, developed by La Jolla Pharmaceuticals, consist of three or four identical antigenic constructs attached to a proprietary carrier platform made of nonimmunogenic polyethylene glycol (Merrill, 2004). Abetimus sodium (LJP 394, Riquent) consists of four deoxyoligonucleotide epitopes selective for antibodies to dsDNA (Lorenz, 2002). Animal studies and clinical trials suggested that LJP-394 is safe and well tolerated. It has been shown to inhibit binding of DNA to anti-dsDNA antibodies and to reduce antibody producing cells in an immunised mouse model and in controlled trials in SLE, it can reduce circulating anti-dsDNA antibody levels without causing generalized immunosuppression. Treatment with LJP 394 in patients with high affinity antibodies seemed to prolong time to renal flare and decreased the number of renal flares (Lorenz, 2002). Similarly, another polyvalent antigenic structure has been developed for the treatment of stroke, deep-vein thrombosis and other conditions associated with antibody-mediated thrombosis. LJP 1082 is aimed at crosslinking specific surface Ig and tolerising B cells to β_2GPI (Merrill, 2004).

Toleragen studies have provided evidence of encouraging clinical benefit however it is likely that they can cause a reduction in the average affinity of the remaining antibodies. If this type of therapeutic intervention results in elimination of a majority of high affinity B cells that specifically recognize an antigen, administration of a small

peptide that targets arginine residues in autoantibodies would be a possible option. My findings have shown that in the two human monoclonal antibodies, B3 and IS4, arginine residues are important in binding to clinically relevant antigens, therefore targeting these arginine residues with an inert epitope can be effective at modulating the immune response and would be expected to influence the amount and affinity of circulating antibody.

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