Auto-Antigenicity of Hemoglobin: Antigenic, Biological and Genetic Analysis

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CERTIFICATE

This is to certify that the thesis entitled "**Auto-Antigenicity of Hemoglobin: Antigenic, Biological and Genetic Analysis**" submitted by **Harshita Bhatnagar** in partial fulfillment of the degree of **Doctor of Philosophy** from **Jawaharlal Nehru University**, embodies the work done by the candidate under my guidance at the **National Institute of Immunology**, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

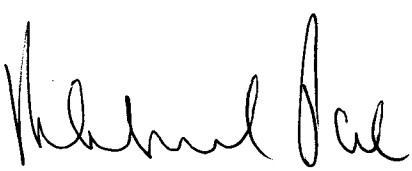

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Table of Contents

Abbreviations	*2-4*
Introduction	*6-8*
Review of Literature	*10-41*
Materials and Methods	*43-57*
Results	*59-77*
Discussion	*79-93*
Summary and Conclusions	*95-97*
Bibliography	*99-134*
Appendix	*136-145*

Abbreviations

ABBREVIATIONS

ACR	American College of Rheumatology
AIHA	Autoimmune Hemolytic Anemia
AMV	Avian Myeloblastosis Virus
ANA	Anti-Nuclear Antibodies
APLS	Anti-Phospholipid Syndrome
APS	Ammonium Persulphate
BAFF	B Cell Activation Factor Belonging To TNF Family
BBB	Blood Brain Barrier
BCA	Bicinchoninic acid
$ß_2$-GPI	$ß_2$-Glycoprotein-I
BSA	Bovine Serum Albumin
CDC	Center for Disease Control
CDR	Complementarity Determining Region
CFA	Complete Freund's Adjuvant
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate
CHB	Congenital Heart Block
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
Cyt c	Cytochrome c
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
EBNA	Epstein Barr Nuclear Antigen-1
EBV	Epstein Barr Virus
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetraacetic Acid

EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
FACS	Fluorescence Assorted Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FWR	Framework Region
gld	Generalized Lymphoproliferation Disease
HAT	Hypoxanthine Aminopterine Thymidine
Hb	Hemoglobin
HBMEC	Human Brain Microvascular Endothelial Cells
hHb	human Hb
Hp	Haptoglobin
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
Hx	Hemopexin
ICAM	Intercellular Adhesion Molecule
IFA	Incomplete Freund's Adjuvant
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVIG	Intravenous Immunoglobulins
LB	Luria Bertani
lpr	Lymphoproliferation
Mb	Myoglobin
MCP	Monocyte Chemoattractant Protein
metHb	methemoglobin
mHb	murine Hb
NMDA	N-methyl-D-aspartate
NPSLE	Neuropsychiatric Lupus Erythematosus
NZB/W F1	F1 generation of New Zealand Black crossed with New Zealand White mice
NZM	New Zealand Mixed
OPD	Ortho-Phenylene Diamine

PB	Phosphate Buffer
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PI	Propidium Iodide
PVA	Polyvinyl Alcohol
RAG	Recombination Activating Gene
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SLE	Systemic Lupus Erythematosus
TEMED	N, N, N', N'-Tetramethylethylenediamine
TFA	Triflouroacetic Acid
TMB	3, 3′, 5, 5′-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
TNP	Trinitrophenyl
TPO	Thyroid Peroxidase
TRAIL	TNF-Related Apoptosis Inducing Ligand
UV	Ultraviolet
VCAM	Vascular-Cell Adhesion Molecule
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
Yaa	Y-linked autoimmune acceleration

Introduction

INTRODUCTION

The immune system serves to protect the organism from invading pathogens. Defects in the functioning of this critical process are often associated with serious pathological consequences; for example, anomalies in the ability to distinguish self from non-self could lead to autoimmune disease. Traditionally, autoimmune diseases have been classified as being either organ-specific or systemic. Systemic Lupus Erythematosus (SLE; also referred to as lupus), is considered the prototypic systemic autoimmune disease. Most patients are women of child-bearing age and symptoms of disease usually include arthritis, scarring rashes and glomerulonephritis. More than a hundred different auto-antibody specificities have been described, including those reactive to nuclear and cytoplasmic proteins, double-stranded DNA, ribonucleoproteins and lipids, with some auto-antibodies arising as early as 9 years before the onset of clinical symptoms. Auto-antibodies form immune complexes leading to complement activation and inflammation. Some auto-antibodies have been associated with end-organ failure and pathology. For example, antibodies to double-stranded DNA (dsDNA) are thought to contribute to kidney malfunction by depositing in the kidneys as antigen-antibody complex or by reacting to kidney antigens such as α-actinin. Anti-dsDNA antibodies can also cross-react with receptors present on cells of the central nervous system, resulting in neuronal loss. Antibodies to the ribonucleoprotein Ro are believed to cause neonatal lupus and congenital heart block. Similarly, auto-antibodies against phospholipids and antibodies directed against the RBC surface antigens (such as Rh antigens E and c proteins), result in autoimmune hemolytic anemia with the release of intracellular hemoglobin (Hb), an event that may have independent pathological consequences.

The etiology of SLE is not very well understood. It has been established that host genetics and the environment both contribute towards pathology. Defects in the induction of central or peripheral tolerance could conceivably result in autoimmunity. Further, molecular mimicry, epitope spreading, crypticity, idiotypy, altered levels of cytokines (such as IL-2, IL-6, IFN-α and TNF-α), hormones, environmental triggers such as sunlight, viral infections, certain drugs, aberrant apoptosis and the defective clearance of apoptotic cells by macrophages have all been associated with disease onset or progression.

Various mouse models have aided in delineating disease progression and pathology, and have greatly enhanced our understanding; traditional models in which lupus-like disease develops spontaneously, such as New Zealand Black (NZB) x New Zealand White (NZW) F1 animals, are the best-studied. Other spontaneous models include MRL$^{lpr/lpr}$ and BXSB mice. Additionally, injection with hydrocarbon oils, and immunization with peptides and auto-antibodies have given rise to other mouse models. Newer models using animals made deficient in single genes (for example, those involved the phagocytic uptake of apoptotic cells) shed fresh light on previously un-appreciated aspects of disease.

Though deficiencies in specific genes (such as C4) are known to result in an SLE-like syndrome in humans, in most instances, the disease is considered a multi-gene disorder. Genetic studies and micro-satellite marker analysis in lupus-prone mice have delineated a number of lupus-susceptibility loci (such as *Sle1*, *Sle2*, *Sle3*, *Lmb1* and *Lmb2*). Analyses in congenic strains bearing these loci, either individually or in combination have revealed their respective roles in disease pathology.

This study focuses on characterization of some aspects of the autoimmune response in lupus. As indicated above, anti-phospholipid antibodies and RBC-reactive antibodies are associated with extensive hemolysis, leading to the release of free Hb. Haptoglobin (Hp), an acute phase serum protein which increases during inflammation, has strong affinity for free Hb. The Hb-Hp receptor (CD163) mediates endocytosis of the Hb-Hp complex and the complex is degraded. In many pathological conditions in which extensive RBC lysis occurs (for example, malaria, sickle cell anemia and lupus), Hp levels may become undetectable and the concentration of free Hb increases. Tetrameric Hb can degrade into dimers which are small enough to be filtered through the kidney, resulting in nephrotoxicity. Hb (subsequent to oxidation from the Fe^{2+} form to the Fe^{3+} form) can then break down to heme and globin. Heme binds hemopexin and is cleared from circulation. Under excess RBC lysis due to autoimmune hemolysis, the heme clearance system may be overwhelmed. Heme, being lipophilic, readily crosses the plasma membrane and results in the generation of reactive oxygen species, causing cytotoxicity. The endothelium, because of it's

proximity to free Hb, suffers deleterious effects. Neuronal death due to Hb and its degradation products is also observed in during episodes of hemorrhage.

Despite the high degree of homology in the structures of Hb of different species, xeno-immunization leads to the production of specific antibodies. B cell epitopes have shown to be present on both alpha and beta subunits. In NZB autoimmune-prone mice, which suffer from extensive autoimmune hemolysis, enhanced T cell proliferation is observed in response to autologous Hb.

During the course of a previous investigation, it was observed that a human monoclonal antibody reactive towards apoptotic cells demonstrated frank cross-reactivity towards hemoglobin. With this lead, this project sought to establish the generality of anti-Hb autoimmune reactivity in both animal models of lupus and in human patients of SLE and in other diseases characterized by significant RBC lysis. Anti-Hb autoimmune antibody responses were characterized in terms of antigenicity and biological activity, employing both polyclonal and monoclonal anti-Hb antibodies. Sequencing of the variable region genes of the monoclonal anti-Hb antibodies permitted genetic characterization as well.

Review of Literature

REVIEW OF LITERATURE

Most living organisms have developed a remarkable system to protect themselves from invading pathogens and neoplastic cells. The immune system in the higher mammals can be broadly characterized as either "innate" or "adaptive". The innate system does not require a prolonged period of exposure to pathogens and can act immediately, but does not generate long-lasting immunity. Phagocytes and Natural Killer cells are important components of innate immunity. When the innate immune system cannot get rid of the pathogen, adaptive immune responses come into play, with the generation of antigen-specific effector cells. These responses are characterized by the generation of immunologic memory and the capacity of self/non-self discrimination. Adaptive immune responses are mainly mediated by two cell types – the T and B lymphocytes. Defects in their functioning are often associated with serious pathological consequences; for example, anomalies in the ability to distinguish self from non-self could lead to autoimmunity, the pathological consequences of which result in autoimmune diseases. Autoimmunity per-se can occur without any overt disease or appearance of symptoms; it is only when the functions of targeted organs are adversely affected do autoimmune diseases occur. Thus, autoimmunity may be common in the general population; incidences of self-limiting, self-reacting T and B cells are high[1], but only a small percentage of these individuals develop full-blown disease. Autoimmune diseases, described as *horror autotoxicus* by Paul Ehrlich, are therefore relatively rare.

Traditionally, autoimmune diseases have been classified as being either organ-specific (where a particular organ or gland is affected) or systemic (where multiple antigens and/or organs are targeted), although there exists an entire spectrum of pathologies between these two extremes. While Grave's Disease is an oft-quoted example of an organ-specific autoimmune disease (where agonistic auto-antibodies target the TSH receptor), Systemic Lupus Erythematosus (SLE) is the prototypic systemic autoimmune disease, characterized by anti-self responses against a wide variety of antigens as well as the presence of multi-organ pathology; amongst the organs targeted are the kidneys, the brain, the lungs, and the heart.

Systemic Lupus Erythematosus

SLE (or lupus, as it is more commonly referred to) is a chronic inflammatory autoimmune disease of unknown etiology, affecting 1 in about 2,000 people[2]. Disease concordance in identical twins is 25-50%, and in dizygotic twins around 5%. There is also a higher risk of disease in the first-degree relatives of patients[3] indicating the role of genetic factors in disease onset, as discussed is more detail below. Lupus predominantly affects women, especially of reproductive age. The female-to-male ratio is around 9:1 for patients between 15-50 years of age. Further, African-American and Hispanic-American women have a two-four times higher risk of developing the disease as compared to Caucasians[4].

The disease progresses through four broad stages - the appearance of auto-antibodies against a number of ubiquitous self-antigens, the deposition of auto-antibodies and immune complexes in various tissues, tissue inflammation followed by tissue damage, and fibrosis affecting organ function, finally resulting in high morbidity and mortality[5].

Lupus is the most heterogeneous autoimmune disease. Patients of SLE can manifest more than a hundred auto-antibody specificities. While some of these may be epi-phenomena, others are thought to cause (or are at least closely associated with) distinctive pathologies. Some individuals exhibit tissue deposition of auto-antibodies but no associated inflammation; others have antibody deposition as well as inflammation but no tissue damage and still others exhibit antibody deposition, tissue inflammation and associated pathology. Because of multi-organ involvement, a variable disease course and protean manifestations, diagnosis of SLE is often difficult. The American College of Rheumatology (ACR) has put forward eleven clinical criteria for diagnosis, which are detailed in Table 1[6,7]. A person found to exhibit four or more of these clinical conditions, either simultaneously or during any period of observation, is diagnosed as being afflicted. Employing these criteria, diagnosis can be achieved with 95% specificity and 85% sensitivity. In specific instances, the presence of three significant indicators (nephritis and the presence of anti-nuclear and anti-DNA antibodies) can also lead to a diagnosis of SLE.

Table 1[6,7]

CATEGORY	S.No	ITEM	DEFINITIONS
Skin category	1	Malar rash	Fixed erythema, flat or raised, over the malar eminences sparing the naso labial fold.
	2	Discoid rash	Erythematosus raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions.
	3	Photosensitivity	Skin rashes as a result of unusual reaction to sunlight, by patient history or physician observation.
	4	Oral ulceration	Oral or nasopharyngeal ulceration usually painless observed by physician.
Systemic criteria	5	Non erosive arthritis	Involving two or more peripheral joints, characterized by tenderness, swelling or effusion.
	6	Pleuritis or pericarditis	Pleuritis: Convincing history of pleuritis or rub heard by a physician or evidence of pleural effusion OR Pericarditis: Documented by ECG or rub or evidence of pericardial effusion.
	7	Renal disorder	Persistent proteinuria greater than 0.5 g/day or greater than +3 if the equivalent not performed OR Cellular casts- may be red cells, hemoglobin, granular, tubular or mixed.
	8	Seizures or psychosis	Seizures- In the absence of offending drugs or known metabolic derangement. E.g. uremia, ketoacidosis or electrolyte imbalance OR Psychosis- In the absence of offending drugs or known metabolic derangement. E.g. uremia, ketoacidosis or electrolyte imbalance.
Laboratory criteria	9	Hematological disorder	Hemolytic anemia with reticulocytosis OR Leucopenia- less than 4000/mm^3 on two occasions OR Lymphopenia-less than 1500 on two occasions OR Thrombocytopenia- less than 100,000/mm^3 in the absence of offending drug.
	10	Immunologic disorder	Anti-DNA: Antibodies to native DNA in abnormal titre OR Anti-Sm: Presence of antibodies to Sm nuclear antigen OR Positive finding of anti-phospholipid antibodies based on (a) An abnormal level of IgG or IgM anti-cardiolipin antibodies. (b) A positive test for lupus anticoagulant. (c) A false positive test for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.
	11	Positive antinuclear antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point of time in the absence of drug.

Auto-antibodies

The most distinctive feature of SLE is the presence of auto-antibodies, mainly of the IgG isotype. Though more than a hundred different auto-antibody specificities have been described, those directed against nuclear antigens (such as dsDNA), phospholipids, ribonucleoproteins and RBCs have been the most extensively studied.

Anti-DNA Antibodies

Almost 99% patients of SLE (with or without nephritis) express anti-nuclear antibodies in their sera. Amongst them, approximately 50-70% patients carry anti-dsDNA antibodies[8]. Though lupus nephritis can develop in absence of anti-DNA antibodies[9], in most of patients, the levels of anti-DNA antibodies increase during disease "flares". These antibodies may form immune complexes with circulating DNA leading to complement activation[10], cross-linking of Fc receptors on leukocytes (as the pathogenic antibodies are mainly of IgG2a or IgG2b isotype) and inflammation. These complexes and/or antibodies are also thought to deposit in various organs like the skin, the lungs, on blood vessels and the kidneys[11]. Deposition in the kidneys is thought to result in the onset of glomerulonephritis and the subsequent development of chronic renal failure. However, not all anti-DNA antibodies are pathogenic i.e. not all induce glomerulonephritis. In some instances, the pathogenic anti-DNA antibodies have been shown to react to glomerular antigens; α-actinin, a 100 KDa protein and a major component of glomerular podocytes, was recognized by such antibodies, while non-pathogenic anti-dsDNA antibodies showed either very weak binding or no binding to the protein. α-actinin mimics the charge characteristics of DNA, itself being very acidic, with many negatively charged amino acids[12]. Sera and kidney eluates from the lupus-prone mice showed the prevalence of anti-α-actinin antibodies[13]. Intravenous immunization in non-autoimmune animals with anti-DNA antibodies resulted in glomerular binding within one hour[14]. The fact that these antibodies can cross-react with other auto-antigens such as Sm[9], may aid in their pathology.

The involvement of the central nervous system in lupus is reflected by the occurrence of cognitive impairment, depression, seizures and psychoses. It has now been shown that the pathogenic anti-DNA antibodies can cross-react with the N-methyl-D-aspartate NMDA subunit NR2 glutamate receptor in the central nervous system (CNS). Such antibodies could also bind neurons of the

cortex *in vivo* and cause excitotoxic death in the neurons by the upregulation of caspase 3[15]. *In vivo,* intentional breakdown of blood-brain barrier permitted these auto-antibodies to gain access to the brain where they bound hippocampal neurons, causing death. Animals displayed performance deficits on tasks that depend on the integrity of the hippocampus. Antibody-mediated damage could be prevented by memantine, an NMDA receptor antagonist which is a potent non-competitive inhibitor of NR2A and NR2B-containing receptors[16].

Although T cells are not thought to play a direct role in tissue damage in SLE, CD4+ T cells seem to be required for the production of pathogenic IgG antibodies[2]. Ample evidence exists describing the importance of somatic mutations in complementarity determining regions (CDR) for antibody specificity. High affinity anti-DNA antibodies (both pathogenic and non-pathogenic) have been shown to contain a relatively higher number of arginine residues in the variable regions of the heavy chain. Addition of arginines in the CDR3 has been shown to increase affinity to DNA; conversely, replacement of arginine by glycine (disrupting hydrogen bonding or electrostatic interactions) resulted in the partial or complete loss of binding[17].

Immunization with anti-dsDNA antibodies bearing the public idiotype 16/6 in non-autoimmune animals resulted in generation of autoimmune responses. This phenomenon has been discussed in greater detail below.

Anti-Ribonucleoprotein (RNP) antibodies

Anti-RNP antibodies are a common occurrence in lupus patients. Such auto-antibody responses can be of the IgG, IgM[18] and the IgA[19] isotype and include antibodies to Ro/SSA, La/SSB and Smith (Sm) proteins. Anti-Ro antibodies are found in around 50% of SLE patients and comprise antibodies directed against two non-homologous auto-antigens - Ro52 and Ro60; these can be found in other autoimmune diseases as well, such as Sjogren's syndrome[20]. Ro60 has a molecular mass of 60 KDa, is an evolutionary conserved molecule and is involved in the quality control of 5S rRNAs. It is present in both the cytoplasm and the nucleus, whereas Ro52 (52 KDa) is mainly found in the cytoplasm[21]. The function of Ro52 is controversial, as is its association with Ro60[20]. It has been shown recently in humans that antibodies to Ro60 can be detected as early as nine years before the symptoms of lupus appear[22], and earlier than the appearance of other auto-antibodies,

such as those against Sm. Children born to mothers harboring anti-Ro antibodies have a higher probability of developing congenital heart block (CHB)[23]; around 2-5% females suffering from SLE deliver new-born babies with CHB. Conversely, nearly all infants with CHB are born to mothers harboring anti-Ro antibodies[24]. Antibodies to Ro52 have been shown to bind cardiomyocytes and cause an intracellular overload of calcium concentrations, leading to loss of contractibility and eventual apoptosis[25]. Reports have shown that Ro60 and La associate together in apoptotic blebs[26]. During developmental processes, the cardiocytes that undergo apoptosis are quickly taken up by healthy cardiocytes. Maternal anti-Ro and anti-La antibodies inhibit uptake. It is postulated that inhibition of apoptotic cell uptake during massive regeneration can have serious pathological implications[27].

The La protein has a molecular mass of 48 KDa and plays a role in the termination of the transcritption by RNA polymerase III and also allows for the multiple re-initiation of transcription by RNA polymerase III. It is mainly found in the nucleus and has been shown to be exported to the cytoplasm under conditions of stress[28]. Auto-antibodies to La are present in around 34% SLE patients and appear later in the disease than anti-Ro antibodies. Like anti-Ro antibodies, anti-La antibodies are implicated in CHB. These antibodies bind La that is translocated to the apoptotic blebs of developing cardiomyocytes that die due to calcium overload (induced by anti-Ro antibodies), thus increasing the antibody deposition in the heart. Mortality due to CHB this is around 30% and more than two-thirds of the patients need life-long pacemaker implants[29].

The Sm ribonucleoproteins are a part of the spliceosomal complex. A number of Sm proteins are known: Sm B, B`, D1, D2, D3, E, F and G. They function to remove the intronic mRNA. The presence of anti-Sm antibodies are specific to lupus and occur in around 30% of patients[30]. These antibodies appear relatively late, just a few months before the diagnosis of the disease[22].

Twelve percent of SLE patients carry serum antibodies to ribosomal (Rib) P proteins; the incidence of this reactivity appears to be higher in patients also expressing anti-Sm antibodies. Anti-Rib P antibodies, like anti-Sm antibodies, are specific to lupus. Antibodies are mainly of IgG2a isotype, though IgG2b, IgG3 and IgG1 isotypes can also exist[30]. Rib P proteins comprise of a family of acidic phosphoproteins P0, P1 and P2 with molecular masses of 38 KDa, 19 KDa and

17 KDa respectively, and auto-antibodies are directed mainly to the carboxy terminal of these proteins. Some reports suggest that the presence of these antibodies correlates with disease activity, and also with neuro-psychiatric manifestations (such as psychosis and depression) seen in lupus[31,32]. Anti-Rib P Protein antibodies can be poly-reactive, demonstrating binding to auto-antigens such as ssDNA, dsDNA and $ß_2$-glycoprotein-I ($ß_2$-GPI). In addition, an association between anti-Rib P Protein antibodies and anti-phospholipid antibodies has been recently demonstrated[33].

Anti-phospholipid antibodies

Auto-antibodies against phospholipids (aPL) are also relatively frequent in lupus[34] and are associated with thrombotic complications[2,35]. In the course of disease, anti-phospholipid antibodies appear at around the same time as anti-Ro and anti-La antibodies and are usually directed against negatively-charged phospholipids like cardiolipin and phosphatidylserine. Lesser auto-antibody reactivity can also be seen against phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. Anti-phospholipid antibodies of the IgG, IgM or IgA isotypes have been described[35,36]. Some antibodies may be directed against PLs complexed with PL binding proteins such as β2-glycoprotein I (β2-GPI), also called apolipoprotein H[37]. Women with aPL suffer preeclampsia, recurrent fetal loss, hemolysis, elevated liver enzymes and low platelet counts[38]. These antibodies are thought to bind to the surface of the platelets and endothelial tissue and in turn, cause decreased secretion of prostacyclin production by endothelium and increased thromboxane production by platelets, resulting in vasoconstriction[37]. Passive administration of monoclonal anti-cardiolipin antibodies in mice resulted in recurrent fetal losses and thrombocytopenia[38]. Auto-reactive β2-GPI specific $CD4^+$ T cells have also been identified that do not recognize the native molecule but the reduced or recombinant β2-GPI produced in bacteria. It is hypothesized that cryptic epitopes (as discussed later) of β2-GPI initiate the auto-reactive response to phospholipids[39]. Hence, animals immunized with foreign β2-GPI develop anti-phospholipid antibodies. This phenotype could be transferred to naive mice by infusing the bone marrow from β2-GPI immunized animals. But when bone marrows were depleted of T cells, no auto-reactive anti-phospholipid responses were observed. This observation further strengthened the involvement of T cells in autoimmune responses to phospholipids. Accumulating reports now suggest that anti-phospholipid antibodies bind the placenta and the decidua, and activate

complement, resulting in the death of these tissues, finally resulting in fetal loss. When sub-optimal doses of heparin were used, binding of auto-antibodies was not affected but apoptosis of the cells could be inhibited by preventing complement activation both *in vitro* and *in vivo*[40].

Antibodies directed at cell surface proteins

Auto-antibodies directed against cell surface molecules result in pulmonary hemorrhage, platelet destruction and endothelial damage[11,37]. The anti-phospholipid antibodies can cross-react with the red blood cell membrane and cause autoimmune hemolytic anemia (AIHA)[41,42]. In patients with high titres of anti-cardiolipin antibodies, hemoglobin (Hb) concentrations were very low, thereby exhibiting significant negative correlation[41]. In addition, antibodies directed against RBC surface antigens (Rh antigens such as E and c proteins[43], and antigens of the Kell blood group[44]) result in AIHA, also associated with the release of intracellular Hb. In the case of NZB mice that spontaneously develop autoimmune hemolytic disease, there exist two major groups of anti-mouse red blood cells (RBC) auto-antibodies. Antibodies of the first group are predominantly IgG and react with an exposed surface determinant (referred to as antigen X) on intact RBC; antibodies of the second group are of IgM class and react with the HB antigen only after treatment of RBCs with proteolytic enzymes[45]. Antibodies directed against the RBC surface cause hemolytic anemia due to Fc receptor-mediated erythrophagocytosis or sequestration of agglutinated RBCs in the spleen and the liver. These auto-antibodies, present on the cell surface of RBCs, can be detected by adding species-specific secondary antibody; agglutination is referred to as a positive "Coombs' test". The monoclonal anti-RBC antibody generated from Coombs'-positive old New Zealand mice (NZB) animals caused autoimmune hemolytic anemia in non-autoimmune animals BALB/c mice[46]. Another commonly targeted molecule is the Band 3 anion transporter protein in humans. Anti-RBC antibodies can be of IgG, IgM or IgA isotype. Though similar auto-antibodies can be found in healthy individuals, higher concentrations of these antibodies can be eluted from the RBC surface in autoimmune patients; thus, there appears to be a defect in the regulation of the natural anti-RBC antibodies in patients[47].

Etiology and Pathology

Host genetics and environment are both thought to contribute towards the development of lupus[48]. Though the primary cause of the disease remains unknown, a number of hypotheses have been put forward in efforts to help delineate its initiation and progression. These include the loss of central or peripheral tolerance, molecular mimicry with infectious agents such as bacteria and viruses, the exposure of cryptic epitopes, epitope spreading to include previously untargeted moieties and dys-regulation of the idiotypic network.

Defects in tolerance mechanisms

Errors in tolerance have been shown to be responsible for various autoimmune manifestations[49]. It has been demonstrated that, in healthy individuals, T cell populations that use the Vβ11 TCR family form a minor population in the thymus and in peripheral lymphoid tissues as a result of clonal deletion. In mice thymectomized one to four days after birth, their numbers in the periphery increase ten-fold, coinciding with the development of autoimmune diseases[50]. Additionally, Aire (Autoimmune Regulator) has been shown to be important for central tolerance. It induces the expression of a number of peripheral-tissue antigens in thymic medullary epithelial cells which results in the negative selection of potentially auto-reactive T effector cells. In the absence of Aire, autoimmunity and ultimately overt autoimmune disease develops[51] (Figure Ia).

Tolerance in the B-cell compartment arises through receptor editing, deletion and anergy. The strength of the B-cell receptor signaling and the nature of the antigen are important in determining the fate of B cells. A defect in any of the mechanisms involved in the maintenance of tolerance can lead to the generation of high number of auto-reactive antibodies in the bone marrow and in germinal centers[52,53,54] (Figure Ib, Ic).

Molecular mimicry

In addition to other triggers, evidence implicating the role of infectious agents as initiators of lupus-specific autoimmune responses has emerged[55]. The role of Epstein Barr virus as the initial insult to the immune system is being increasingly suspected. In a group of one hundred and seventeen SLE patients, it was found that one hundred and sixteen were sero-positive for EBV, whereas viral DNA was detectable in peripheral cells in all one hundred and seventeen patients[56].

When an Epstein Barr Nuclear Antigen-1 (EBNA) expression vector was injected into mice, the animals generated antibodies not only against EBNA-1 but also against dsDNA and the auto-antigen Sm; as indicated above, both reactivities are specific to lupus[57]. It is believed that molecular mimicry between the EBNA-1 and the Sm nucleoprotein is responsible for these observations; a proline-rich epitope (PPPGMRPP) in the C-terminus of SmB`/B is similar to an epitope (PPPGRRP) in EBNA-1; the latter peptide is recognized by human anti-Sm sera[58]. It is believed that EBV works by latently infecting B cells, promoting their proliferation and hyper-activation and encoding proteins that inhibit apoptosis (another hypothesized defect in SLE). In further evidence implicating EBV in lupus, antibodies directed towards a peptide representing amino acids 169-180 of the auto-antigen Ro60 cross-react with a peptide representing amino acids 58-72 of EBNA, even though the two peptides exhibit no sequence homology[59].

Crypticity

In some instances, tissue-specific antigens are sequestered and are thus unavailable to the immune system. In addition, while T cells against dominant self-determinants are eliminated or rendered tolerant, those against other epitopes may escape tolerance. Determinants which are quiescent when present as part the whole molecule, but immunogenic when presented by themselves, are referred to as "cryptic". It is postulated that viral infection (along with attendant inflammation and the enhancement of co-stimulatory functions) may result in the effective presentation of previously sequestered or cryptic antigens, leading to the activation of previously quiescent T and B cells[48,49]. Indeed, immunization with a cryptic peptide can lead to autoimmunity[60], a phenomenon which usually requires inflammatory conditions[1].

Epitope spreading

As indicated above, it has been shown in human SLE patients that several years before the onset of the disease, there occur auto-reactive antibody responses directed against amino acids 169-180 of Ro60. As time progresses, other regions of the molecule are targeted, a phenomenon known as intra-molecular epitope spreading[59]. Additionally, immunization of rabbits with the EBNA-1 peptide cross-reactive this Ro60 peptide (described above) also leads to epitope spreading to the other Ro60 peptides. Immunization with a dominant T cell epitope of La led to the appearance of antibodies to both Ro60 and La[61]. Immunization with either Ro60 or La led to the appearance of

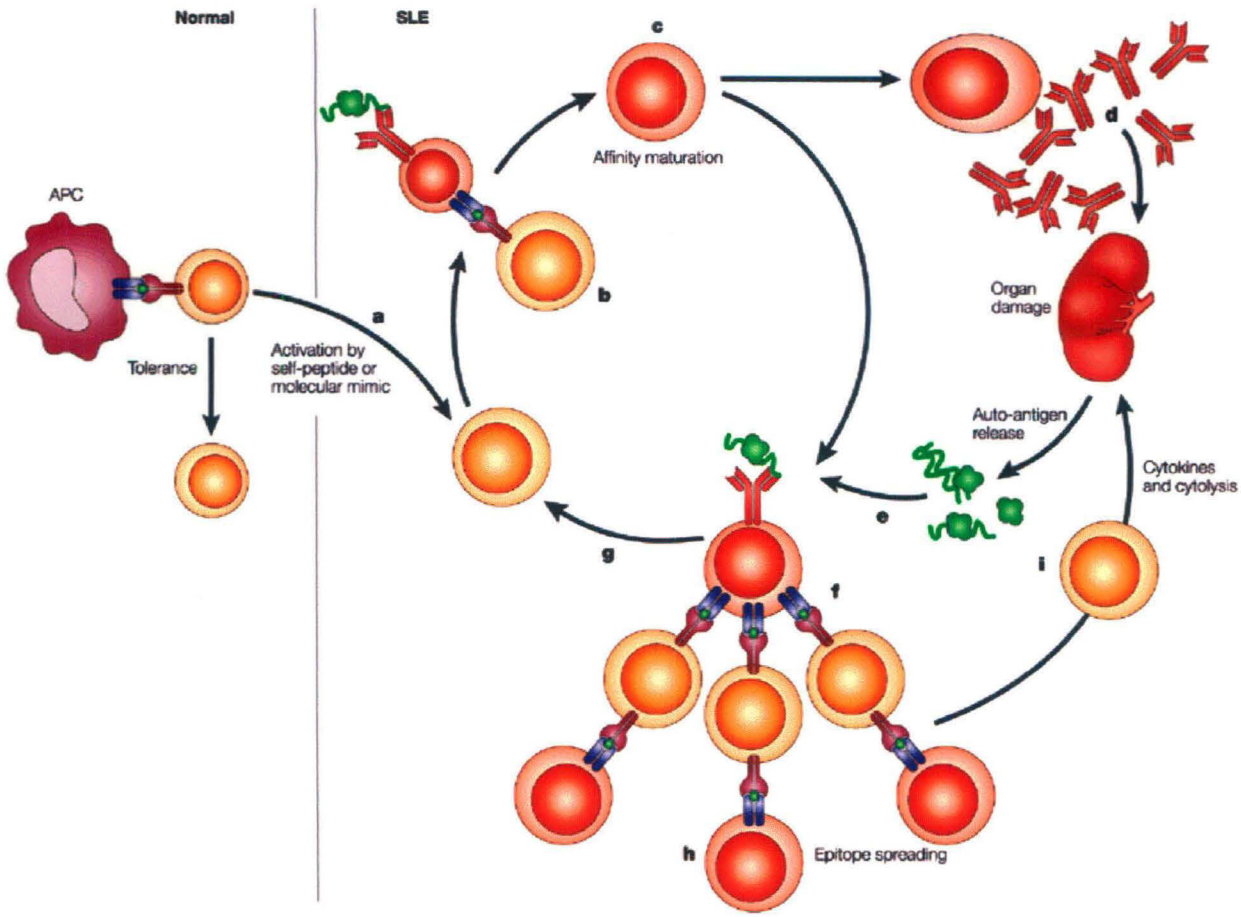
autoimmune responses to both La and Ro60[62]. In addition, immunization of mice with a dominant epitope of the Sm antigen lead to the appearance of antibodies directed to other parts of the molecule[63]. The phenomenon is explained thus: Were B cells of different specificities to internalize multi-component protein complexes (after recognition of their respective cognate epitopes) and then receive help from a single T cell against which tolerance has been broken, antibodies of different specificities would be induced (Figure Ih). However, emerging data indicate that this model may be overly simplistic.

Idiotypy

In 1974, Jerne proposed that the immune response might be regulated by antigenic determinants on the immunoglobulin variable region (idiotypes)[64]. Data has revealed that the immune system may be involved in the progression of autoimmune disease. Immunization with anti-DNA antibodies (Ab1) bearing the "pathogenic idiotype" 16/6 generated Ab2, which in turn endogenously generated Ab3; Ab3 bore the 16/6 idiotype and demonstrated anti-DNA specificity, thus perpetuating disease. Unexpectedly, immunization also led to high titers of anti-ssDNA, anti-dsDNA, anti-Sm, anti-RNP, anti-Ro and anti-La auto-antibodies[65]. Immunization with anti-cardiolipin antibodies was shown to lead to anti-phospholipid syndrome (APLS)[66]. Immunization of rats with a poly-reactive human monoclonal antibody directed against apoptotic cells led to the generation of anti-idiotypic antibodies targeting molecules distinct from those recognized by the immunizing antibody[67].

Generation of Neo-Epitopes

Another theory suggests that autoimmunity can arise upon the formation of neo-self determinants, generated through the binding of drugs or other haptenic groups to self molecules, as well as through molecular modifications via gene mutations[49]. A study on monocytes from SLE patients indicated impairment in the removal of 8-oxodG formed due to oxidation by reactive oxygen species (ROS) in the DNA[68]. It has been reported that anti-DNA antibodies found in SLE demonstrate enhanced recognition of ROS-modified DNA[69].



Shlomchik, M. J. *et. al.* Nature Reviews Immunology, 2001, 1, 147.

Figure I: (a) Although normal T cells exposed to self-antigen in the periphery become tolerized, lupus-prone T cells are sensitive to lower thresholds of activation by agonist or weak-agonist peptides. **(b)** Once activated, T cells provide primary stimulation to genetically hyper-responsive B cells. **(c)** These auto-antigen-stimulated B cells undergo somatic hypermutation and affinity maturation. **(d)** On the synthesis of pathogenic auto-antibodies, tissue damage results in the release of self-antigen, **(e)**, **(f)** which is taken up and presented by specific antigen-presenting B cells in a second round of T-cell activation, **(g)** leading to a positive-feedback cycle. **(h)** Autoimmune T-and B-cell responses are diversified, which results in epitope spreading. This continuing and cyclic process of B cell-T cell cognate interaction serves to amplify the ensuing autoimmune processes. **(i)** Activated T cells can also directly cause tissue pathology by migrating to the target organ and releasing cytokines and by mediating direct cytotoxicity. T cells are shown orange; B cells are red.

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Defects in apoptosis and apoptotic cell uptake

Under normal circumstances, apoptotic cells are efficiently engulfed by macrophages in the early stages of the apoptotic cascade, the process being anti-inflammatory in nature[70]. Several lines of evidence indicate that defects in apoptosis and/or clearance mechanisms are pre-disposing factors for lupus. Reduced clearance of apoptotic bodies is observed in lupus patients, an effect attributed to intrinsic phagocytic defects[71,72]. In addition, lupus patients demonstrate a higher percentage of apoptotic lymphocytes in circulation[73] and higher rates of apoptosis are observed in short-term *in vitro* lymphocyte cultures, leading to increased release of nucleosomal material[74].

C1q deficient mice develop lupus-like symptoms and demonstrate a higher preponderance of apoptotic bodies in the glomeruli, indicating either increased apoptosis and/or inefficient clearance of apoptotic bodies[71]. Consequent to excess apoptosis and impaired clearance, post-apoptotic necrosis occurs, leading to modification of molecules present in the apoptotic bodies and the creation of neo-epitopes[70]. Proteolytic cleavage of these neo-antigens may lead to the exposure of cryptic epitopes, breakdown of tolerance, causing the spread of immune responses to other auto-antigens[75].

In animals treated with clodronate liposomes to induce the apoptosis of macrophages, the kinetics of the appearance of proteinuria and severe glomerulonephritis were enhanced[76]. These animals also had higher levels of total IgG levels. Somewhat paradoxically, defects in the apoptotic cascade due to the loss-of-function mutations in the *fas* gene or the *gld* gene (which encodes for Fas-ligand) also results in a lupus-like syndrome in mice and humans.

Role of hormones

It has been established that host genetics and the environment both contribute towards pathological sequelae. As mentioned earlier, the incidence of disease is the highest in women in the reproductive age group, leading to the hypothesis that lupus onset and progression may, at least in part, be influenced by the hormonal milieu.

SLE male patients have significantly higher levels of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). In addition, some subjects also demonstrate lower

testosterone levels[77]. Female lupus patients exhibit decreased levels of other androgens (androstenedione, dehydroepiandrosterone and dehydroepiandrosterone sulfate). Significantly, the lowest levels of these hormones were found in female patients with active disease[78]. Abnormal estrogen metabolism (conversion of estrogen to 16α hydroxylated estrogen) has also been reported; 16α hydroxylated estrogen metabolites are the more potent and feminizing estrogens[79]. An increase in the aromatase activity has also been observed, which would contribute to the imbalance between androgen and estrogen levels, since aromatase is involved in the synthesis of estrogen; higher aromatase levels are directly correlated to plasma levels of estrogen[80]. Thus, excess estrogenic activity and low androgenic activity might contribute to disease pathology, a hypothesis strengthened by the fact that disease "flares" occur during periods of high circulating estrogen, for example during pregnancy and after exogenous administration of the steroid[81].

Prolactin levels were also found to be higher in the non-stimulated peripheral blood mononuclear cells (PBMCs) from lupus patients when compared with the healthy controls[82].

Environmental factors

Amongst the environmental factors that might be responsible for the initiation of the disease are the infectious agents (as discussed earlier), dietary supplements such as alfalfa sprouts which contain L-canavanine[37], drugs such as procainamide and hydralazine and isoniazid in individuals that are slow acetylators[83,84] and exposure to ultraviolet (UV) rays. It was observed that exposure of keratinocytes to UV-B (but not UV-A) led to the binding of antibodies present in the sera lupus patient sera to the surface of cells; monospecific anti-Ro and anti-La antibodies also bound to such cells, implying exposure of lupus-related auto-antigens[85].

A single environmental factor is not thought sufficient to cause disease. Evidence indicates that a combination of factors, coupled with a susceptible host genotype, may be responsible. A number of murine models of lupus exist. Extensive work using mice which express a lupus-like phenotype has helped further delineation of disease pathology and genetics.

Mouse Models

Several animal models of SLE exist, and have proved invaluable in investigations of disease processes. Autoimmune-prone mice are particularly relevant, since 99% of the mouse genome is homologous to the human genome[86] and most of the immunological abnormalities apparently fundamental to the human disease are also operative in the mouse[87]. A mouse chromosomal region containing a disease-susceptibility locus will generally have a syntenic region in the human genome[88]. Three basic types of animal models have been used in autoimmune research:

1. Spontaneous models
2. Induced models
3. Genetically engineered models

The third category has been further divided into transgenic and knockout mice[89].

Spontaneous Models

These models are produced fortuitously; some were first described more than thirty years ago. For example, the chance crossing between New Zealand Black (NZB) female and New Zealand White (NZW) male mice produced one of the best-studied murine models for lupus-(NZB x NZW) F1[90] and the related New Zealand Mixed (NZM) 2410 congenic recombinant strain. Neither the NZB nor the NZW parents develop severe lupus-like symptoms. Other well-characterized models include MRL$^{lpr/lpr}$ and BXSB mice[91]. In (NZB x NZW) F1, the onset of autoimmunity involves multiple genes. In MRL$^{lpr/lpr}$ and BXSB mice, immune dysfunction appears to be mediated by single-gene defects, though background genes may contribute to the ultimate phenotype; the former carry a mutation in the Fas gene, and the latter contain the Y-linked *Yaa* gene (discussed in detail later)[88]. These genes were identified by chance; they were inherited across generations in a simple Mendelian fashion and affected apoptosis in mice with lymphoproliferation (for MRL$^{lpr/lpr}$) or were linked with sexual dichotomy (for *Yaa*). Although the specific features of disease vary among these models, all are characterized by the development of high titres of IgG auto-antibodies to nuclear antigens, including dsDNA. Mice suffer from glomerulonephritis and extra-renal manifestations such as thymic atrophy, splenomegaly, hemolytic anemia, vasculitis and arthritis[91].

(a) (NZB x W) F1: This was the first lupus model to be developed. NZB and NZW were derived independently in New Zealand in 1950s; NZB animals were initially noted to develop hemolytic anemia[92]. NZW were first described as being disease-free; glomerulonephritis was documented in females in subsequent studies, in the absence of increased mortality[90]. In 1963, F1 hybrids between these two strains were reported, which developed renal pathology very similar to that seen in human lupus. Disease shows a strong female bias, and the kinetics of the development of auto-antibodies mimics that seen in human lupus; end-organ failure due to tissue deposition of antigen-antibody complex is frequently seen. Recent studies have shown that more than twenty loci are involved in the development of disease[93]. Glomerulonephritis first occurs at four-five months, along with proteinuria. F1 mice are heterozygous at *H2* locus ($H2^{d/z}$), inherited from NZB ($H2^{d}$) female and NZW ($H2^{z}$) male and it is believed heterozygosity is associated with auto-antibody production[94] and increased frequency of glomerulonephritis[95]. Genes from both the parents contribute to the development of disease[94,96]. Various disease susceptibility loci have been identified; *sle1, sle2, sle3, sle5* and *sle6*. Four suppressor loci have also been mapped - *sles1*, *sles2* and *sles3* and *sles4*. These loci appear to interact with each other as well as with other loci in an epistatic fashion to contribute towards final disease outcome[4,97,98].

The NZM strain was derived in 1980 by the breeding between NZB/W F1 hybrid female with an NZW male. Due to recombination of the ancestral gene, a litter of mixed coat colour was developed. Some of the descendents succumbed to early renal failure. A number of lines were generated, such as NZM 2410, NZM 64 and NZM 2758, with NZM 2410 being closest in terms of disease symptoms to human lupus - animals develop nephritis, proteinuria, and immune complex deposition in the brain, along with other associated pathologies[99].

(b) $MRL^{lpr/lpr}$: *lpr* was identified as a spontaneous mutation of the Fas (CD95) molecule and *gld* (Generalized Lymphoproliferation Disease) a mutation in the Fas ligand (FasL) in mice. Fas and FasL belong to tumor necrosis factor (TNF) and TNF-receptor (TNF-R) receptor family respectively. Active FasL exists as a homotrimerized complex which, on binding membrane-bound Fas, causing the latter's oligomerization; apoptotic death of Fas-bearing cells follows[100]. Homozygosity of either the *lpr* and *gld* mutations results in lupus-like disease, as well as in the accumulation of $CD4^{-}CD8^{-}$ $CD3^{+}$ T cells[101]. The autoimmune phenotype is most apparent when

the Fas mutation is on an MRL background, which is an admixture of LG/J (75%), AKR/J (12.6%), C3H (12.1%) and C57BL/6 (0.3%)[89]. In 1967, a similar mutation was described in a human patient, leading to disease now known as autoimmune lymphoproliferative syndrome (ALPS)[100]. Self-reactive T and B cells, arising due to defects in the apoptosis pathway caused by these mutations, are thought to contribute to disease[102]. Though "background" genes are clearly important (for example, MHC haplotypes which help in the shaping autoimmune T cell repertoire), such genes by themselves are insufficient to cause lupus, as they can be shared by non-autoimmune individuals[103]. The Fas mutation has been mapped to chromosome nineteen, and arises due to the insertion of a transposable element between exons two and three, causing aberrant RNA splicing and premature termination of *Fas* gene transcription[104]. Aberrant *FasL* expression, on the other hand, is associated with normal transcription of the *gld* gene, but the lack of a functional *FasL* product due to a single amino acid change at the C-terminal portion of the molecule[103]. Defects in functional *lpr* and *gld* protein expression should be considered accelerators; though mutations on an otherwise normal (non-autoimmune) background do induce the generation of auto-antibodies, minimal histopathology is observed. In addition, aging $MRL^{+/+}$ mice demonstrate the presence of auto-antibodies as well. Other genes that might be implicated in the pathogenesis associated with lupus in MRL/*lpr* are now being slowly unraveled; experiments with chemokine receptor *Ccr2* deficient MRL/lpr mice show that these animals developed less adenopathy and demonstrate a lower percentage of CD4+ and CD8+ peripheral T cells[105]. Recent reports have implicated various loci such as *Lprm4* on chromosome five, *Ldrm1* on chromosome seven and *Asm* on chromosome ten[106].

(c) BXSB: These mice are derived from a cross between a C57BL/6J ($H\text{-}2^b$) female and a SB/Le ($H\text{-}2^b$) male[88]. They have a mutation in the *Y-linked autoimmune acceleration* (*Yaa*) gene that is responsible for the accelerated onset of lupus-like disease[107]. Since males develop disease earlier than females, it was hypothesized that the autoimmune-enhancing phenotype was being encoded by the Y chromosome derived from the SB/Le strain[108]. It has been shown that the genetic lesion underlying *Yaa* is an X to Y translocation of a telomeric region of more than 1 Mb, resulting in the duplication of around sixteen genes, including the gene for *Tlr7*[109], which has been implicated in the development of lupus. Addition of *Yaa* gene to other autoimmune backgrounds by cross-

breeding results in worsening of disease. The detailed molecular mechanisms related to disease onset are still under study.

Induced Models

These models helped further elucidate various aspects of lupus pathogenesis.

(a) Peptide-induced models: Injection of dsDNA (a major auto-antigen) does not result in auto-antibody production[110]. In an effort to identify a peptide surrogate of dsDNA, panning of a random phage display library using a murine anti-dsDNA antibody identified the peptide DWEYSVWLSN. Immunization of non-autoimmune BALB/c mice with this peptide resulted in the generation of high titres of IgG antibodies (mainly IgG1) against dsDNA. Auto-antibodies were also generated against histones and cardiolipin. In addition, increased immunoglobulin deposition was observed in renal glomeruli[111]. When peptides aa26-40 and aa56-70 of the auto-antigen snRNP D protein were used to immunize normal mice, strong autoimmune T cell and B cell responses were observed against other snRNPs[112]. The auto-antibodies also demonstrated anti-nuclear reactivity. Interestingly, no autoimmune responses were observed when the whole protein was used for immunization, indicating crypticity of the peptides. Similar responses were also seen with Ro60 peptides. When $Ro60_{316-335}$ peptide was used for immunization, immune responses were seen against other regions of Ro60 and also against other ribonucleoproteins such as La, SmD, 70-KDa U1RNP and the Golgi apparatus, which could not be absorbed by the immunizing peptide[113]. Peptide PPPGMRPP (which is repeated four times in Sm B/B`, and is a major auto-antigenic epitope in human patients) when immunized in a number of mice strains, resulted in the development of high titers of anti-Sm antibodies, with epitope spreading observed to other regions of Sm B/B` and Sm D, and the appearance of anti-nuclear antibodies as well[114].

(b) Pristane-induced autoimmunity: Pristane (2, 6, 10, 14-tetramethylpentadecane), when injected intraperitoneally in non-autoimmune BALB/c mice, induced auto-antibodies characteristic of lupus, such as IgM anti-ssDNA antibodies and anti-histone antibodies. However, no anti-dsDNA antibodies were observed. Injected animals also demonstrated anti-Sm responses, in conjunction with significant proteinuria, supporting the idea that the lupus phenotype arises as a result of interplay between genetic and environmental factors[115]. In SJL mice injected with pristane, auto-

antibodies were generated against ribosomal protein P and animals went on to exhibit extensive glomerulonephritis[116].

(c) Idiotypy: A lupus-like syndrome resulted when a human monoclonal anti-dsDNA antibody (bearing the "public" 16/6 idiotype, discussed previously) was immunized in non-autoimmune C3H.SW female mice; high levels of anti-16/6 and anti-anti-16/6 antibodies were observed, with concomitant increase in anti-DNA antibodies. Elevated titres of auto-antibodies reacting with the ssDNA and ribonucleoprotein auto-antigens Sm, Ro and La, as well as with cardiolipin were observed. Increased leucopenia and proteinuria were also noted. Additionally, immune complexes of IgG and IgM that were deposited in the kidneys contained the 16/6 idiotype[110]. Immunizations with 4B4 (an anti-Sm human monoclonal antibody) led to the production of antibodies against dsDNA, ssDNA, Sm and the mouse Fc fragment, though no antibodies could be detected against Ro and La. No inflammatory changes were observed in kidneys[117]. In another study, immunization with peptides derived from the variable region of the heavy chain of the anti-DNA antibody Id540 and A6.1 were found to increase the survival, delay the development of nephritis and anti-double stranded antibodies[118]. Immunization of an apoptotic cell-specific antibody resulted in the generation of anti-idiotypic antibodies exhibiting reactivity to antigens different from that of the immunizing auto-antibody, further indicating that the idiotypic network could be potentially be involved in antigen spreading and disease progression[67].

Genetically engineered models

These models are used for the investigation of molecules of potential relevance to lupus progression and pathogenesis; genes of interest are either transgenically expressed or knocked out. Some of these are:

(a) B-Cell Lymphoma Protein-2 (Bcl-2): Bcl-2 is 24 KDa protein which promotes cell survival by the suppression of apoptosis. Bcl-2 transgenic mice, where expression is restricted to B lymphocytes, exhibit excess B cells, pre-B cells and plasma cells (attributed to their increased longevity) associated with heightened serum immunoglobulins. Plasma cells were detected in cultures even after 3 weeks; cells from non-transgenic strains died within 6 days. Animals had

amplified and protracted antibody responses and by one year of age, 60% of the animals exhibited anti-nuclear antibodies, glomerulonephritis, lymphadenopathy and myocardial infarction[119].

(b) FcγRIIB: This molecule acts as a negative regulator of immune-complex triggered activation, and functions *in vivo* to suppress autoimmunity by regulating B-cell function. Loss of this inhibitory receptor is a B-cell autonomous defect. These animals showed increase in the number of IgG^+ plasma cells leading to development of pathogenic IgG anti-DNA antibodies, autoimmune glomerulonephritis with C3 and antibody deposition in kidneys in a strain-specific manner[120]. Deletion of the gene in the C57BL/6 background resulted in elevated immunoglobulin responses to DNA, increased inflammatory response to immune complexes, glomerulonephritis and decreased survival[121]. In human lupus patients, the memory B cells have significantly lower surface expression of FcγRIIB[122].

(c) IFN-γR: Reports indicate that the production of anti-nuclear antibodies, glomerulonephritis and mortality is decreased by administration of neutralizing IFNγ antibodies[123] or soluble IFNγ receptor[124] in NZB/NZW F1 mice. IFNγR knockout animals also had lowered levels of pathogenic IgG2a and IgG3, in addition to suppressed IgM and IgG1, less Ig and C3 deposition in kidneys, and an absence of renal failure[125]. In a contrasting report, another group demonstrated that IFN-γR deficient $MRL^{lpr/lpr}$ mice, which expressed colony stimulating factor-1 (CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF) in tubular epithelial cells, had four to five fold higher macrophage accumulation in the kidneys due to more rapid proliferation and decreased apoptosis, when compared with IFN-γR intact mice. There was also increased migration of dual negative ($CD4^-$, $CD8^-$) T cells[126].

(d) TNF-R1: Fas and TNF receptor-1 (TNF-R1) belong to the same family of genes[127]. *Tnfr1* receptor knockout mice do not develop autoimmunity. However, when these mice were crossed with mice of the $MRL^{lpr/lpr}$ strain, F1 animals exhibited a five-ten fold increase in lymph node weight. The presence of auto-antibodies was observed; early-onset autoimmune disease accompanied by lesions in the kidney, lungs, liver and knee joints was associated with high mortality[128].

(e) TGF-β: Mice deficient in TGF-β exhibit massive lymphocytic and monocytic infiltration in the lungs, salivary glands, liver and the heart. Serum antibodies against dsDNA, ssDNA, Sm ribonuclear protein, collagen type I and collagen type V were present and Ig glomerular deposits were observed. There were no antibodies to the Ro and La ribonucleoproteins. Auto-antibodies to dsDNA and Sm were predominantly of the IgG isotype. These results support the concept that absence of suppressor cytokines can lead to systemic autoimmune disease[129].

(f) C1q: Homozygous deficiency of the first component of complement pathway in humans is very strongly associated with the development of SLE[130]. As discussed earlier, C1q has been shown to bind to apoptotic cells and promote their clearance, and homozygous deficiency in mice leads to the generation of anti-nuclear and anti-histone antibodies, immune complex deposition in kidneys, the presence of multiple apoptotic bodies in diseased glomeruli, and increased mortality[71]. Crossing of $C1q^{-/-}$ and $MRL^{+/+}$ animals (which have an intact *Fas* gene) resulted in increased disease in F1 animal in comparison with $MRL^{+/+}$ animals; higher titers anti-nuclear antibodies (ANA) and anti-ssDNA antibodies were observed, along with kidney failure and proteinuria. Prevalence of glomerulonephritis was higher in females than in males[131]. It has recently been shown that C1q deficiency increases the positive selection of auto-reactive B1b cells leading to the generation of high titres of IgM auto-antibodies directed against intracellular antigens[132].

(g) C4: C4 deficiency in mice lead to impaired antibody production. However, by five-six months of age, more than half of the animals demonstrated significant titres of ANA, with higher reactivity observed towards ssDNA than dsDNA. Glomerular pathology was observed at a higher incidence in females, with the demonstrated presence of IgG3 and C3 in the mesangia[133]. Animals demonstrate elevated levels of circulating IgM-dsDNA complexes. Deposition of apoptotic dsDNA in splenic marginal zones is believed to induce secretion of IFN-α by $CD11b^{+}$ cells[134]. C4 deficiency in conjunction with *Fas* deficiency results in significantly higher cervical lymph node mass on the C57BL/6 background; such animals also exhibited heightened glomerulonephritis, with increased deposition of IgG in the kidneys and ANA titer[135].

(h) c-Mer: The membrane tyrosine kinase domain of c-Mer helps in uptake of apoptotic cells. The animals lacking the kinase domain demonstrate low in-vivo clearance of exogenously administered apoptotic cells. There is a progressively increased level of auto-antibodies to chromatin and animals spontaneously develop auto-antibodies to IgG, chromatin ssDNA, dsDNA and display features of moderate renal pathology. Autoimmunity is not accompanied by polyclonal B cell activation in these animals[136].

(i) Lyn: Lyn is a protein kinase of the Src family expressed in B cells, monocytes and macrophages. It is involved in B cell receptor (BCR) mediated signaling. *Lyn*$^{-/-}$ deficient mice have reduced numbers of circulating lymphocytes and smaller or undetectable Peyer's patches but paradoxically also suffered from splenomegaly and enlarged lymph nodes, in addition to having high concentrations of IgM and IgA in sera. Conversely, the numbers of Mac^{+} positive cells (macrophages, neutrophils, NK cells and activated CD8^{+} cells) were increased in knockout animals, both in the spleen and lymph nodes. High titres of anti-dsDNA antibodies were observed, most of which were mediated by IgM[137]. IgG immune complex deposition in kidneys was associated with glomerulonephritis[138,139].

(j) BAFF: B cell activation factor belonging to TNF family (BAFF) is a 285 amino acid transmembrane protein of TNF ligand super-family. It is cleaved on the cell surface to form a biologically active 17 KDa molecule and is involved in suppression of B cell apoptosis. Increased levels of circulating BAFF is observed in SLE patients. Over-expression of BAFF in mice was shown to lead to defects in B cell tolerance and SLE-like autoimmunity associated with increased antibody production to dsDNA, ssDNA and rheumatoid factor. The total serum levels of IgM, IgG2c, IgG2b, and IgG3 were significantly increased, leading to antibody deposition in the kidneys[140]. In *baff*$^{-/-}$ mice generated upon the NZM 2328 background, the number of splenic B cells and CD4^{+} T cells were reduced by more than 90% and 70% respectively. The number of circulating mature B cells was reduced (though immature B cells numbers remained unaltered), resulting in a reduction in the level of circulating IgG. At six to seven months, kidney deposition of IgG was greatly reduced and glomerular C3 was barely discernible[141].

(k) Dnase1: Dnase1 is a major nuclease, involved in the degradation of DNA at sites of high cell turnover. Dnase1 deficient mice develop symptoms of severe lupus (including high ANA titres and glomerulonephritis) and die at six to eight months. Animals expressed high levels of antibodies against nucleosomes, ssDNA and dsDNA, in addition to antibodies to ribosomal protein P and histones. IgG and complement deposition was also evident in the kidneys[142].

(l) PD-1: PD-1 is a transmembrane protein belonging to the immunoglobulin super-family and contains the immunoreceptor tyrosine-based inhibitory motif (ITIM). It has been found to be strongly induced in lymphocytes following activation. Animals lacking the PD-1 gene develop mild proliferative glomerulonephritis at six months, significant deposition of IgG3 and IgM the glomeruli. By fourteen months, significant glomerulonephritis and arthritis is observed. PD-1 deficient animals additionally harboring the lpr/lpr genotype exhibit accelerated onset of disease.[143].

(m) Serum Amyloid Protein (SAP): SAP is a highly conserved plasma protein that shows specific, calcium-dependent binding to DNA and chromatin; it displaces H1-type histones, thereby solubilizing native chromatin. Binding reduces inter-nucleosomal cleavage; in the absence of SAP, DNA is rapidly cleaved. SAP deficient mice spontaneously produced high titres of antibodies against chromatin, DNA and histones. Female mice suffer from a high incidence of severe proliferative glomerulonephritis, immune deposition and mortality[144].

(n) Secretory IgM: Mice in which B cells lack the ability to secrete IgM develop lupus-like disease. These mice at four to six months demonstrate higher lymphoid organ mass and total cell numbers. This was accompanied by nephritis with significant cellular infiltrates comprising mainly of T cells[145]

Cytokines

From the human and animal studies mentioned above, as well as from other reports, it is clear that cytokines play key roles in the pathogenesis of disease[146]. Altered levels of IL-1, IL-4, IL-6[146], IL-2[147], IL-10[148], IL-12[149], IFN-α[150], TNF-α[151,152], IFN-γ[153] have been associated with lupus; alterations in type I interferons seem particularly significant[148].

Overproduction of IL-1 has been linked to pathogenicity in SLE. In Il-1$\beta^{-/-}$ BALB/c animals, when lupus was induced by immunization with 16/6 idiotype, lower levels of anti-dsDNA antibodies and diminished disease was observed. Knockout animals showed lower levels of IL-2, IL-4, IL-1, IFN-γ and TNF-α secretion[154]. IL-1ß inhibition has been shown to suppress the spontaneous production of IgG from PBMCs from SLE patients. When PBMCs from healthy individuals are cultured with sera obtained from SLE patients, an upregulation of IL-1ß mRNA is observed[148,155].

Apparently paradoxically, neutralization of IL-2 by anti-IL-2 antibody results in development of autoimmunity characterized by autoimmune hemolytic anemia, gastritis, splenomegaly, lymphadenopathy and multi-organ lymphocyte infiltration[156]. Treatment reduces the numbers of FoxP3 expressing regulatory T cells; the resulting disease can be reversed by adoptive transfer of $CD25^+$ $CD4^+$ FoxP3 cells. Decreased *in vitro* secretion of IL-2 by $CD4^+$ cells from SLE patients is often seen and correlates with high IgG levels in the serum[157]. The hyposecretion of IL-2 can be reversed by resting T cells from the SLE patients for two-three days in culture before stimulation with mitogen[158]. This correlates with the higher concentration of cAMP response element modulator (CREM) in the nuclei of the T lymphocytes from the SLE patients. CREM, which normally binds to the -180 site of *IL-2* promoter, leads to decreased promoter activity and thus transcriptional repression of *IL-2* [159]. When the normal T cells were treated with the sera from SLE patients, there was increase in the concentration of CREM mRNA and protein level. In another study, blocking of IL-2 mediated signal in the mice lacking *FoxP3* (and thus the regulatory T cells) partially inhibited the lymphoproliferation and autoimmunity associated with the lack of regulatory T cells and resulted in prolonged life span versus the *FoxP3* null animals[160].

IL-4, when constitutively expressed as a transgene in B cells, completely prevented the nephritis and mortality associated with lupus, probably by lowering of the levels of pathogenic IgG2a and IgG3 isotypes and increasing IgG1; levels of the glycoprotein (gp)70-anti-gp70 complex were also reduced[161]. However, contradictory reports suggests that IL-4 knockout animals, while expressing reduced titers of IgG1 (with no changes in IgG2a and IgG2b) demonstrated reduced renal and salivary gland disease and decreased lymphadenopathy[153].

Lupus mouse models as well as SLE patients have high serum concentration of IL-6[162,163]. Reports suggest that the IL-6 receptor is constitutively present on B cells from SLE patients, in contrast to B cells from healthy controls[164]. Elevated levels of IL-6 were found in the cerebrospinal fluid (CSF) from patients with neuropsychiatric lupus erythematosus (NPSLE)[165,166]. Oligodeoxynucleotide CpG, derived from sequences found in circulating DNA from SLE patients, caused enhancement of IL-6 and IL-8 mRNA from the endothelial cells *in vitro*[167]. Treatment of $MRL^{lpr/lpr}$ animals with anti-IL-6 antibodies markedly decreased glomerulonephritis and caused a temporary reduction in anti-dsDNA antibodies[168].

IL-8 is a chemokine that appears to be a useful marker for central nervous system involvement in lupus; levels decrease during remission[166]. Some reports suggest that the presence of IL-8 can be used for diagnosis of NPSLE[165].

Serum levels of IL-10 too have been found to be elevated in SLE patients, probably due to increased secretion by monocytes[168]. The levels of IL-10 significantly correlate with disease severity; higher levels of the cytokine are seen in murine lupus as well[169]. High IL-10 results in increased apoptosis in $CD4^+$ cells, an effect blocked by anti-IL-10 antibodies[170]. Monocytes from the SLE patients had decreased IL-12 production[171]; levels of IL-12 go up during remission[168].

Plasma levels of IL-17 and IL-23 were higher in patients suffering from lupus than the healthy controls and there was a positive correlation with the disease severity. The *ex-vivo* induction of PBMCs from SLE patients by IL-23 could significantly induce and augment the release of IL-17[172].

Patients with active SLE have high concentration of circulating TNF-α in circulation and in kidney biopsies[163,168]; levels drop down to normal during periods of remission. *Fas* deficient animals also exhibit high levels of TNF-α[128]. Surprisingly, treatment of NZB/W F1 animals with TNF-α, when started at 14 weeks, resulted in disease amelioration, whereas such a beneficial effect was not seen when treatment was initiated at twenty seven weeks[173]. The cytokine may thus have contrasting effects, depending on the stage of disease.

Interferon-α appears to be a key cytokine in SLE pathogenesis as high levels of this cytokine are frequently encountered. When IFN-α is administered therapeutically for other conditions, auto-antibodies typical of SLE appear[174]. Elevated levels of the cytokine are also observed in C4 knockout mice[134]. The presence of IFN-α in young mice before any signs of disease suggests that the activation of IFN-α system in SLE is a prerequisite rather than a result of disease. Upon microarray analysis of the PBMCs from SLE patients, it was observed that fifteen genes were up-regulated, out of which fourteen were targets of IFN-α. Analysis of PBMCs from a patient in remission demonstrated no significant differences in gene expression from controls[175], indicating relevance of the observations to active disease. Anti-dsDNA and DNA-containing immune complexes and/or apoptotic bodies have been shown to induce the generation IFN-α from plasmacytoid dendritic cells, resulting in the maturation of monocytes to dendritic cells. Such phenomena may constitute major contributing factors in lupus pathology[176].

Genetic involvement

SLE rarely occurs as a single gene defect. It is a polygenic trait in which a number of MHC and non-MHC genes interact with environmental factors to create varied disease phenotypes. More than fifty loci have been found to affect the susceptibility to murine lupus. These loci are dispersed on chromosomes one, four, seven, ten and seventeen[177].

In genetic mapping studies in MRL$^{lpr/lpr}$ mice, four loci were found to have varying degrees of influence on lupus predisposition[178]. *Lmb1* congenic animals had enlarged spleen and lymph nodes. *Lmb2* and *Lmb4* loci caused slightly larger lymph nodes but enhanced proliferation in the spleen was not observed. *Lmb4* appeared to be involved in the induction of glomerulonephritis at the late stages of disease, in addition to aiding in lymphoproliferation. *Lmb3,* which was mapped to a 0.9 Mb interval[179] of the chromosome seven, is believed to have a major influence on lymphoproliferation and the induction of anti-chromatin antibodies.

Other important loci are *sle1*, *sle2*, *sle3* on chromosomes one, four and seven respectively. *Sle1* by itself is sufficient to generate high-titre antibody response to dsDNA, chromatin and histones (H2A/H2B/DNA complex). It is strongly associated with glomerulonephritis and induces a high serum concentration of IgG. The T cells demonstrate proliferative responses to histones in *sle1*

congenic animals[180]. When this locus was further divided into 4 loci- *sle1a*, *sle1b*, *sle1c*, *sle1d*, it was found that *sle1a* and *sle1b* are the most potent loci, each being capable of mediating fatal lupus when combined with *yaa* or *lpr*. There was a significant increase in the levels mRNA and protein of IL-6 in these animals[181].

Sle2 is associated with B cell hyperactivity, polyclonal B cell activation and an increase in the B-1a subset of B cells (with a phenotype of IgM^+ $B220^{low}$ $CD5^+$ $CD23^-$ $CD43^+$ $CD11b^+$)[182]. These cells are the major source of serum IgM and positive selection by auto-antigens plays an important role in their development. The expansion in the B-1a compartment is due to increased proliferation, decreased apoptosis and increased cell production by the fetal liver. This locus is further subdivided into *Sle2a, Sle2b* and *Sle2c*. Even though *Sle2c* contributes to an increase in B-1a cells, it does not appear to accelerate lupus pathogenesis. *Sle2a* and *Sle2b* have been shown to increase the lymphocytic expansion and kidney pathology. A combination of *Sle2a* with *Sle1* and *Sle3* resulted in significantly higher proteinuria than when only *Sle1* and *Sle3* where present[183].

Sle3 is responsible for increased serum levels of IgM and IgG antibodies, causing immune complex-mediated glomerulonephritis, lymphadenopathy and anti-dsDNA antibody production. Expression of CD69 on T cells increased with age, indicating an activated phenotype. On stimulation with anti-CD3 and anti-CD28, T cells from congenic animals showed significantly enhanced proliferation, accompanied by a reduction in the apoptosis of activated T cells[184]. A number of genes identified as contributing to lupus susceptibility in mouse models lie in close vicinity of this locus.

Other loci such as *Sbw1* and *Sbw2* also contribute to splenomegaly, glomerulonephritis and mortality[185].

In humans, no associations have been found with the MHC I locus, though there is evidence implicating the MHC II region. DR-B1 alleles, DR2 and DR3 have been consistently found to be associated with disease in European-Caucasian lupus patients and DR and DQ alleles show a stronger association with the presence of auto-antibodies like anti-Ro and anti-La[4]. In African-American patients, HLA-DR2 and HLA-DR7 have been associated with SLE[88].

Therapies

The first therapies, initiated in the 1950s, consisted of corticosteroids, immunosuppressive agents like cyclosporin and anti-malarial medicines. Hydroxychloroquine for the skin and joint manifestations has since then been successfully used. It works by interfering with antigen processing, inhibiting phagocytosis and neutrophil migration. Corticosteroids have anti-inflammatory and immunosuppressive effects, but their use is usually associated with multiple side-effects, necessitating the use of other medications. The efficacy of the combination of cyclophosphamide and prednisolone has been reported. Additionally, intravenous immunoglobulins (IVIG) have also been successfully used[186]. Drugs such as mycophenolate mofetil, which suppresses T and B cell proliferation, have been shown to be efficacious in the lupus mouse models and in human patients, and fewer side effects have been reported[187].

B cells are known to play a key role in lupus. Thus, it was hypothesized that B cell removal would lead to lupus amelioration. Rituximab is a chimeric monoclonal antibody reagent consisting of human IgG1 and kappa constant regions from a murine hybridoma directed at human CD20. Treatment with this molecule results in almost complete depletion of B cells from the periphery, an effect that lasts for more than 6 months[188]. Within one week of treatment, Rituximab causes down-regulation of co-stimulatory molecules CD40 and CD80 on the $CD19^+$ B cells. Rituximab does not however, lower the numbers of plasma cells, as they do not express CD20. Even after B cell numbers in the circulation return to normal levels after treatment, patients remain in remission[189].

Hemoglobin

Hemoglobin (Hb) is the most extensively studied protein in vertebrates. It is always enclosed the red blood cells, and its major function is to transport oxygen throughout the body. The quaternary structure of Hb is composed of two alpha and two beta subunits which are very similar to each other. Both subunits have high structural homology to myoglobin, a monomeric oxygen-binding protein present in the muscles. Each subunit of Hb is associated with heme (which is protoporphyrin IX) with an iron atom in the centre (Figure II a); it is the presence of heme that gives Hb its red colour. Normally, the iron atom is always in the ferrous oxidation state, whether it is bound to oxygen or not. The heme moiety is present in other proteins as well, such as catalase, the cytochromes and peroxidase. Heme has a planar structure. Fe^{2+} binds to the four porphyrin

rings through coordinate bonds (Figure II b). The fifth coordination bond which is below the plane, is to the histidine side chain of the globin moiety of the protein. When heme is bound to oxygen, it forms the sixth coordination bond above the plane.

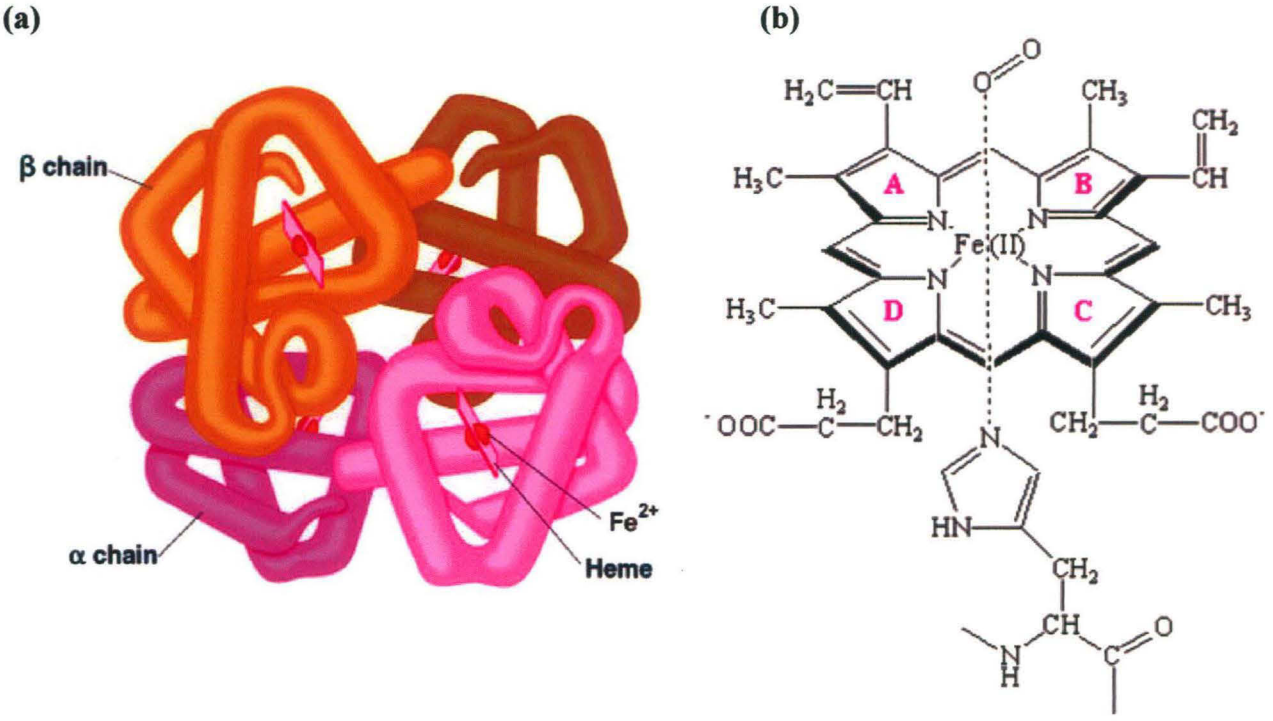


Figure II: a) Hemoglobin structure. **b)** Planar structure of heme, showing 4 coordination bonds to nitrogen (N) of four porphyrin rings (labeled A to D) and one to nitrogen (N) of histidine side chain of the protein (below the plane). The sixth coordination bond is to the oxygen molecule that it carries.

In RBCs, a small percentage of Hb is always oxidized from the ferrous (Fe^{2+}) form to ferric (Fe^{3+}) form, resulting in the formation of methemoglobin (metHb). MetHb cannot bind oxygen as it is octahedrally coordinated to a water molecule. Erythrocytes contain methemoglobin reductase which functions to reduce metHb[190,191].

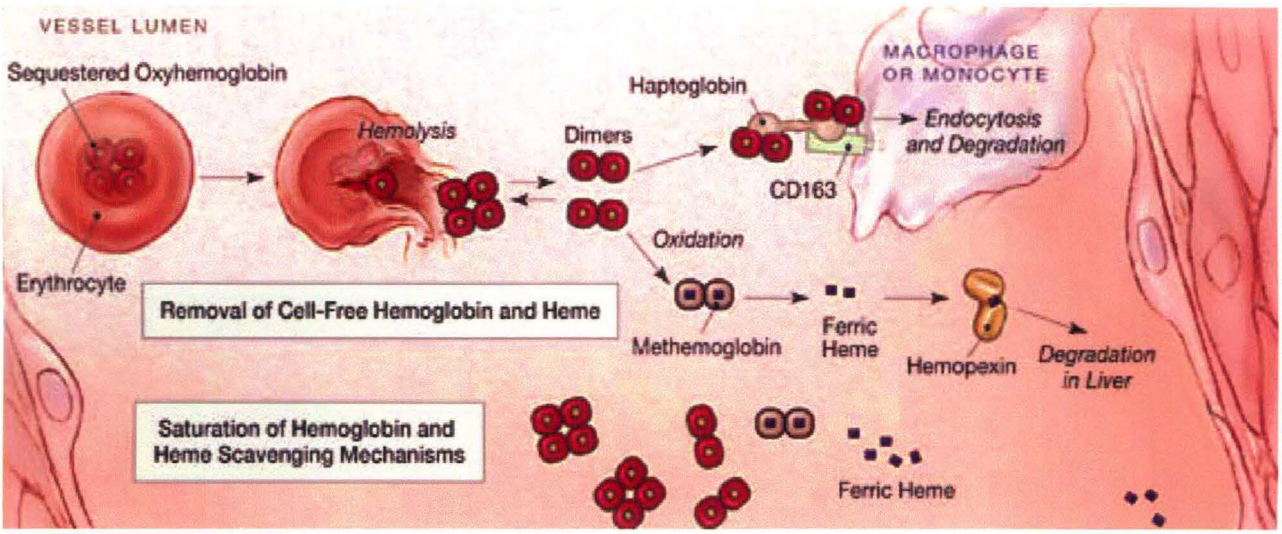
When Hb is released in the plasma due to intravascular lysis of RBCs, the quaternary structure breaks down into dimers[192] which bind Haptoglobin (Hp), an acute phase protein[193]. Hp is a serum glycoprotein that binds Hb by a strong, irreversible, non-covalent bond with dissociation constant being 10^{-15} moles/litre[194]. It is synthesized as a single polypeptide that is cleaved proteolytically

into a smaller alpha chain of around 10 KDa and longer beta chain of mass around 39 KDa. These then connect through disulphide bonds[195]. Binding of Hb to Hp results in stabilization of the former, preventing the release of toxic heme in the circulation (see below).

Formation of Hb-Hp complex exposes a neo-epitope, permitting high affinity binding to the CD163 molecule, a scavenger receptor, with a molecular mass of 130 KDa[196]. The Hb-Hp complex is taken up and degraded, mainly in the liver and the spleen; recent evidence indicates the presence of CD163 in circulating monocytes as well[197]. Binding is believed to induce intracellular signaling and mediate anti-inflammatory responses. Soluble CD163, formed upon proteolytic cleavage under inflammatory conditions has been found to be high in the patients suffering from rheumatoid arthritis[198]. CD163 also acts as immuno-modulator, because clearance of Hb results in the formation of carbon monoxide which is anti-inflammatory (see below). Additionally, CD163 binding results in increased secretion of IL-6 and IL-10[196].

If, upon release into circulation, Hb is not bound by Hp (under disease conditions discussed below), free heme is released. Heme binds to hemopexin (Hx) with a very high binding capacity (K_d being less than 1 pM). The Heme-Hx complex is then degraded in the liver. Uncomplexed heme can intercalate into the lipid membranes of cells as it is very lipophillic[199]. Intracellularly, heme is broken down to iron, biliverdin and carbon monoxide via the action of heme-oxygenase I. Ferritin synthesis is also up-modulated; the protein serves to sequester free iron and so protects the cell from the ROS-mediated damage that may result due its presence. Ferritin is multimeric protein composed of twenty four subunits of two types – a heavy chain and a light chain, and demonstrates a very high binding capacity for iron (4500 moles of iron per mole of Ferritin)[199, 200].

Under conditions of extensive RBC lysis as malaria, sickle cell anemia and autoimmune hemolytic anemia, Hp levels decrease below detectable levels. Low levels of unconjugated hemopexin levels have also been reported[192,201,202,203]. Excessive hemolysis therefore saturates and overwhelms these Hb removal systems and leads to build up of Hb (to concentrations in the range of 20-150 μM)[204] and heme in plasma[192] (Figure III).



Rother, R. P. *et al.* J. Am. Med. Ass. 2005, 293, 1653.

Figure III: The fate of free Hb in blood

Ferrous Hb has very high affinity for Nitrous Oxide (NO), which acts as a muscle relaxant; removal of this gas by free Hb causes vascular constriction[205] and endothelial dysfunction such as disruption of smooth muscle tone and platelet activation[192]. By virtue of direct contact with the bloodstream, endothelial cells are the first target of Hb-mediated damage[206]. Hb induces caspase-mediated apoptosis in cultured endothelial cells[207]. Additionally, free Hb disrupts the endothelial cell integrity via oxidative modification of the plasma membrane[208].

These observations may be of special relevance to lupus. As indicated above, free Hb in circulation (arising because of circulating anti-RBC antibodies) rapidly breaks down to dimers[209] which could be oxidized in the presence of ROS[192] (a condition that characterizes the inflammatory environment seen in SLE[210,211]). The oxidized dimers then break down to release free heme, and globin (as shown in Figure III). As indicated, free heme is extremely toxic as it can easily enter the cells because of its lipophillic nature. In cells, the release of free iron causes oxidative damage by generation of other ROS by Fenton reaction[212]; lipid peroxidation may also occur[213]. Hb dimers and heme are also avidly taken up by the kidneys resulting in nephrotoxicity[211,214].

Hb causes brain injury, both by itself and its degradation products; brain edema results within twenty-four hrs of exposure. Hb acts by inhibiting the Na^+/K^+ adenosine triphosphate activity,

causes lipid peroxidation[215], brain swelling, increase in brain water content, excitotoxic death of neurons in the cortex and induces depolarization of hippocampal neurons[216]. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-libeling (TUNEL)-positive neurons and astrocytes are found around the area of hemorrhage in the brain[217]. When primary neurons are exposed to Hb, time and dose dependent cytotoxicity is observed.

Some evidence of the antigenicity and immunogenicity of Hb exists. Despite the high degree of homology in the amino acid sequences of Hb of different species, xeno-immunization leads to the production of specific antibodies. Five major B cell epitopes have shown to be present on both alpha and beta subunits of human Hb[218,219]. Antibody reactivity against human Hb has been reported in patients of sickle cell anemia, possibly related to repeated transfusion[220]; antibodies target the mutation site. Aberrant T cell reactivity to the Hb-Hp complex has been reported in the NZB animals that have extensive hemolytic anemia. Autologous RBC antigens appeared to be a stimulus for $CD4^+$ T cell proliferative responses[221].

Materials and Methods

MATERIALS AND METHODS

Tissue Culture

Cell cultures were maintained at 37^0C in 5% CO_2 in a humidified incubator. Murine cells were grown in DMEM (GIBCO-BRL) and human cell lines were cultured in RPMI (GIBCO-BRL). In both cases, medium was supplemented with 10% FBS (Biological Industries) and 1% antibiotic-antimycotic cocktail (GIBCO). HBMEC (Human Brain Microvascular Endothelial cells) cells were cultured in RPMI media additionally supplemented with 10% Nu serum (Becton Dickinson), 1% non-essential amino acid solution (GIBCO) and 1mM sodium pyruvate (SIGMA).

In subsequent descriptions, while "complete" medium refers to supplemented medium described above, "incomplete" medium refers to medium without FCS.

For subculture, adherent cells were "flushed" using a pipette or were trypsinized using Trypsin-EDTA (GIBCO) and washed once by centrifugation at 400 g at 4^0C for 5 mins. Cells were then transferred to new flasks at an appropriate density. At different times, cells were cryopreserved after resuspension in mouse or human freezing medium (Appendix) at a density of 2-20 million cells per vial. Cells were held at -70^0C for one day before being transferred to a liquid nitrogen container for long term storage.

For recovery of cryo-preserved cells, the cryovials were "quick-thawed", and cells were layered on cold, incomplete medium. The cells were centrifuged at 400 g at 4^0C for 5 mins and the supernatant discarded. After two additional washes under the same conditions, cells were dispensed in 24-well plates in complete medium.

Animals

The animals were housed at the centralized animal facility at NII. The following mouse strains were employed in this study: NZB/W F1, C57BL/$6^{lpr/lpr}$, BALB/c, and NZB/W F1 x BALB/c. All procedures were approved by the Institutional Ethics Committee.

Human Sera

Sera from SLE patients were obtained from the Department of Medicine of All India Institute of Medical Sciences, New Delhi, India. Sera from malaria patients were kindly gifted by Dr. Ashis Das, Birla Institute of Technology and Science, Pilani and the sera from patients suffering from leishmaniasis were a kind gift from Prof. Rentala Madhubala, Jawaharlal Nehru University, New Delhi. Autoimmune sera of defined specificity were obtained from the Center for Disease Control and Prevention, Atlanta. Control sera were collected from healthy volunteers. The blood was collected in heparinized tubes and centrifuged at 3220 g at 4^0C for 10 mins. Plasma was diluted 1:10 in PBS (Appendix), and aliquots were stored at -70^0C.

Mouse sera

400 µl of blood was collected via the retro-orbital vein using capillaries. The blood was kept at room temperature to allow clot formation. The clot was disturbed and the tubes centrifuged at 3220 g for 10 mins at 4^0C in swinging bucket rotor. Serum was withdrawn, diluted 1:10 in PBS and stored at -70^0C.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood was collected in heparinized tubes, diluted two-fold in complete DMEM and transferred to a 50 ml Falcon tube. At room temperature, an equal volume of Ficoll (Amersham) was gently layered below the blood. The suspension was the centrifuged at 400 g at 25^0C for 20 mins. The interface, comprising agranular mononuclear cells, was collected and washed three times with complete DMEM to remove traces of Ficoll.

Monoclonal Antibody Generation

Human Hybridoma

Human lymphoblastoid cell lines (previously generated in the laboratory by Epstein Barr Virus mediated transformation of B cells obtained from SLE patients in the midst of a disease "flare") were thawed after extraction from liquid nitrogen storage and cultured in complete RPMI. Cell lines secreting antibodies to human Hb were harvested and washed twice with incomplete medium by centrifugation at 400 g at 4^0C for 5 mins. Cell viability

was determined by Trypan Blue (Sigma) exclusion. K_6H_6/B_5 (a mouse-human heteromyeloma) cells were similarly processed. The two cell types were mixed (K_6H_6/B_5: lymphoblastoid cells::2:1) and centrifuged. 1 ml of a solution of 38% Polyethylene Glycol (PEG; molecular weight 1600 Daltons; Sigma) made in incomplete media was then added onto the cell pellet over a 30 sec period. The cell suspension was gently mixed for 30 secs and an incubation was carried out for a further 30 secs. 5 ml of incomplete medium was then added over a period of 90 secs, followed by an additional 5 ml. The cell suspension was gently agitated for 5 mins and then centrifuged at 400 g at 20^0C for 5 mins. Cells were washed twice under the same conditions to remove PEG and resuspended in complete medium containing 0.1 mM hypoxanthine (Sigma) and 0.016 mM thymidine (Sigma). The cells were counted and plated at cell density of 3000-5000 cells/well. One day later, cultures were supplemented with 0.0004 mM aminopterin (Sigma) and 0.001 mM Ouabain (Sigma). Two weeks subsequently, cells were transferred to complete medium supplemented with HT for a fortnight, and henceforth cultured in complete medium.

At appropriate times, cells from independent cultures were transferred to 24-well plates and then to 25 cm^2 flasks. Cells were cryopreserved as described above and supernatants stored for further analysis.

Mouse Hybridoma

Old (ten to twelve months old) NZB/W F1 animals were euthanized under sterile conditions. The spleens were excised, briefly suspended in incomplete medium, and then teased between autoclaved frosted slides. A single cell suspension was made by gently passing the suspension through a syringe attached to a 26G needle. The cells were centrifuged at 400 g at 4^0C for 5 mins. Red blood cells (RBCs) were lysed by hypotonic shock: Nine ml of distilled water was added to the splenocyte pellet. Five secs later, 1 ml of 10X PBS was added to restore osmolarity. SP2/O cells were harvested by centrifugation as described above. Cells were mixed (Splenocyte: SP2/O:: 4:1) and fusion was carried out as described above, except that a 50% solution of PEG was employed. Subsequently, cells were resuspended in complete supplemented with HAT (100μM

Hypoxanthine, 400 nM Aminopterin, 16 μM Thymidine) and plated at a density of 50,000 cells/well. Two weeks subsequently, cells were transferred to complete medium supplemented with HT for a fortnight and henceforth cultured in complete medium.

At appropriate times, cells from independent cultures were transferred to 24-well plates and then to 25 cm^2 flasks. Cells were cryopreserved as described and supernatants stored for further analysis.

Subcloning

Supernatants derived from independent murine or human hybridoma lines were tested for reactivity to Hb by ELISA (as described below). Cells secreting antibodies of interest were quick thawed after extraction from liquid nitrogen storage. Cells were then cultured at appropriate density, and the resulting supernatant re-assessed for the presence of antibodies to Hb. The number of viable cells was determined by Trypan Blue exclusion. Viable cells were dispensed at concentrations of 5, 2.5, 1.25 and 0.625 cells/well in a 96-well tissue culture plate. As cells began to multiply, wells containing single clones were visually identified and supernatants from such wells were screened for the presence of anti-Hb antibodies. Cultures containing cells secreting antibodies of interest were "expanded" into 6-well plates or 25cm^2 flasks. Each cloned cell line was sub-cloned at least two additional times in a similar fashion to ensure monoclonality. Cells were cryopreserved multiple times during these procedures.

Generation of ascites

NZB/W F1 x BALB/c (F1) animals, which mimicked the genotype of the hybridoma cells, were employed for this purpose. Animals were administered intra-peritoneal injections of 500 μl of Incomplete Fruend's Adjuvant (DIFCO). One week later, each animal received an intra-peritoneal injection of 20 million murine hybridoma cells resuspended in incomplete medium. When appropriate, ascites fluid was "tapped" using an 18G needle under sterile conditions. The fluid was centrifuged at 400g at 4^0C for 5 mins to precipitate cells and debris, and the supernatant was aliquoted and stored at -70^0C. In some instances, cells derived from the fluid were cryopreserved as described above.

Hemoglobin Purification and Characterization

Purification

Hb was purified by protocol modified from Antonini *et. al.*[222] 10 ml of blood was collected from a human volunteer in a heparinized tube. The blood was mixed with 30 ml cold PBS and a centrifugation was carried out at 3220 g at 4^0C for 15 mins. The supernatant was discarded and the RBCs were gently washed three times with cold PBS by centrifugation under the same conditions. Cells were then resuspended in 30 ml of water and incubation was carried out at room temperature for 20 mins, causing the RBCs to lyse. The lysate was dialyzed extensively against chilled PBS and centrifuged at 1575 g for 45 mins at 4^0C in a fixed angle rotor. The supernatant was loaded onto the CM-52 (Whatman) column, previously calibrated with 10 mM Phosphate Buffer, pH 6.5. After Hb "entered" the column, a pH gradient of pH6.5 to pH8.5 and salt gradient of 10 mM to 15 mM was applied. Fractions containing Hb were pooled and concentrated using concentrators (30KDa cut-off) by centrifugation at 1800 g at 4^0C for 15 mins. Hb was quantified at 540 nm: An O.D. of 0.88 corresponded to a concentration of 1.0 mg/ml. Human Hb, in the form of metHb, was obtained from Sigma.

An analogous procedure was followed for the purification of murine hemoglobin from NZB/W F1 animals.

HPLC, N-terminal Sequencing And Mass Spectrometry

Hb was dissolved in 0.1% TFA and "loaded" onto an analytical C-19 reverse phase column. A gradient was created using two solutions - 0.1% TFA (Solution A, the aqueous phase) and acetonitrile containing 0.1% TFA (Solution B, the organic phase). The gradient ranged from 5 to 90% (Solution B in Solution A) over a period of 130 mins. Protein present in "peak" fractions was characterized by mass spectrometry and its identity confirmed by N-terminal sequencing at in-house facilities at NII.

Generation of Methemoglobin (metHb)

An equimolar concentration of H_2O_2 was added to Hb previously dissolved in PBS. At different times, oxidation of ferrous (Fe^{2+}) Hb to ferric (Fe^{3+}) Hb (or metHb) was assessed spectrophotometrically. As expected, ferrous Hb displayed three absorbance maxima – 410 nm (referred to as the Soret Band, a characteristic of all heme-containing proteins), 540 nm and 577 nm. When the Hb was oxidized to the ferric form, these latter maxima decreased and new maxima at 623 nm was observed. After analysis, a buffer exchange was carried out against PBS using Amicon concentrators (Millipore) with a molecular weight cut-off of 30 KDa.

Fluorescence Activated Cell Scanning (FACS)

$2x10^5$ cells were dispensed per well in 96-well round-bottomed plates and washed three times with FACS Buffer (Appendix) by repeated low-speed centrifugation (400g, 4°C, 5 min). For permeabilisation, the cells were previously incubated in Permeabilisation Buffer (Appendix) for 90 secs and then washed with FACS buffer. The cells were then incubated with 100 µl of primary antibody at appropriate dilution at 4^0C for one hr. Cells incubated with FACS buffer comprised a negative control. After three washes with FACS buffer, 20 µl of appropriately diluted Fluoroscein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch) was dispensed. Cells incubated with FACS buffer comprised another negative control. After another incubation at 4^0C for one hr, cells were washed three times with FACS buffer and then re-suspended in 0.2% paraformaldehyde (Sigma) at 4^0C. Data was acquired on a LSR flowcytometer (Becton-Dickenson).

Immunofluorescence and confocal microscopy

The cells were dispensed into 24-well plates 12 hrs prior to the experiment; seeding densities varied with cell type. The cells were then repeatedly "rinsed" with FACS buffer. In parallel wells, cells were permeabilised as described above, and then "rinsed" with FACS buffer. Primary antibodies were dispensed, and an incubation was carried out at 4^0C for 1 hr. Following three "rinses" with FACS buffer, appropriately diluted FITC-conjugated secondary antibody was added and another incubation carried out at 4^0C for 1

hr. The cells were then rinsed with FACS buffer and then fixed with 0.2% paraformaldehyde. Digital images were acquired on a Nikon or an Olympus microscope. For confocal microscopy, cells were dispensed onto sterile cover slips placed in 6-well tissue culture plates. Twelve hrs later, staining procedures were carried out as described above. Cover slips were then glycerol-mounted on slides in an inverted orientation. Digital images were acquired on an Olympus microscope.

Induction of Apoptosis

Cells were incubated in complete DMEM medium supplemented with 5 μM camptothecin. At different times post-apoptosis induction, cells were incubated with primary and secondary antibodies described above. Subsequently, cells were transferred to Annexin Binding Buffer (Appendix). The prescribed quantity of fluorochrome-conjugated Annexin-V was added and an incubation carried out at 4^0C for 20 mins. Alternatively, Propidium Iodide (PI) was added immediately prior to analysis. Two-colour flow cytometery was then carried out.

Enzyme Linked Immunosorbant Assay (ELISA)

Direct-binding ELISA on proteins

0.5 μg of respective proteins in Carbonate Buffer (Appendix) were dispensed into each well of an ELISA plate and an incubation carried out at 4^0C for 16 hrs. After rinsing the wells with PBST (Appendix), vacant sites were "blocked" by dispensing a solution of 5% BSA (Sigma) and an incubation for 2 hrs at 37^0C. After three rinses with PBST, primary antibodies, diluted in PBST supplemented with 5% BSA, were dispensed into the wells. For each antibody, non-antigen coated wells served as negative controls; positive controls consisted of commercially procured antibodies against the specific proteins. Following an incubation at 37^0C for 2 hrs, wells were rinsed five times with PBST at two minute incubation intervals. 100 μl of Horse Radish Peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch), appropriately diluted in PBST supplemented with 5% BSA, was added and another incubation carried at 37^0C for 2 hrs. After further rinses as above, 100 μl of Ortho-Phenylene Diamine (OPD) substrate (Sigma) or 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate (BD Biosciences) solution was added to each well.

The enzymatic reaction was stopped by the addition of 50 µl of 2N H_2SO_4 (Merck). Absorbance was determined at either 490 nm or at 450 nm (for OPD and TMB substrates respectively) on a BioTek ELISA reader.

Direct-binding ELISA on Hb peptides

Contiguous peptides representing mHb alpha (seven peptides) and mHb beta (eight peptides) were commercially procured. Peptide lengths varied from seven to twenty-two amino acids. Hb peptides were dissolved in water or a minimum quantity of DMSO as appropriate. A concentration of 2 µg peptide per well was employed; other assay conditions were as described above.

Competition ELISA

Assays were designed to investigate whether the anti-Hb monoclonal antibodies generated during the course of this study bound sites on Hb which over-lapped with those recognized by the Hb-binding protein Haptoglobin (Hp). ELISA wells were individually adsorbed with 0.5 µg Hb or 1 µg Hp under conditions described above. Wells were 'blocked" by incubation with 0.5% poly-vinyl alcohol (PVA) for 2 hrs at 37°C. After subsequent rinses with PBST, 2 µg Hp was added to wells containing Hb. Primary antibody (anti-Hb monoclonal antibodies, or anti-Hp polyclonal antibodies) and secondary antibody incubations were carried out as mentioned previously.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

Cell Lysate

Cells were harvested, counted and washed three times by low speed centrifugation with PBS to remove FCS-associated moieties. 100 µl of TKM Buffer (Appendix) was added per 5 million cells and in incubation carried out at 4^0C for 40 mins. The cells were then snap-frozen in liquid nitrogen and quick-thawed for three consecutive cycles. The lysate was centrifuged at 10,500 g for 10 mins at 4^0C. The supernatant was aliquoted and stored at -70^0C.

Protein Estimation

Protein content in the lysate was estimated by the BCA method (Pierce). BSA was employed as the standard and dilutions of the protein were made in TKM buffer. Data was subjected to linear regression, and protein concentrations were determined by comparisons with a standard curve.

Electrophoresis

Resolving gel and stacking gels (Appendix) were cast and allowed to polymerize at room temperature. Sample Buffer (Appendix) was added to the samples (either cell lysate procured as described above, or purified proteins), and the mixture boiled for 10 min. Samples were then centrifuged at maximum speed in a microfuge in order to precipitate debris. The supernatant was transferred into wells of the gel and eletrophoresed at 50V; once the sample dye entered the resolving gel, the voltage was increased to 100V. Upon completion of the run, the gel was processed for visualization of the resolved protein moieties (by Coomassie Blue or Silver Staining) or for Western blot.

Silver Staining

The gel was incubated in fixative (Appendix) for 16 hrs at room temperature, as it was gently agitated. It then underwent two 15-min incubations in 50% ethanol (Fluka), followed by a 5 min incubation in sodium thiosulphate (Appendix) (Sigma). The gel was then washed by three 15-min incubations in water. An incubation was then carried out in a solution of silver nitrate (Appendix) (Sigma) for 20 mins in the dark. The gel was washed with three changes of water and was then immersed in developer till protein bands became visible. The reaction was terminated using 1% acetic acid (Merck).

Western Transfer

The gel was immersed in the Transfer Buffer (Appendix). Nitrocellulose membrane (mdi), cut to the same size as the gel, was soaked in Transfer Buffer. The nitrocellulose paper was then placed on top of the gel, taking care to exclude air bubbles. After appropriate padding on both sides with Whatmann paper and supportive sponges, the sandwich was loaded into the transfer cassette, which was then lowered into the transfer chamber

containing Transfer Buffer, taking care to ensure that the gel faced the anode. Transfer of proteins to the nitrocellulose membrane occurred upon application of a current of at 60 mA for 16 hrs at 4^0C or at 250 mA for 2-3 hrs at 4^0C.

Immunoblot

The efficiency of transfer was assessed by visualization of the protein bands with Ponceau S stain (Appendix) (Sigma). The proteins were destained with PBS, and the vacant sites on the membrane were "blocked" by incubation in PBS containing 5% BSA for 2 hrs at room temperature. The nitrocellulose was washed by three ten-minute incubations in PBST at room temperature, accompanied by gentle agitation. Primary antibodies were diluted in 5% BSA in PBST and an incubation carried out at room temperature for 2 hrs. Extensive washing was the carried out with PBST to remove unbound antibodies. Appropriately diluted secondary antibody was then added, and another incubation carried out for 2 hrs at room temperature. After further washes, reactive bands were visualized on X-ray film using enhanced chemiluminescence (ECL; Biological Industries).

Two Dimensional SDS-PAGE

The cells were lysed in M2 Lysis Buffer (Appendix). The solution was centrifuged at 10,500 g for 10 mins at 4^0C. The protein in the supernatant was quantified by BCA as described above. To a solution of 200 µg of protein in 200 µl PBS, 50 µl of 10% SDS was added to achieve final concentration of 2% SDS. The sample was boiled for 5 mins and the tube contents mixed by external agitation. 1.2 ml of chilled acetone was then added. After intense mixing over the course of a minute, an incubation was carried out at -20^0C for 30 mins. Subsequently, centrifugation was carried out at 18,000 g for 20 mins at 4^0C. The precipitate was air-dried and resuspended in 260 µl of 2D Sample Buffer (Appendix). 2.6 µl of IPG Buffer (pH 3-10) (Appendix; Amersham) was added. Samples were dispensed into sample "coffins" (Amersham); taking care to ensure a uniform spread and absence of trapped air bubbles. A 13 cm strip (pH 3-10) (Amersham) was gently placed in the coffin, once again avoiding air bubbles, with the gel side of the strip facing the protein sample. The strip was then covered with 1 ml of mineral oil

(Amersham) to prevent evaporation. The coffin was placed on the multiphor (Amersham) with the positive side of the strip facing the positive side of the multiphor. Isoelectric focusing was carried out as per the following parameters:
Initial rehydration: 9 hrs; Rehydration: 50V for 5 hrs; 500V for 2 hrs; 1000V for 30 mins; 4000V for 1 hr, 5000V for 4 hrs.

After the run, the strip was removed, wiped free of oil and incubated with 5 ml Equilibration Buffer (Appendix) supplemented with 50 mg DTT (Sigma) for 15 mins. A second equilibration was carried out in 5 ml Equilibration Buffer supplemented with 125 mg iodoacetamide (Fluka) for 15 mins. Upon a cast 10% SDS-PAGE gel (without "stacking" gel), a small piece of Whatman paper containing the protein marker was inserted in between the two gel plates, such that it touched the surface of the gel. The strip was then overlaid on the top of the gel surface, taking care to avoid the entrapment of air bubbles between the resolving gel and the strip. The gel-strip contact was sealed using 1% agarose (Pronadisa) made in stacking gel buffer. Electrophoresis was carried out 40 mA. When the tracking dye reached the bottom, the gel was either processed for silver staining or for Western transfer, as described above.

Immunization

Eight week old NZB/W F1 animals were subcutaneously immunized with 50 μg mHb (Fe^{2+}) or mHb (Fe^{3+}) emulsified in Complete Fruend's Adjuvant (Difco). Two booster injections, with the respective antigen at the same dose emulsified in Incomplete Fruend's Adjuvant (Difco), were administered at fortnightly intervals. Control animals received adjuvant alone. Blood samples were collected prior to immunization and one week following each injection. Sera were stored at -20^{0}C.

Antibody Elution from Organs

Low pH elution of antibodies was done by protocol modified from Koffler *et. al.*[223] Mice (BALB/c, NZB/W F1) were sacrificed at different ages. Different organs were dissected and immersed in chilled PBS containing 20 μl protease inhibitor per ml. Organs were homogenized in an ice environment. The homogenate was washed once with PBS by

centrifugation at 12,800 g for 10 mins at 4^0C. The supernatant was discarded and the pellet resuspended in 0.02M citrate phosphate buffer, pH 3.2. An incubation was carried out at room temperature for 2 hrs with gentle agitation. The suspension was then centrifuged at 12,800 g at 4°C for 10 mins. The pH of the supernatant was then neutralized by the addition of 1N NaOH. A buffer exchange against PBS was then carried out using centrifugal concentrator, and the processed organ eluates were stored at -70^0C.

Cytokine Stimulation

Viable THP-1 cells (obtained after Ficoll density centrifugation) were dispensed at density of $2x10^5$ cells per well in a 96-well round bottomed plates. Hb, the individual anti-Hb monoclonal antibodies, or Hb plus the individual antibodies were added to the wells. Negative controls consisted of medium alone, or medium containing isotype matched, non-Hb reactive antibodies. Lipopolysaccharide (LPS, Sigma) was added as the positive control. Incubation was carried out for 16 hrs at 37^0C and 5% CO_2. Cells were then centrifuged at 400 g for 10 mins at 4°C and the supernatants collected. TNF-α, IL-6 and IL-8 levels were estimated by ELISA using OptEIA kits (Becton Dickinson).

Transmigration

Viable HBMEC cells (obtained after Ficoll density centrifugation) were dispensed at $2x10^5$ cells per well in a 24-well plate. Hb, the individual anti-Hb monoclonal antibodies, or Hb plus the individual antibodies were added to the wells. Negative controls consisted of medium alone, or medium containing isotype matched, non-Hb reactive antibodies. Incubation was carried for 16 hrs at 37°C and 5% CO_2. 800 µl of supernatant was then transferred to transwell "bottom" 24-well plates. Transwell cell culture inserts (8.0 µ pore size, Becton Dickinson) placed inside the wells, into which $2x10^5$ viable THP-1 cells were added. Incubation was carried out for 5 hrs at 37°C and 5% CO_2. Subsequently, the transwells were removed and cells in the bottom chambers were counted.

Cloning and Sequencing of Immunoglobulin Variable Regions

RNA Isolation

Hybridoma cells, isolated by repeatedly "flushing" medium along the sides of the tissue culture flask, were centrifuged at 400 g at 4°C for 10 mins. 1 ml Trizol reagent (Invitrogen) was added per $5x10^6$ cells and cells were lysed by repetitive pipetting. An incubation was carried out for 5 mins at room temperature. 0.2 ml of chloroform per ml Trizol was then added, followed by vigorous mixing for 15 secs and further incubation for 3 mins at room temperature. The samples were then centrifuged at 11,000 g at 4^0C for 15 mins. The aqueous phase was collected, to which 0.5 ml of isopropanol was added. The samples were incubated at room temperature for 10 mins and centrifuged at 12,000 g at 4^0C for 10 mins. The supernatant was discarded and the RNA pellet was washed with 75% ethanol, vortexed and centrifuged at 7500 g for 5 mins at 4^0C. The RNA pellet was partially air dried and dissolved in 50 µl Diethylpyrocarbonate (DEPC) treated water (Appendix). RNA was stored at -70^0C.

Generation of cDNA

To 12 µl of RNA, 15 µl of DEPC treated water and 2 µl of oligo dT primers (Promega) were added. The mix was incubated for 10 mins at 70^0C and was then chilled instantaneously. 1.5 µl RNAse inhibitor, 10µl 5X RT buffer, 2.5µl of 10 mM dNTP mix, 5µl of 100 mM DTT and 2.0µl AMV RT (Promega) were then added. Incubation was carried out at 45^0C for 1 hr followed by 70^0C for 5 mins. 1 µl of RNAse H (Promega) was then added and an incubation carried out at 37^0C for 30 mins.

Polymerase Chain Reaction (PCR)

All the reagents were obtained from Promega. Primers were obtained from Novagene. To 2.5 µl of cDNA, the reaction mix was added as mentioned in the Appendix. The final volume was made to 25 µl with water.

Following cycling parameters were used:

	95^0C	5 mins (Hot start)	
	94^0C	45 secs	25 cycles
	60^0C	45 secs	
	72^0C	1 min	
Final extension	72^0C	7 min	

Upon completion of the run, samples were stored at -20^0C.

Gel Elution

Electrophoreses was carried out on 1% agarose gel and the PCR product was visualized on a transilluminator upon ethidium bromide staining. The region of agarose surrounding the band was excised and DNA was purified using the eluted using the Qiagen gel elution kit. The eluted product was ligated using pGEMT ligation kit (Promega) into the pGEMT vector.

Generation of Competent Cells

5 ml LB broth (Himedia) was inoculated with *E.coli* DH5α strain and an incubation carried out at 37^0C for 16 hrs, with cultures agitated at 225 rpm. Growing cells were used to re-inoculate 100 ml of broth. The bacteria were allowed to grow till the O.D. reached 0.3-0.4. The cells were chilled on ice for 10 mins and then centrifuged at 3220 g for 10 mins at 4^0C and the supernatant discarded. The cells were resuspended in one-tenth the original volume (10 ml) of ice cold 100 mM $CaCl_2$ (which was freshly made and autoclaved) and incubated on ice for 30 mins. Cells were harvested by centrifugation at 3220 g for 10 mins at 4^0C and resuspended in 6.7 ml (one-fifteenth the volume of LB media) of a solution of 15% glycerol in 100mM $CaCl_2$. Cells were then aliquoted in pre-chilled autoclaved Eppendorf vials and stored at -70^0C.

Transformation

1-2 µl of ligated plasmid was added to 100 µl of competent cells and an incubation carried out for 30 mins on ice. Subsequently, cells were exposed to a heat shock of 42^0C for 2 mins. The cells were then immediately transferred to ice. 1 ml of LB media was

added and a static incubation carried out at 37^0C for 10 mins, and another for 30-50 mins with gentle agitation. The cells were then centrifuged at 2600 g for 15 mins. The supernatant was discarded except for the last 100 µl. Cells were spread on LB-ampicillin plates supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal). The plates were incubated at 37^0C for 12-16 hrs.

Plasmid Purification and Sequencing

White colonies were "picked" and inoculated into 5 ml of LB media. Cultures were incubated overnight. Plasmids were purified using the Qiagen plasmid purification kit. The presence of the cloned insert of appropriate size was verified after incubation of plasmid DNA with the restriction enzyme EcoRI and subsequent agarose electrophoresis. The plasmids were then sequenced at a commercial facility or at the in-house sequencing facility at NII. The analysis of the variable region was done using NCBI Ig BLAST.

Statistics

Statistical analysis was carried out using the Student's t-Test.

Results

RESULTS

Anti-Hb reactivity in human auto-reactive antibody responses

Anti-Hb reactivity of an apoptotic-cell specific human monoclonal antibody

A human monoclonal antibody (RN86) had previously been established in the laboratory[67]. Briefly, B cells from an SLE patient in the midst of a disease "flare" were transformed with Epstein Barr Virus to generate several hundred independent lymphoblastoid cells lines. Cells secreting antibodies specifically targeting apoptotic cells (a reactivity that formed the focus of the previous study) were "fused" with the heteromyeloma K_6H_6/B_5 and monoclonal antibodies were generated after repeated sub-cloning procedures. Upon the induction of drug-induced apoptosis, the antibody demonstrated preferential localization on cells also binding Annexin-V (Figure 1a). Upon the surface of apoptotic cells, the antibody co-localized with Propidium Iodide on putative apoptotic blebs (Figure 1b). During the course of characterization of this antibody, Western blots were carried out using lysates from different cell lineages as targets. Upon red blood cell (RBC) lysate, reactivity was observed towards a protein of molecular weight of approximately 64 KDa (Figure 1c). Given the molecular weight, and the fact that hemoglobin (Hb) comprises more than 90% of the protein in RBC[222,224], ELISA was carried out, using commercial human Hb. Results indicate that antibody RN86 was indeed reactive to human Hb (hHb; Figure 1d).

Anti-hHb reactivity in patients of SLE, Malaria and Leishmaniasis

Sera were obtained from SLE patients visiting the Rheumatology Clinic at the All India Institute of Medical Sciences, New Delhi. Care was taken to choose patients in the midst of a disease "flare". Control sera were obtained from healthy volunteers. A percentage of SLE sera demonstrated the presence of antibodies reactive to commercial hHb by ELISA; dose-dependent binding was observed (Figure 2a). Control sera were poorly reactive to hHb. Further confirmation of anti-hHb reactivity in SLE sera was obtained upon Western blots carried out using hHb as target. The negative control consisted of incubations of hHb-containing strips with enzyme-linked secondary antibody. Patients' sera demonstrated reactivity with hHb; reactive bands were observed corresponding to the

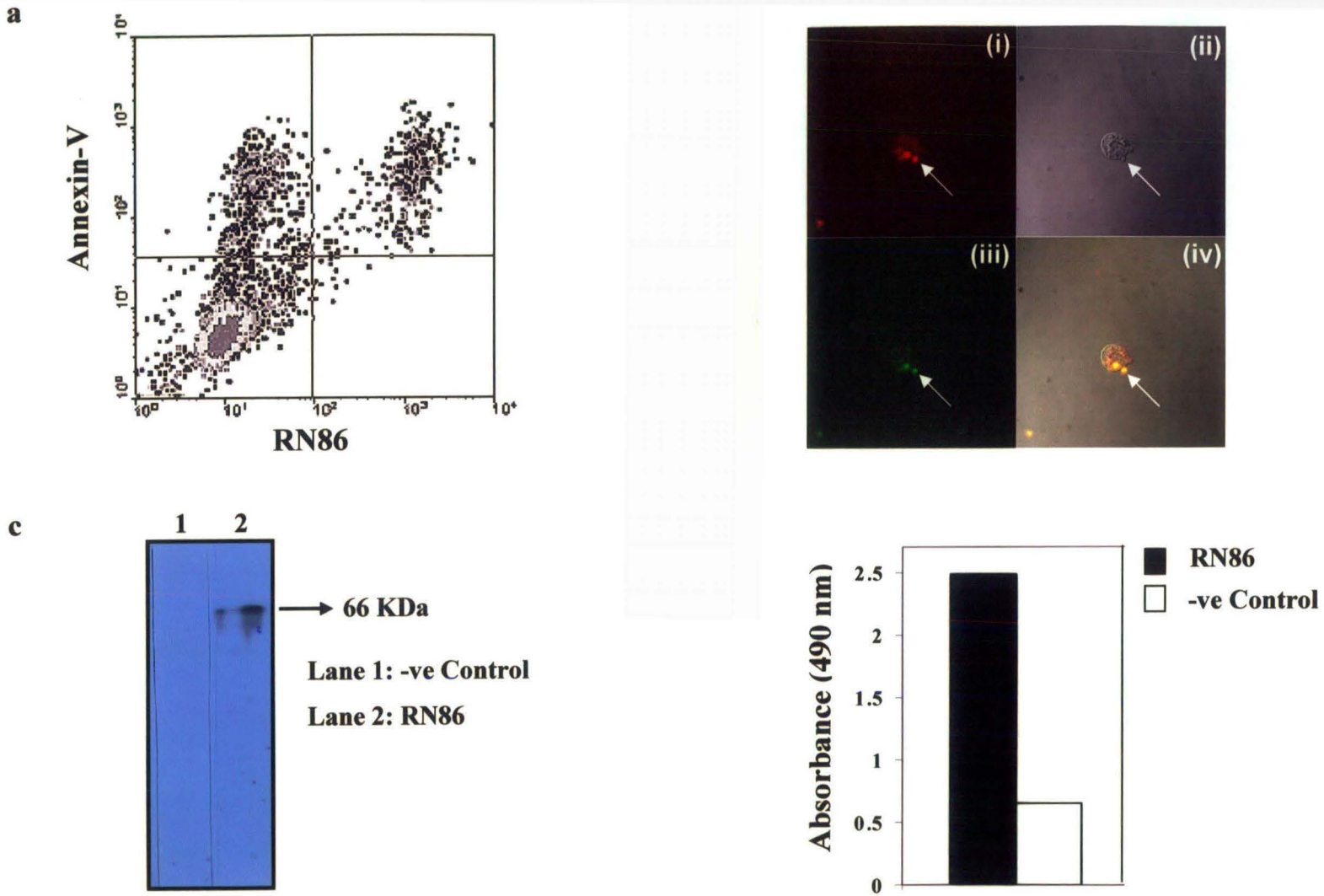


Figure 1: Characterization of RN86. (**a**) Reactivity towards Annexin-V-positive apoptotic cells (**b**) Co-localization with PI on apoptotic blebs (arrows). (i) PI (ii) Phase contrast (iii) RN86 (iv) Colocalization of PI and RN86. (**c**) Western blot on RBC cell lysate. –ve Control: Secondary antibody (**d**) ELISA on commercial human Hb. -ve Control: Secondary antibody.

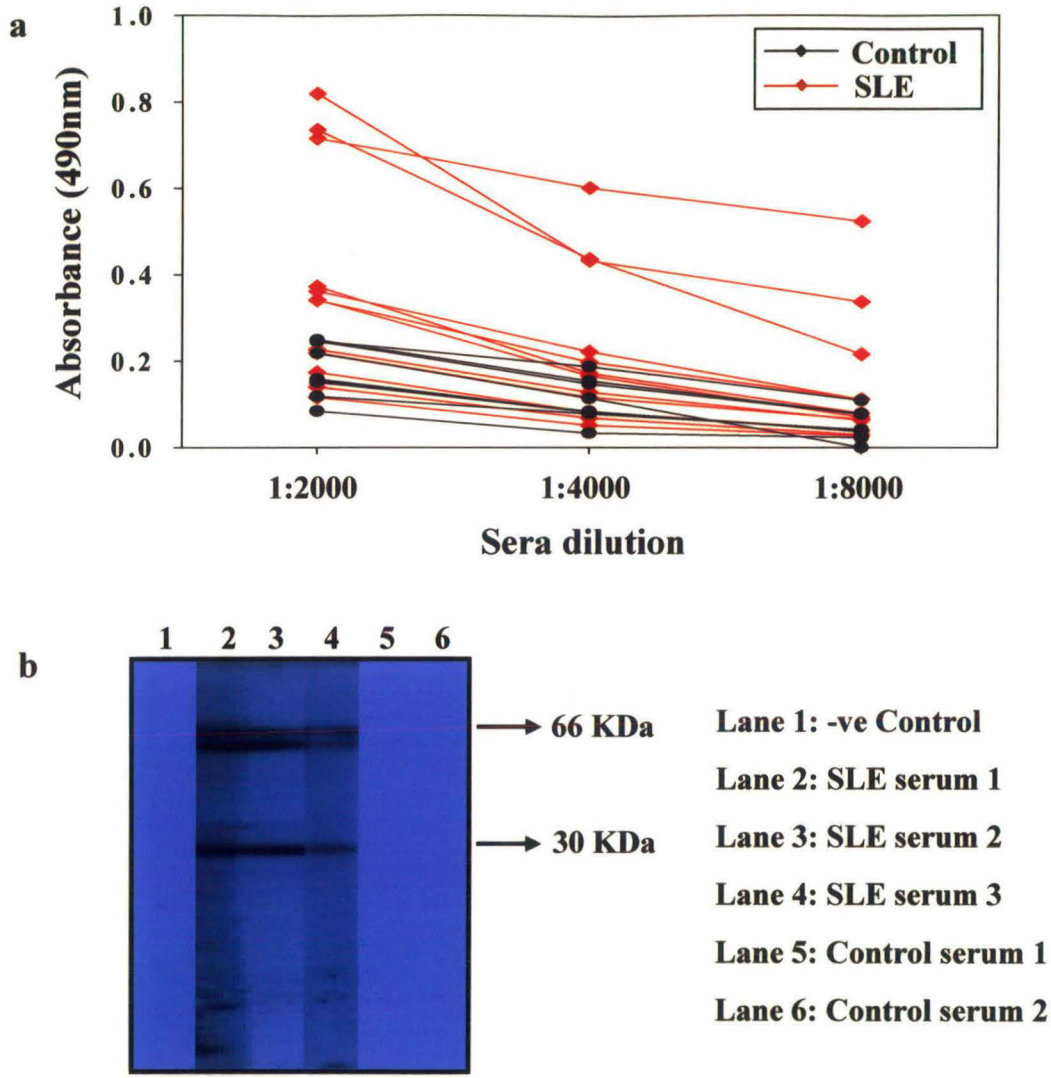


Figure 2: Anti-hHb reactivity in SLE patients. (**a**) Reactivity of sera from control subjects and SLE patients by ELISA. (**b**) Reactivity of sera from control subjects and SLE patients by Western blot. Bands seen with SLE sera correspond to hHb dimer (~30 KDa) and tetramer (~64 KDa). -ve Control: Secondary antibody.

hHb dimer (~30 KDa) and tetramer (~64 KDa). Control sera demonstrated no such reactivity (Figure 2b).

Studies were then extended to include a panel of auto-reactive sera obtained from the Center for Disease Control and Prevention (CDC), Atlanta, USA, which had been characterized for the presence of various autoimmune reactivities (ANA (3); La, U1-RNP; Sm; nucleolar; Ro; Scl70; Jo-1, Scl; Ribosomal-P). ELISA results showed that only sera designated as "anti-Sm" contained antibodies reactive to hHb (Figure 3a). The association of anti-hHb and anti-Sm reactivity was further confirmed on three individual sets of CDC sera obtained over three years (Figure 3b). Western blots on commercial hHb using these sera as probes further extended these findings; reactivity corresponding to hHb dimer was obtained only when anti-Sm serum was employed (Figure 3c).

In the light of these findings, sera from Indian SLE patients (along with sera from several controls) were simultaneously re-assessed for hHb and Sm reactivity; Figure 4a depicts hHb reactivity and Figure 4b Sm reactivity. While three sera contained antibodies reactive towards hHb, two of these sera (and no others) contained antibodies also binding Sm. Control sera from healthy individuals were essentially non-reactive towards both antigens. These studies indicate that anti-Sm reactivity was probably sufficient but not necessary for anti-hHb reactivity. The isotype of anti-hHb antibodies varied from subject to subject (Figure 4c); while antibodies in CDC sera were mainly of the IgG isotype, IgM antibodies were predominant in an Indian SLE patient (AN).

As in SLE, excessive apoptosis and hemolysis have also been reported in patients of malaria and leishmania[225]. Auto-antibodies are also a common occurrence in these diseases[226-232]. It was therefore of interest to determine if anti-hHb reactivity could be detected in these non-autoimmune pathologies as well. ELISAs were carried out using hHb as the target, employing sera obtained from patients of leishmaniasis and malaria (*Plasmodium vivax* or *Plasmodium falciparum*). Significantly higher reactivity to hHb was obtained in the sera of patients, in comparison with sera obtained from control subjects. As expected, sera from these patients did not contain antibodies reactive with

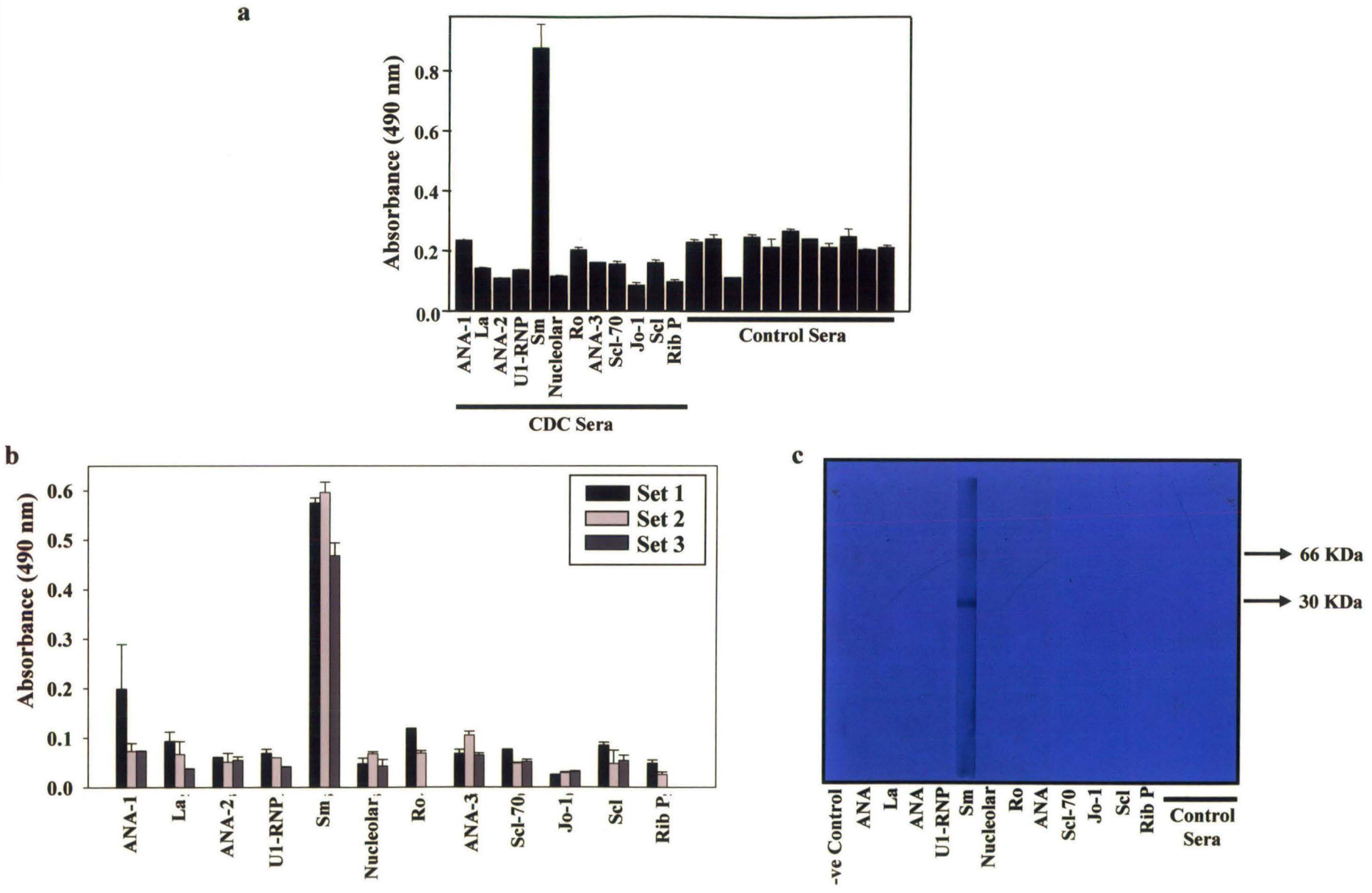


Figure 3: Reactivity towards hHb (**a**) Reactivity of CDC and control sera (**b**) Reactivity of three individual sets of CDC sera (**c**) Reactivity of CDC and control sera by Western blot. – ve Control: Secondary antibody.

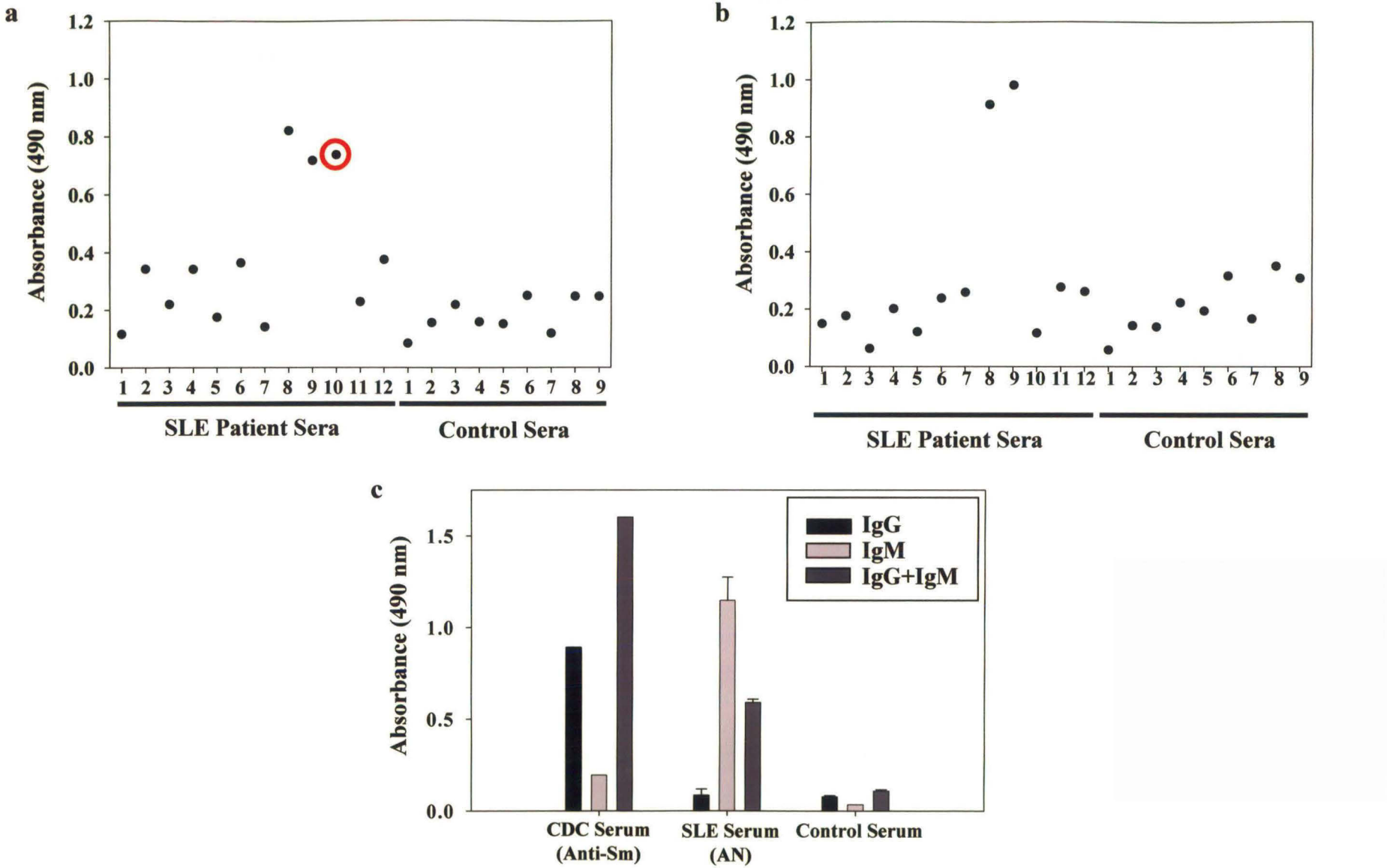


Figure 4: (a,b) Relationship between anti-hHb and anti-Sm reactivity. Reactivity of sera from control subjects and SLE patients to (**a**) hHb and (**b**) Sm. The red circle delineates serum reactive to hHb and not Sm. (**c**) Isoytpes of anti-hHb antibodies in CDC (anti-Sm) serum and serum from an Indian SLE patient (AN).

Sm (Figure 5a), a reactivity known to be specific for lupus, once more emphasizing that anti-Sm may not be a necessary criteria for anti-hHb reactivity. While anti-hHb antibodies in patients of leishmaniasis and *P. vivax* malaria were mainly of the IgG isotype, those of *P. falciparum* malaria expressed equivalent levels of the IgG and IgM anti-hHb isotypes (Figure 5b).

Anti-murine Hb auto-reactivity in the sera and organs of NZB/W F1 mice

The presence, characteristics and relevance of anti-Hb reactivity in NZB/W F1 mice were then investigated. These mice, which represent a spontaneous autoimmune murine model of lupus in which the disease course closely resembles that seen in the human disease, have contributed immensely in the elucidation of immunopathology[96].

Purification of murine Hb (mHb)

Blood was withdrawn from NZB/W F1 mice from the retro-orbital vein and collected in heparinized tubes. Purification of mHb was carried out by ion-exchange chromatography, as described in the Methods section. A typical purification profile is shown in Figure 6a. Fractions corresponding to the second "peak" were collected and pooled; fractions corresponding to the first "peak" comprised of mHb that did not bind the column and had probably been modified due to exposure to low pH. The integrity and purity of mHb was assessed by SDS-PAGE analysis, and protein bands were revealed by silver staining (Appendix); commercially procured hHb was run in parallel as a reference. Figure 6b depicts the results. Protein bands corresponding to the Hb monomer (~15KDa) were observed for both hHb and mHb; no additional protein moieties were observed. Samples were analyzed by HPLC as an additional assessment of purity. Three "peaks" were obtained, designated Peak 1, Peak 1` and Peak 2 (Figure 6c); no other "peaks" were observed. N-terminal sequencing, conducted on individual "peaks", revealed that amino acids of Peak 1 and Peak 1` demonstrated identity to the alpha subunit of mHb and Peak 2 to the beta subunit of mHb (Figure 6d). Mass spectrometry analysis was then carried out on the subunits in order to verify molecular mass; for Peak 1, a mass of 14,987.84$\pm$3.21 Daltons was obtained, matching the reported mass of the alpha subunit of mHb (Figure 7). Peak 1` demonstrated a mass of 14,979.24$\pm$6.45 Daltons (Figure 8),

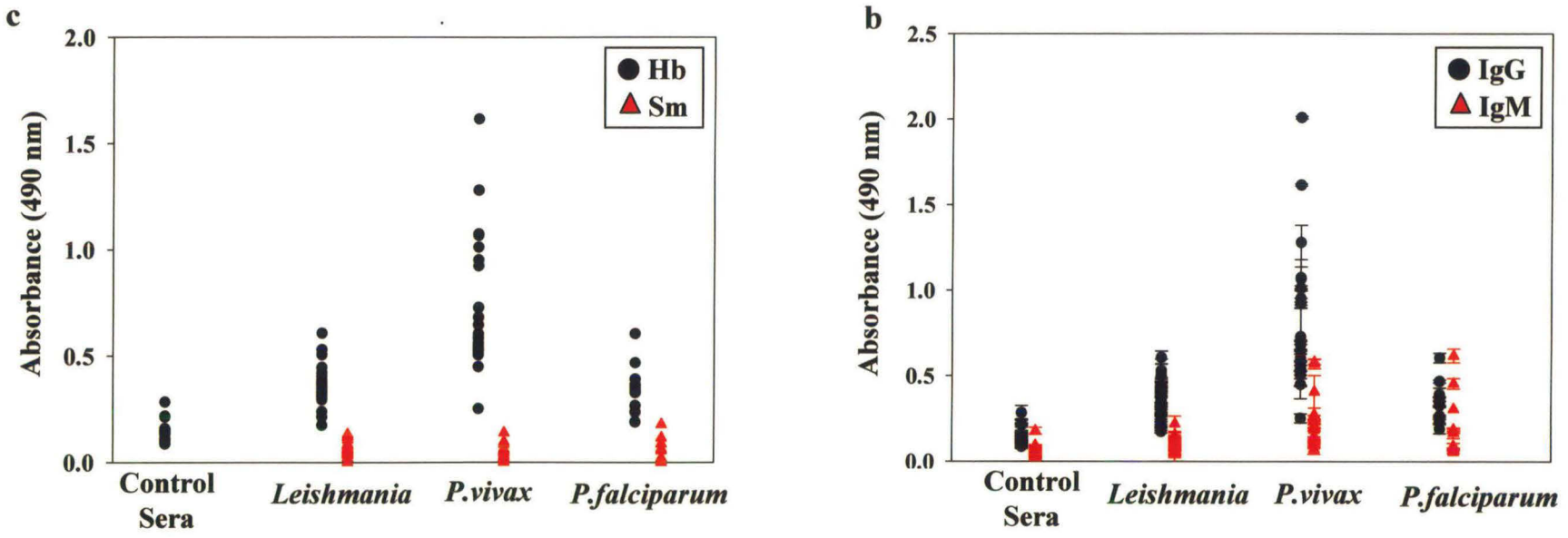


Figure 5: (**a**) Reactivity of antibodies in the sera of human patients harboring *Leishmania*, *P. vivax* and *P. falciparum* to hHb and Sm. (**b**) Isotypes of anti-hHb antibodies in sera from Leishmaniasis and malaria patients.

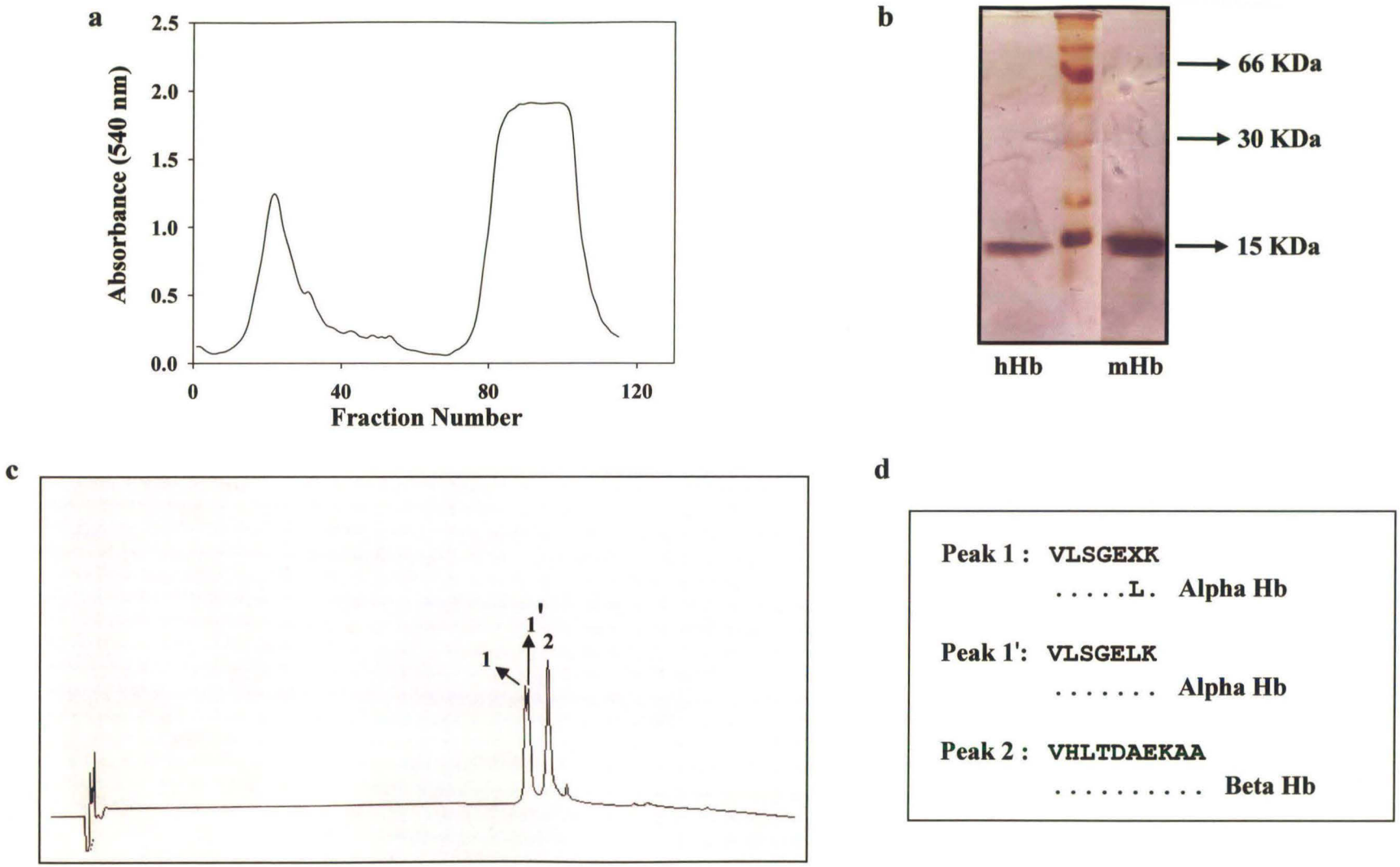


Figure 6: Purification and characterization of mHb. (**a**) Purification profile upon ion exchange chromatography. (**b**) Integrity and purity of mHb as assessed by silver staining subsequent to SDS-PAGE. A commercial preparation of hHb was employed as reference. (**c**) HPLC profile; three peaks were obtained. (**d**) N-terminal sequencing of the three peaks of mHb obtained upon HPLC. Dots signify identity to the indicated subunits. X indicated a mis-read residue.

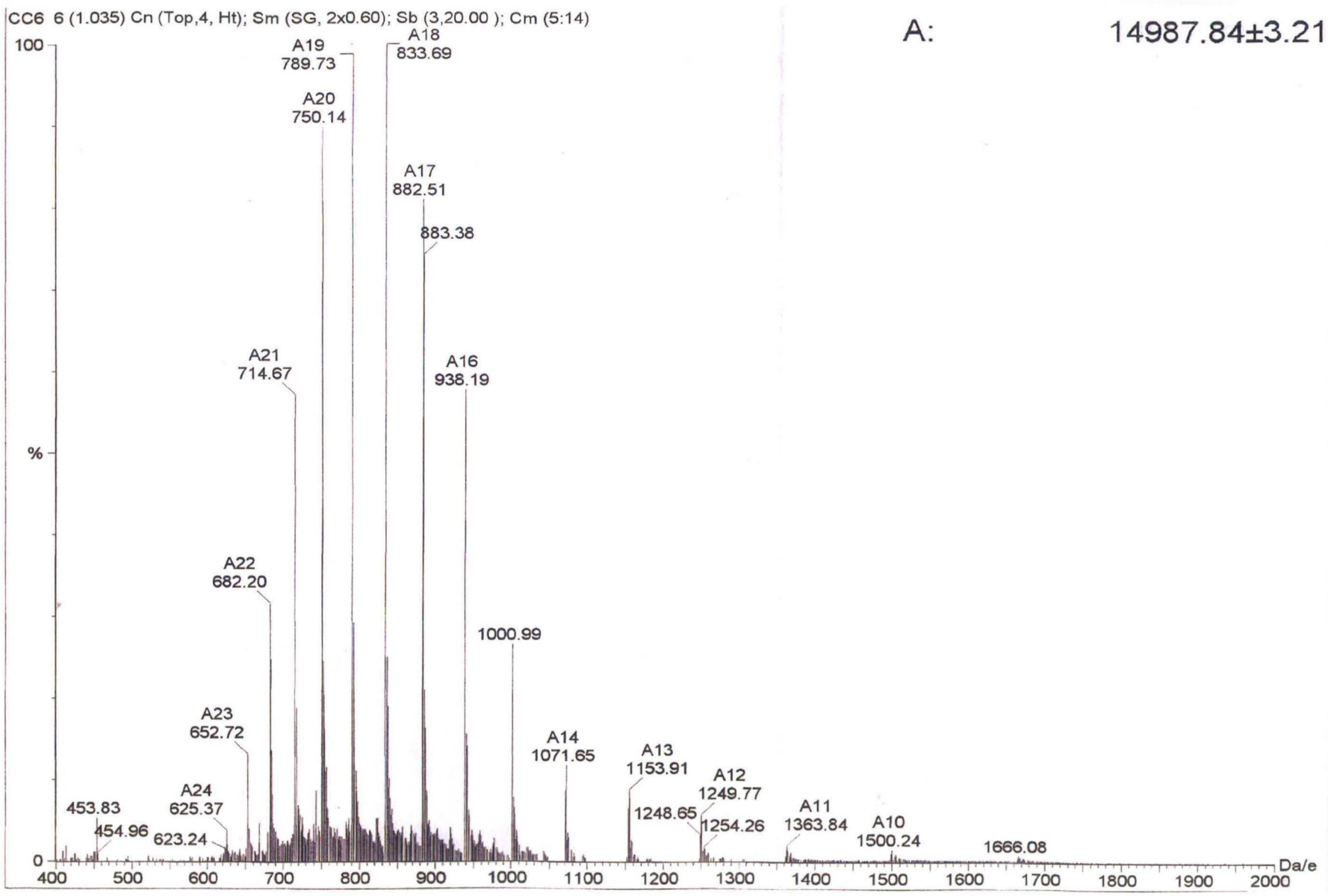


Figure 7: Mass spectrum of the Hb-alpha subunit (Peak 1)

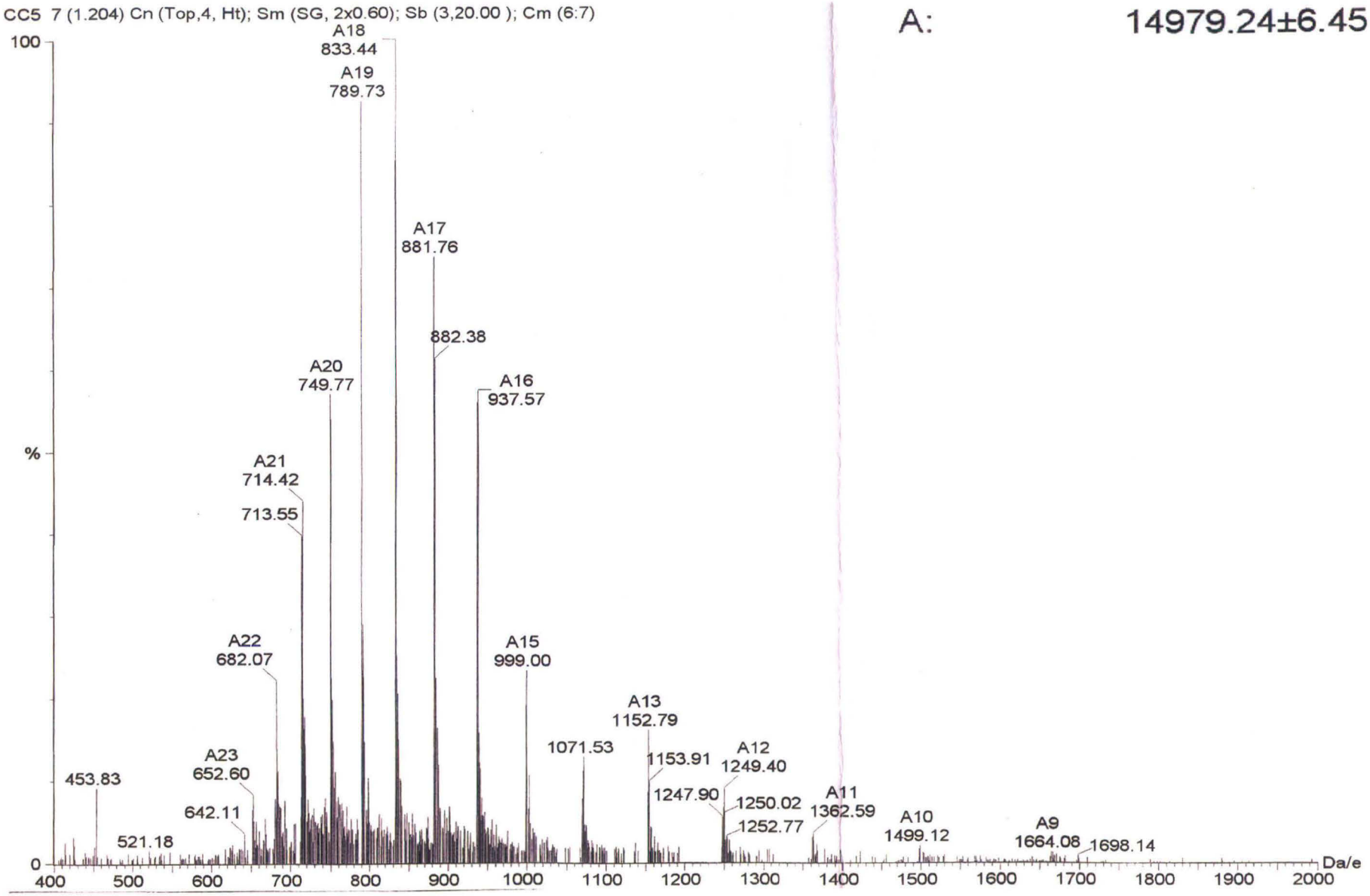


Figure 8: Mass spectrum of Hb-alpha subunit (Peak 1')

slightly higher than for Peak 1, and possibly corresponding to one of the variants of the alpha chain which have been described[233,234]. The mass of Peak 2 was determined to be 15,617.88±5.15 Daltons, matching the reported mass of the beta subunit of mHb (Figure 9). SDS-PAGE, N-terminal sequencing and mass spectrometric analysis thus indicated the preparation of mHb to be pure and homogenous. This preparation was employed in further experiments.

Reactivity to cellular antigens

In order to verify the appearance of auto-reactive antibodies, sera were obtained from NZB/W F1 animals of different ages. Upon FACS analysis, an age-dependent increase of auto-antibody reactivity was observed towards permeabilised Jurkat cells, indicating the spontaneous onset of autoimmune responses against internal antigens (Figure 10a). A similar increase was observed upon immunofluorescence analysis on HeLa cells; while antibodies in the sera of young animals displayed no discernable cellular specificity, those from old animal was frankly reactive, often exhibiting recognition of nuclear antigens (Figure 10b).

Reactivity to mHb

The sera of NZB/W F1 animals were then analyzed for the presence of antibodies to mHb, in order to assess whether the specificity exists in murine lupus as well, as it does in human lupus. ELISAs were performed using mHb as the target; an age-dependent increase in anti-mHb reactivity was observed (Figure 10c). Significantly enhanced reactivity was already apparent at about three months of age.

Reactivity to Sm

Given the apparent association of anti-Sm and anti-hHb reactivity in human lupus, it was of interest to determine the kinetics of anti-Sm response in aging NZB/W F1 mice. As with anti-mHb reactivity, increasing titres of anti-Sm antibodies were observed as mice aged. Increased reactivity was first observed at seven months of age, considerably after anti-mHb antibodies appeared. In addition, anti-Sm titres appeared to plateau at around 10 months, unlike auto-reactivity towards mHb (Figure 10d).

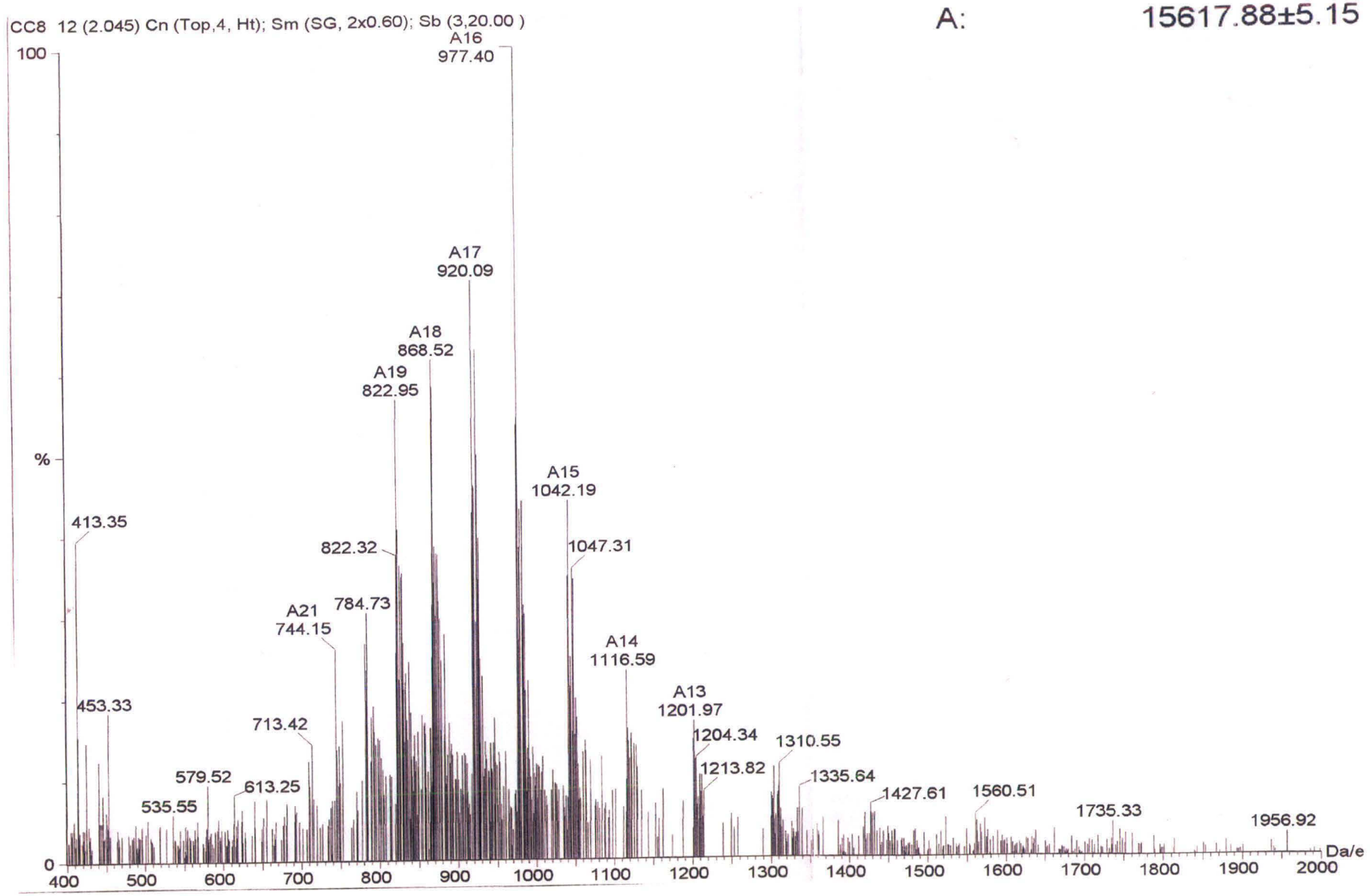


Figure 9: Mass spectrum of Hb-beta subunit (Peak 2)

[redacted]

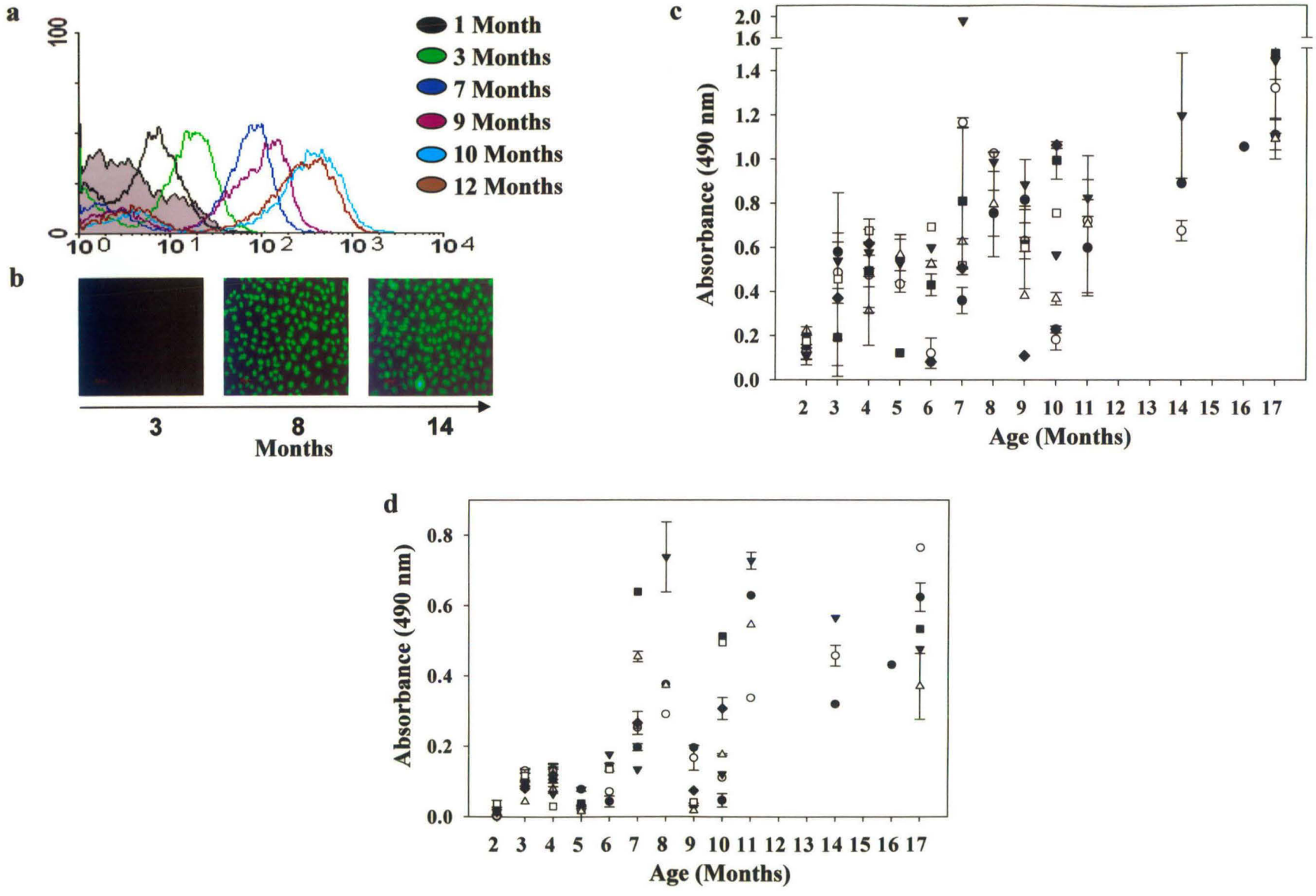


Figure 10: Kinetics of the appearance of auto-antibodies in the serum of NZB/W F1 mice. (**a**) FACS and (**b**) immunoflourescence depict anti-self reactivity. (**c**) Antibodies to mHb. (**d**) Antibodies to Sm.

Reactivity to Hb-alpha and Hb-beta subunits

Whether both alpha and beta subunits of Hb were targets of the spontaneous autoimmune response was then assessed by employing the individual subunits as targets in ELISAs. An increasing trend in reactivity against both subunits was observed as animals aged, with higher binding seen towards beta Hb. In both cases, significant reactivity was first observed at about seven months of age (Figure 11a, b).

Reactivity to mHb of low pH organ eluates

Immune complex deposition in various organs is thought to contribute to lupus pathology[14]. Whether different murine organs harbored anti-mHb antibodies was then investigated. Low pH eluates were obtained from the lungs, brain and kidneys from NZB/W F1 mice at different ages. While minimal reactivity was observed at two months of age, at ten months, animals demonstrated an increased presence of anti-mHb auto-antibodies in the lungs. At fourteen months, all three organs were demonstrated to contain adhered anti-mHb antibodies. Eluates obtained from fourteen month old BALB/c mice showed no such reactivity (Figure 11c). Thus, there appeared to be an age-dependent increase in the sequestration of auto-antibodies to mHb in the organs of lupus-prone mice.

Generation of monoclonal antibodies to Hb

Human monoclonal antibody

ELISAs were performed on the supernatants of 456 Epstein Barr Virus-transformed lymphoblastoid cell lines. Ten of these supernatants demonstrated the presence to antibodies reactive to hHb with RN43, RN86, RN57 and KV2C8 demonstrating the highest binding (Figure 12a). KV2C8 (hereafter referred to as KV) was chosen for further study. As described in the Methods section, "fusions" of these cells were carried out with the mouse-human heteromyeloma K_6H_6/B_5. The number of clones obtained and the number of clones reactive to hHb are enumerated in Figure 12b. After three sub-cloning procedures, the hybridoma secreting monoclonal antibody KV was generated. Isotyping assays revealed the antibody to be of the IgMλ isotype. Western blot on hHb

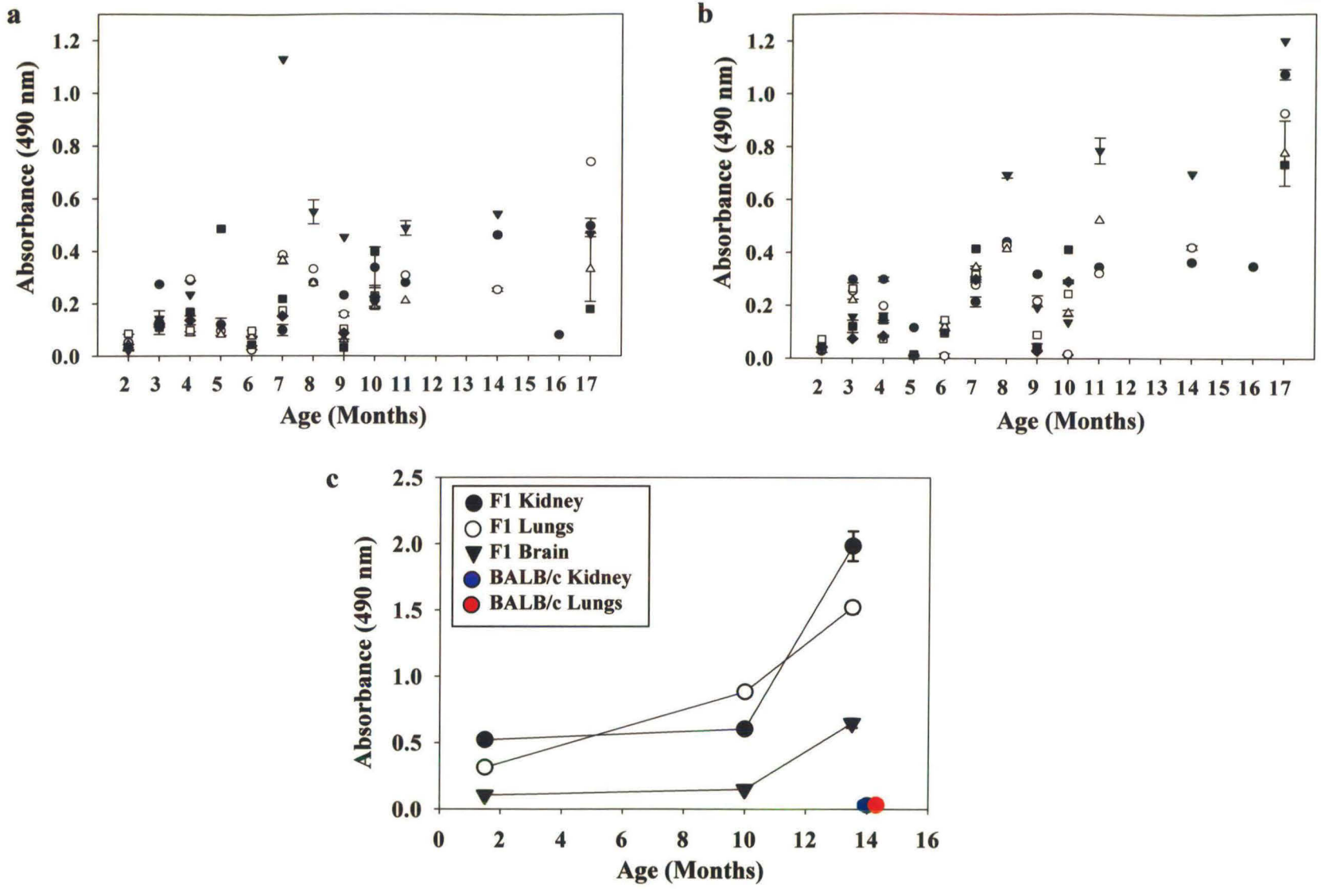
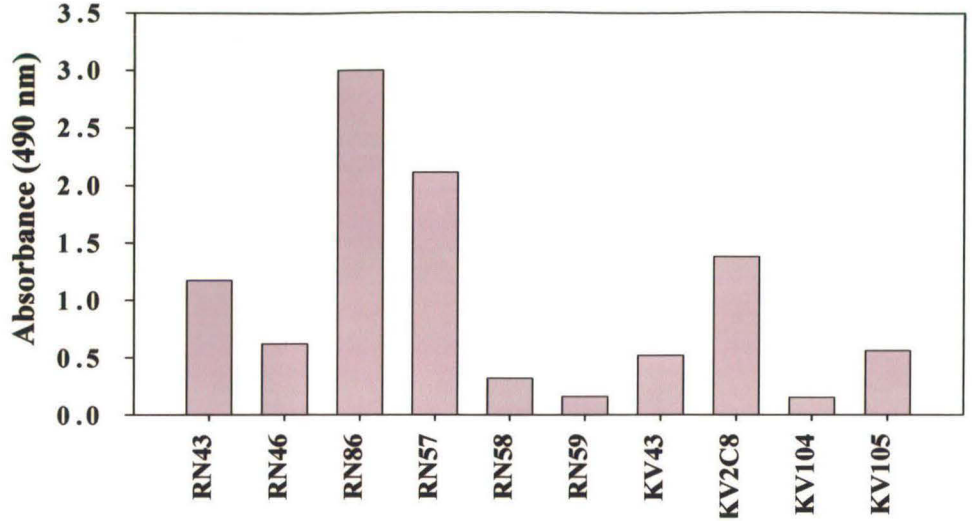


Figure 11: Kinetics of the appearance of antibodies in the serum of NZB/W F1 mice to (**a**) the Hb-alpha subunit and (**b**) the Hb-beta subunit. (**c**) Kinetics of anti-Hb reactivity of low-pH organ eluates from NZB/W F1 and BALB/c mice.

a



b

Fusions	**2**
Clones	**64**
Anti - hHb	**8**
Monoclonal Anti - hHb	**1**
Isotype	**IgMλ**

c

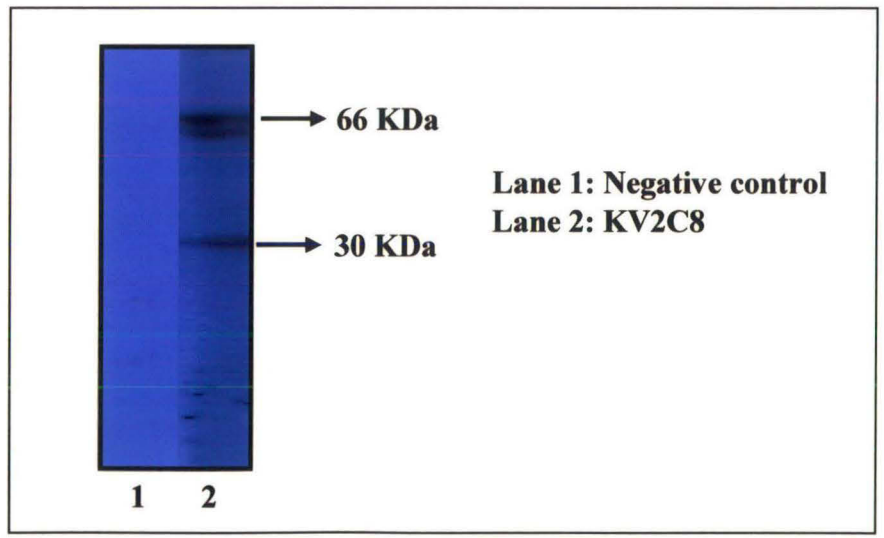


Figure 12: Generation of human anti-hHb monoclonal antibody. (**a**) Anti-hHb reactivity of antibodies in supernatants of EBV transformed B cells from SLE patients. (**b**) Details of fusions performed. (**c**) Western blot on commercial hHb employing supernatant from the human hybridoma (KV2C8). Negative control: Secondary antibody.

demonstrated reactivity of Antibody KV towards the hHb dimer and tetramer, further confirming its reactivity (Figure 12c).

Murine monoclonal antibodies

Splenocytes from old (>12 months) C57BL/6$^{lpr/lpr}$ and NZB/W F1 were "fused" with the mouse myeloma SP2/O as detailed in the Methods section. Myeloma cells had previously been treated with BM-Cyclin (Sigma) for three weeks to counter potential *Mycoplasma* infection. Details of the clones obtained have been enumerated in Figure 13a. The efficiency of the fusion event seemed relatively poor for C57BL/6$^{lpr/lpr}$ splenocytes, and hybridomas secreting anti-mHb antibodies were not obtained. Of the twenty hybridomas secreting anti-mHb antibodies obtained from NZB/W F1 animals, nine monoclonal anti-mHb secreting hybridomas were obtained after sub-cloning procedures and six stable clones were established, the nomenclature and isotypes of which are listed in Figure 13b. Western blots demonstrated that all antibodies bound mHb (Figure 13c); interestingly, Antibody 1C1 appeared to bind the tetramer whereas the other antibodies bound the dimer.

Evaluation of anti-Hb monoclonal antibody reactivity

Recognition of Hb (Fe^{2+}) and Hb (Fe^{3+})

Oxy-Hb (Fe^{2+}, ferrous) may be oxidized to metHb (Fe^{3+}, ferric) under the oxidizing and inflammatory environment that exists in SLE[192,210,211]. It was therefore important to establish whether monoclonal anti-Hb antibodies could differentiate between the two forms of Hb. The spectrum for Oxy Hb consists of three maxima in the visible range; at ~410nm (which is characteristic of all heme-containing proteins and is known as the Soret band), and two maxima at ~540 nm and ~570 nm. As oxidation of Hb proceeds, the maxima at ~540 nm and ~570 nm are substantially attenuated, and maxima at 630 nm emerges, which is characteristic of metHb.

mHb was incubated with H_2O_2 to generate metHb as detailed in the Methods section. Figure 14a reveals spectra recorded at two-minute intervals over a 70 minute period. A gradual, time-dependent decrease in the maxima at ~540 nm and ~570 nm were observed,

a

	C57BL/6$^{lpr/lpr}$	NZB/W F1
Fusions	3	4
Clones	20	180
Anti - mHb	0	20
Monoclonal Anti-mHb	0	9

b

ANTIBODIES	ISOTYPE
1C1	IgAκ
1B5	IgMκ
2A1	IgMκ
2C1	IgMκ
3C4	IgMκ
3A1	IgMκ

c

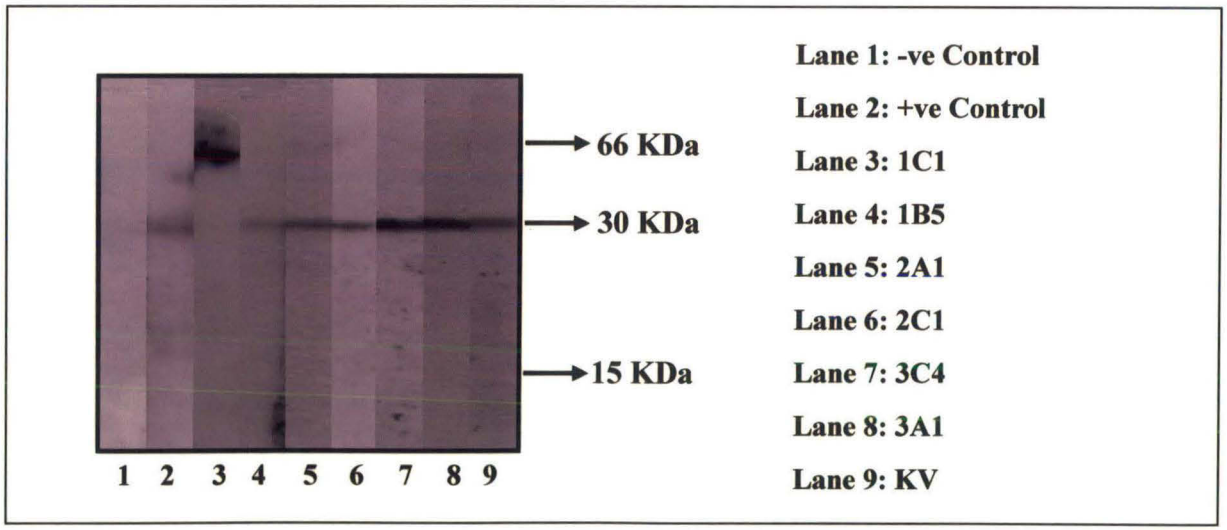


Figure 13: Generation of murine anti-mHb monoclonal antibody. (**a**) Details of fusion performed. (**b**) Details of murine anti-mHb monoclonal antibodies generated. (**c**) Western blot on mHb employing one human (KV) and six murine monoclonal anti-Hb antibodies. –ve Control: Secondary antibody; +ve Control: Commercial anti-hHb polyclonal serum.

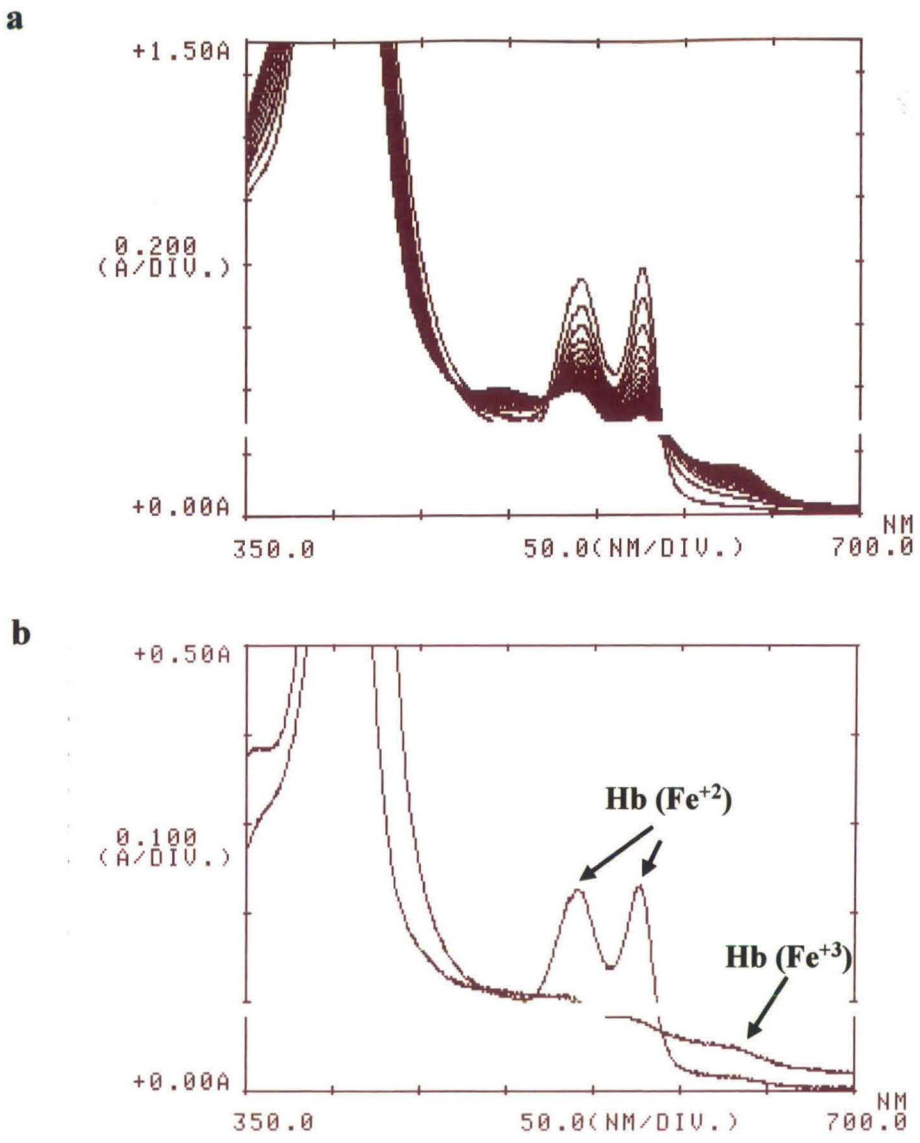


Figure 14: Oxidation of mHb (Fe^{+2}) to metHb (Fe^{+3}) by H_2O_2. (**a**) Spectral analysis at two-minute intervals over a 70-minute period. (**b**) Overlay of mHb and metHb at 24 hrs. The scans show the disappearance of maxima at 540 nm and 570 nm corresponding to the ferrous mHb and appearance of a maxima at 630 nm, characteristic of metHb.

along with the appearance of maxima at 630 nm. For more clarity, Figure 14b depicts the profile overlays of unoxidized mHb and mHb that had been oxidized for 24 hrs, essentially showing the same results. Similarly, oxidation was also carried out for hHb that had been purified from the blood of a healthy human donor.

Figure 15a shows results obtained when Fe^{2+} (ferrous) and Fe^{3+} (ferric) mouse and human Hb were employed as antigens in ELISAs to assess the reactivity of the monoclonal anti-Hb antibodies. Antibodies appeared to behave differently in terms of recognition; while Antibodies 1C1, 3A1 and KV could not distinguish between ferrous and ferric forms of Hb, Antibodies 1B5, 2A1 and 2C1 bound ferric Hb (metHb) much better than ferrous Hb (Figure 15a). Antibody 3C4 too appeared to demonstrate a slight preference for metHb.

Recognition of heme and other heme-containing proteins

Reactivity of the anti-Hb monoclonal antibodies to heme as well as to two other heme-containing proteins [cytochrome c (cyt c) and myoglobin (Mb)] was then assessed to determine if the heme group played a role in recognition (Figure 15b). Commercially procured antibodies to the Hb-alpha and beta subunits were used as controls. Antibody 2C1 demonstrated poor recognition of heme, cyt c and Mb. Antibodies 1C1 and KV exhibited equivalent recognition of heme and Hb, whereas Antibodies 2A1, 1B5 and 3A1 bound free heme better than they did Hb. Relative to Hb reactivity, except for Antibodies KV and 1C1, antibodies bound cyt c poorly. Antibody 3A1 demonstrated minimal reactivity towards Mb, in spite of the high degree of structural homology between the molecule and the β subunit of Hb[191]; the other antibodies exhibited a variable extent of reactivity. While the commercial reagents were generally specific, unexpected and unexplained reactivity was observed for anti-Hb-α antibody towards Mb.

Recognition of Hb and the Hb-Haptoglobin (Hp) complex

Hb, when released from RBCs due to intravascular lysis, is quickly complexed to haptoglobin (Hp), an acute phase serum protein which has a strong affinity for free Hb[194]. Uptake of the Hb-Hp complex occurs in the macrophages of liver, spleen and in circulation, where the complex is degraded upon internalization; Hp is not recycled.

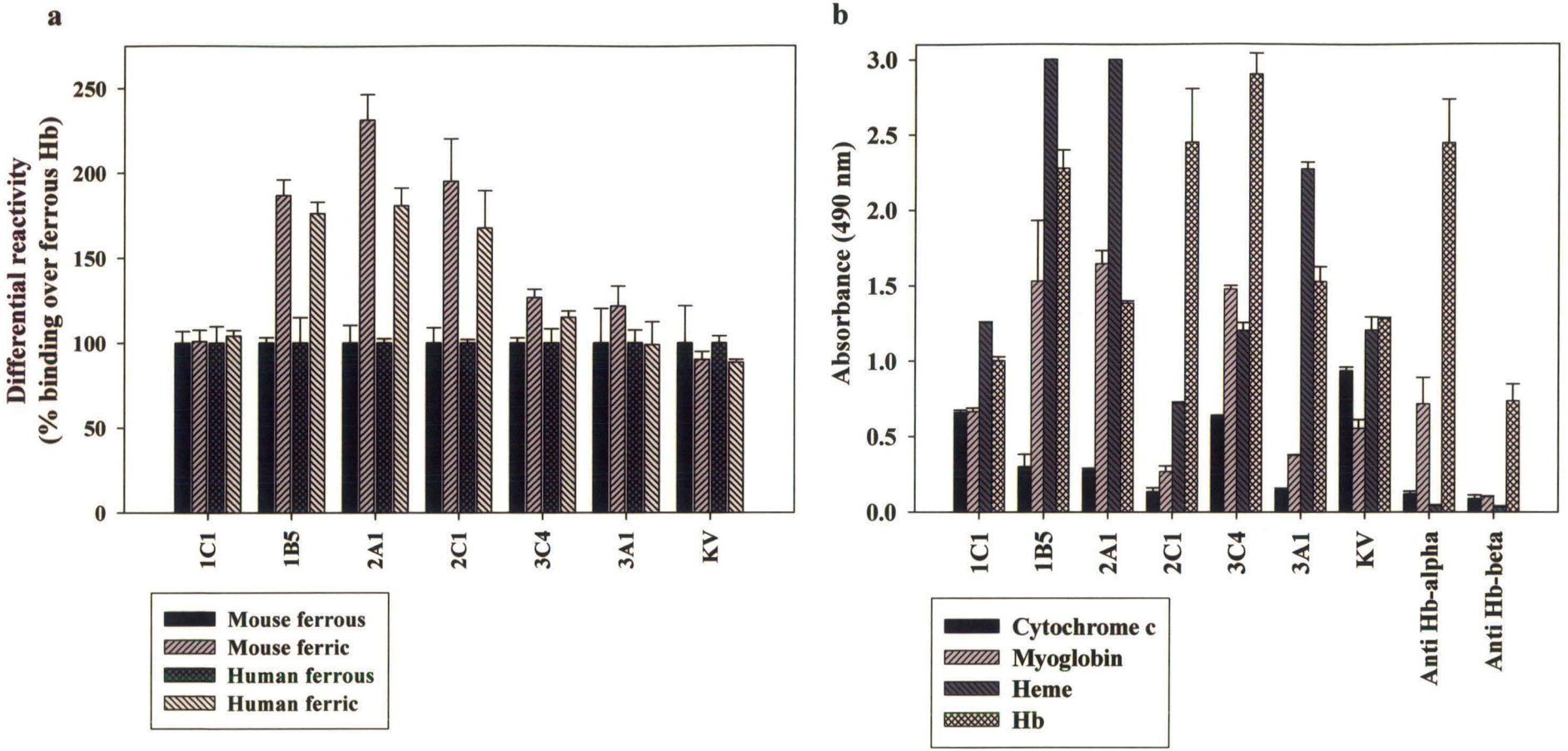


Figure 15: (**a**) Differential reactivity of human and murine monoclonal antibodies to human and mouse (ferrous and ferric) Hb. (**b**) Reactivity of the antibodies to heme-containing proteins and heme. Commercial anti-Hb-alpha and anti-Hb-beta antibodies were employed as controls.

It was important to determine if the epitopes bound on Hb by Hp were the same or distinct than those recognized by the autoimmune anti-Hb monoclonal antibodies. An *in vitro* Hb-Hp association assay was first standardized using commercial anti-Hp antibodies, as detailed in the Methods section (Figure 16a). Demonstration of the interaction of anti-Hp antibodies with Hp served as the positive control. Hb-Hp binding was observed when both molecules were present. The lack of interaction between anti-Hp antibody and Hb attested to the specific reactivity of the commercial anti-Hp reagent and the assay. Another important control included the "coating" of Hp subsequent to "saturation" of the ELISA wells by PVA; lack of significant absorbance under these conditions further testified to assay specificity.

Using conditions in which Hb-Hp association was demonstrated to occur, the ability of Hp to interfere with antibody-Hb interaction was assessed. In parallel, reactivity to Hp was also determined, as such reactivity, if present, could confound the interpretation of results. Results are shown in Figure 16b. Two significant observations were made; firstly, none of the murine anti-Hb antibodies recognized Hp, and secondly, all antibodies appeared to bind the Hb and Hb-Hp complex with equal efficiency, implying that the binding of Hp to Hb did not interfere with the binding the autoimmune monoclonal antibodies to Hb, thereby indicating distinct binding sites. Data obtained using the commercial anti-Hb-alpha and anti-Hb-beta antibodies were inconclusive, primarily because the antibodies themselves unexpectedly demonstrated reactivity towards Hp. Unacceptably higher "background" reactivity of the anti-human Ig-HRP conjugate towards Hp precluded interpretation of Antibody KV reactivity patterns as well.

Recognition of Hb-alpha and Hb-beta and synthetic, contiguous peptides

Heme-containing Hb subunits were used as targets in ELISAs in order to determine if the antibodies exhibited differential subunit specificity. Results indicated that subunit preference did indeed exist for some antibodies; Antibodies 1C1, 2A1, 3C4, 3A1 and KV demonstrated higher recognition of the beta subunit, while Antibody 1B5 bound both alpha and beta subunits equally, and Antibody 2C1 recognized the alpha subunit better (Figure 16c).

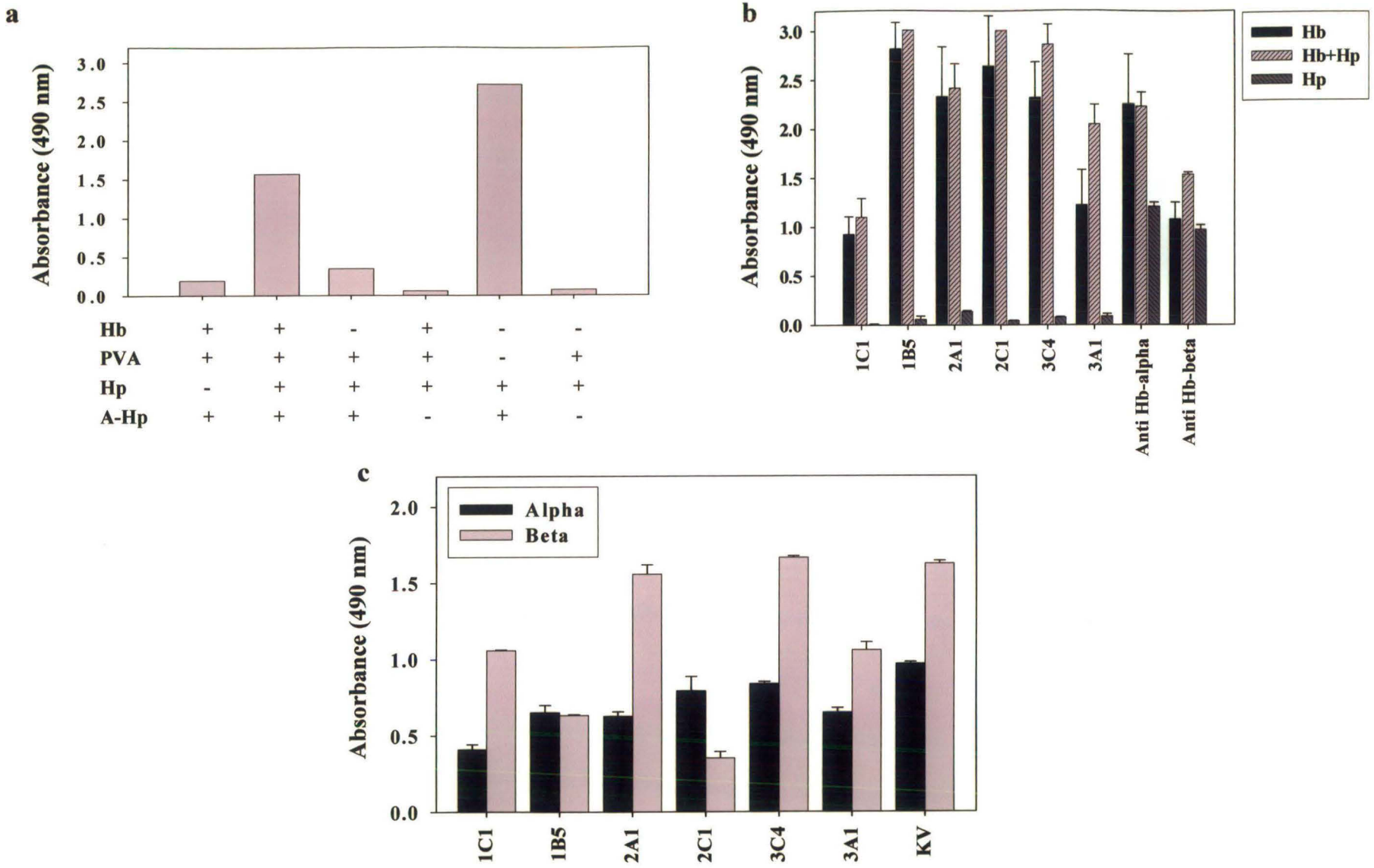


Figure 16: Reactivity of anti-Hb monoclonal antibodies. (**a**) *In vitro* Hb-Hp association assay. A-Hp: anti-haptoglobin antibody. (**b**) Reactivity of monoclonal antibodies to Hb, Hp and the Hb-Hp complex. (**c**) Binding of monoclonal antibodies to Hb-alpha and Hb-beta subunits.

Contiguous peptides representing mHb-alpha (seven peptides) and mHb-beta (eight peptides) were synthesized and used as targets in ELISAs (Figures 17, 18). Intra-molecular polyreactivity was apparent; Antibodies 1C1 and 1B5 bound several peptides representing both alpha and beta subunits (Figure 17a, b). In the case of the latter, the highest reactivity was seen towards peptides 100-119 and 120-141 of the alpha subunit and to 80-99 and 100-119 of the beta subunit. Antibody 2A1 demonstrated dominant reactivity to peptides 100-119 of the alpha and 100-119 peptide of the beta subunit (Figure 17c). Antibody 2C1 bound strongly to just peptide 100-119 of the alpha subunit (Figure 17d). Antibody 3C4 also appeared polyreactive, with predominant (but relatively less intense) reactivity directed against 100-119 of the alpha subunit and 20-39 of the beta subunit (Figure 17e). Antibodies 3A1 and KV did not bind to any peptide, though binding to whole Hb was apparent in the same assay (Figure 18a, b). Similar results were obtained when commercial anti-Hb-alpha and anti-Hb-beta antisera were employed (Figure 18c, d). Lack of reactivity could be interpreted as follows: Either the antibodies bound a structural epitope on Hb not represented by the peptides or their epitopes may have inadvertently been "broken", since the peptides were designed to be non-overlapping.

Reactivity to cellular antigens – FACS and confocal microcropy

As previously described, the anti-apoptotic cell-specific human monoclonal antibody RN86 cross-reacted with hHb. It was therefore of interest to determine whether the anti-Hb monoclonal antibodies, in turn, bound non-Hb cell-associated antigens as well. FACS analysis was carried out on both permeabilised and non-permeabilised cells, and on several cell lines - Jurkat (human T cell line), SP2/O (murine B cell line) and J774A.1 (murine macrophage cell line). Similar results were obtained in all cases. Representative results on Jurkat cells are shown in Figure 19. Figure 19a depicts the binding of anti-Hb monoclonal antibodies to non-permeabilised cells; none of the antibodies demonstrated significant recognition. The positive control employed in this experiment was Antibody 1D1 (IgG3κ), previously generated in the lab and demonstrated to recognize healthy, non-permeabilised cells. Most antibodies demonstrated frank recognition of permeabilised cells, indicating binding to an internal, cross-reactive non-Hb antigen(s).

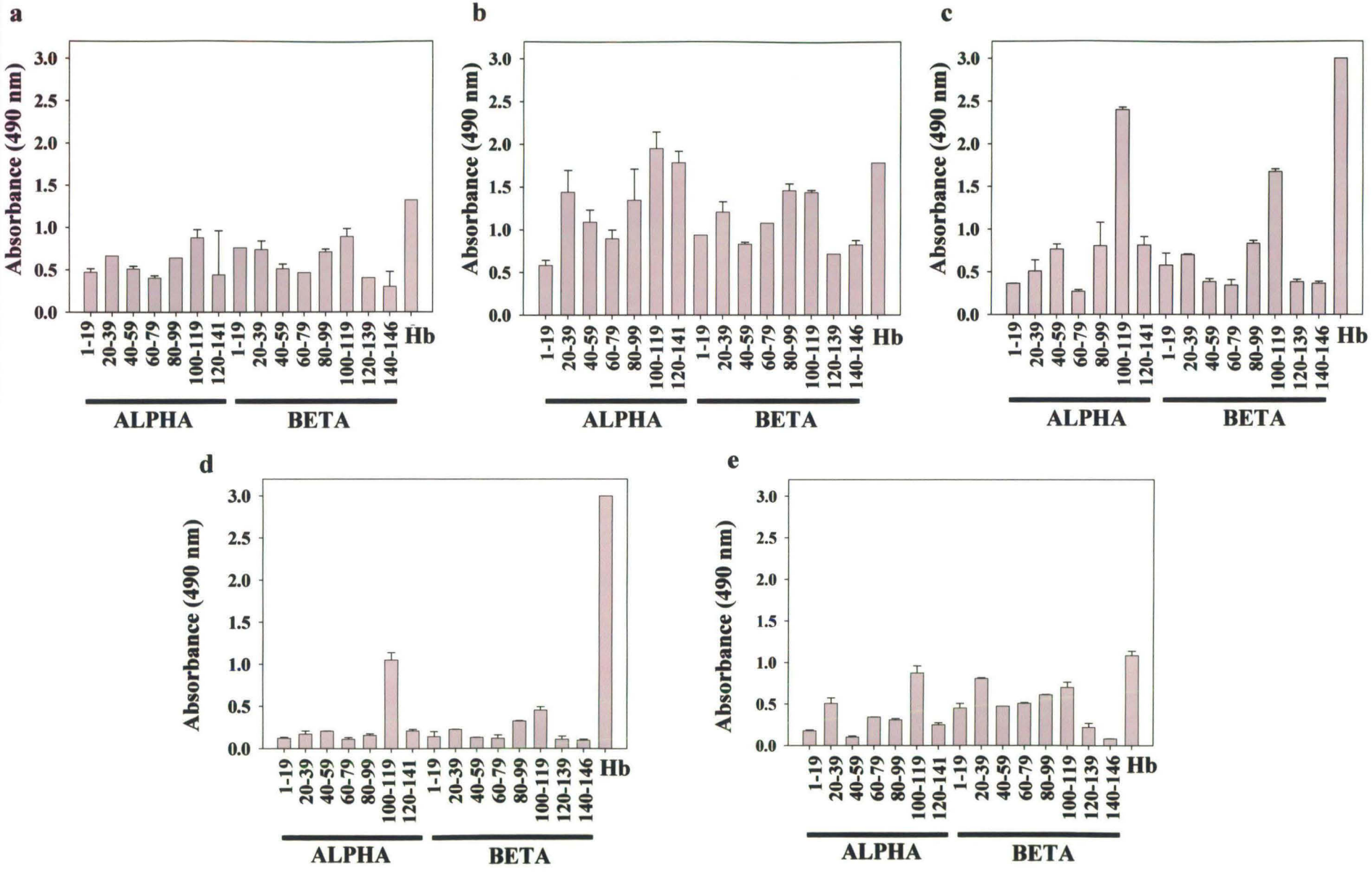


Figure 17: Reactivity of anti-Hb monoclonal antibodies towards sequential peptides representing the Hb-alpha and Hb-beta subunits. (**a**) 1C1; (**b**) 1B5; (**c**) 2A1; (**d**); 2C1; (**e**) 3C4.

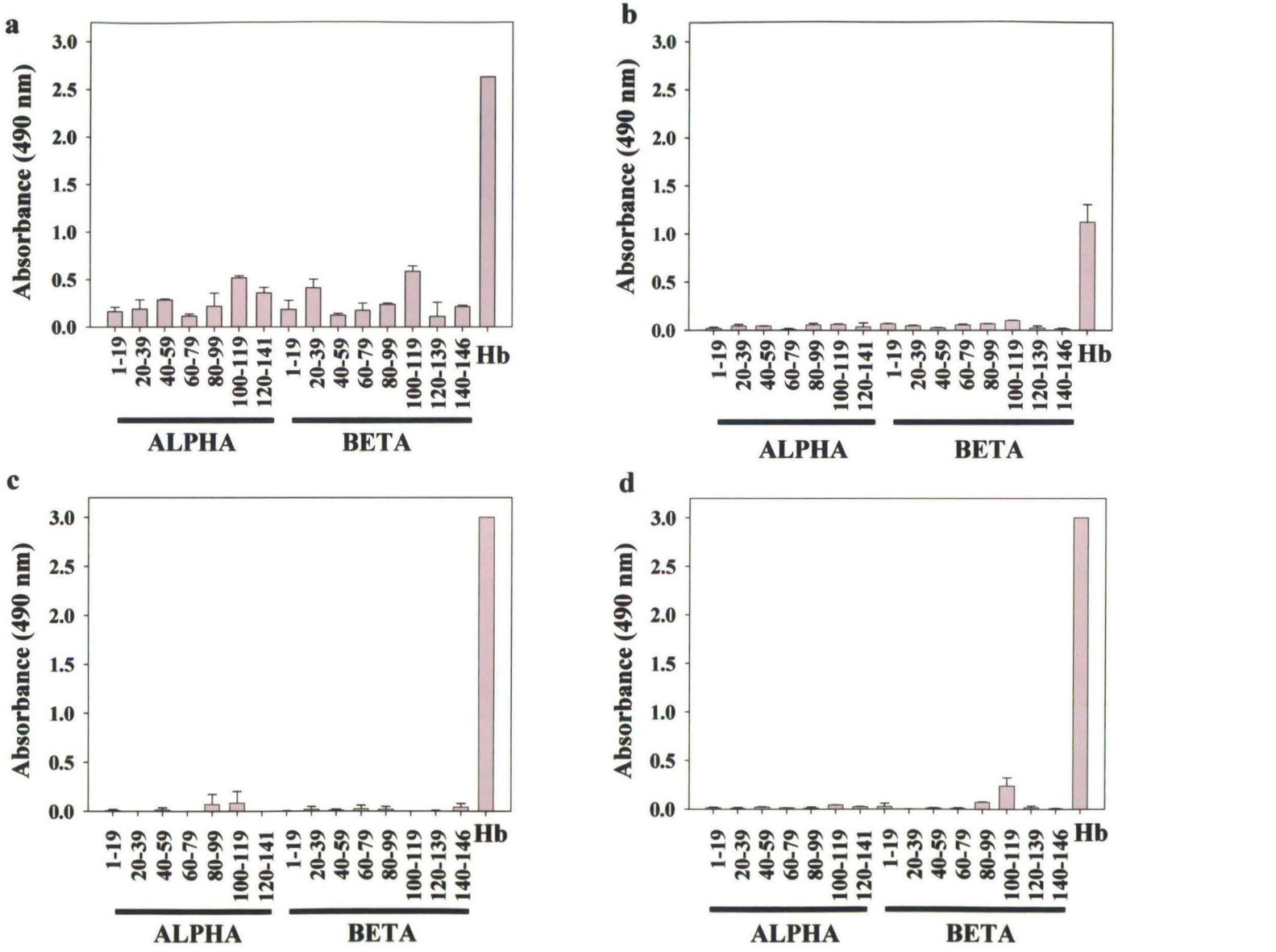


Figure 18: Reactivity of anti-Hb monoclonal antibodies and commercial anti-Hb antisera towards sequential peptides representing the Hb-alpha and Hb-beta subunits. (**a**) 3A1; (**b**) KV; (**c**) Commercial anti-Hb-alpha antibodies; (**d**) Commercial anti-Hb-beta antibodies.

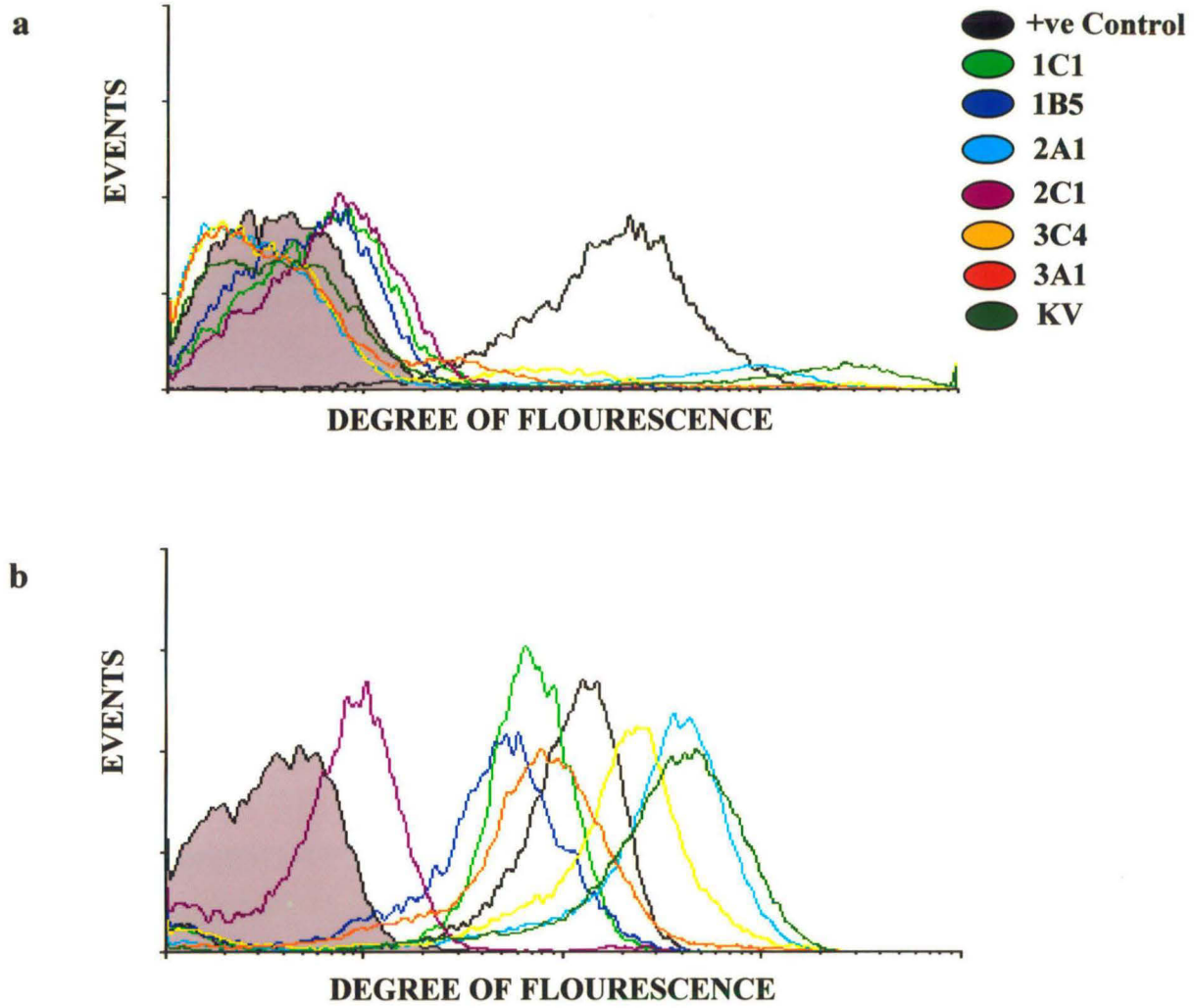


Figure 19: Reactivity of anti-Hb monoclonal antibodies towards (**a**) non-permeabilised and (**b**) permeabilised Jurkat cells by FACS. Grey profiles: negative controls, cells incubated with secondary antibody. +ve Control: Antibody 1D1 for non-permeabilised cells and Antibody 2C11 for permeabilised cells.

Antibody 2C1 was poorly reactive in this assay, and in other assays (not shown) demonstrated insignificant binding (Figure 19b), indicating a lower degree of cross-reactivity to cellular moieties. Antibody 2C11, a previously established monoclonal antibody known to bind permeabilised cells, was employed as a positive control.

Confocal microscopy was then carried out on permeabilised and non-permeabilised HeLa cells to verify these results and to assess cellular localization of the antibodies. Antibody 2C11 was employed as a positive control for these studies; the antibody predominantly bound cytoplasmic antigens, with enhanced perinuclear recognition (Figure 20a). Figure 20b depicts the negative control, where only the second antibody was employed. For both figures (and for all subsequent confocal figures which follow), right panels depict phase contrast images. On permeabilised cells, Antibody 1C1 exhibited recognition of antigens in the perinuclear region, along with some reactivity in the cytoplasm (Figure 21a); the antibody did not bind to non-permeabilised cells (Figure 21b). Antibody 1B5 bound the perinuclear region, with cytoplasmic "tailing" in some cells (Figure 21c); a lack of recognition was shown on non-permeabilised cells (Figure 21d). Antibody 2A1 bound cytoplasmic antigens, with enhanced, punctate binding was observed along the periphery of the cells, marking the cell boundary (Figure 22a); the antibody did not bind non-permeable cells (Figure 22b). Antibody 2C1 was non-reactive to both permeable and non-permeable cells (Figure 22c and 22d), reiterating results obtained upon FACS. Antibody 3C4 reactivity was directed almost exclusively to the perinuclear region, with some filamentous, cytoplasmic "tailing" (Figure 23a); once again, lack of binding to non-permeable cells was observed (Figure 23b). Both nuclear and cytoplasmic reactivity was evident for Antibody 3A1 (Figure 23c), with no recognition of non-permeabilised cells (Figure 23d). The human monoclonal antibody KV displayed a near-homogeneous cytoplasmic reactivity pattern, on permeabilised cells (Figure 24a); once again, non-permeable cells were not recognized (Figure 24b).

Interestingly, commercial (non-autoimmune) anti-Hb-alpha and anti-Hb-beta antibodies did not demonstrate reactivity towards either permeabilised or non-permeabilised cells (Figure 25a-d), indicating that the reactivity profiles of at least some anti-Hb autoimmune

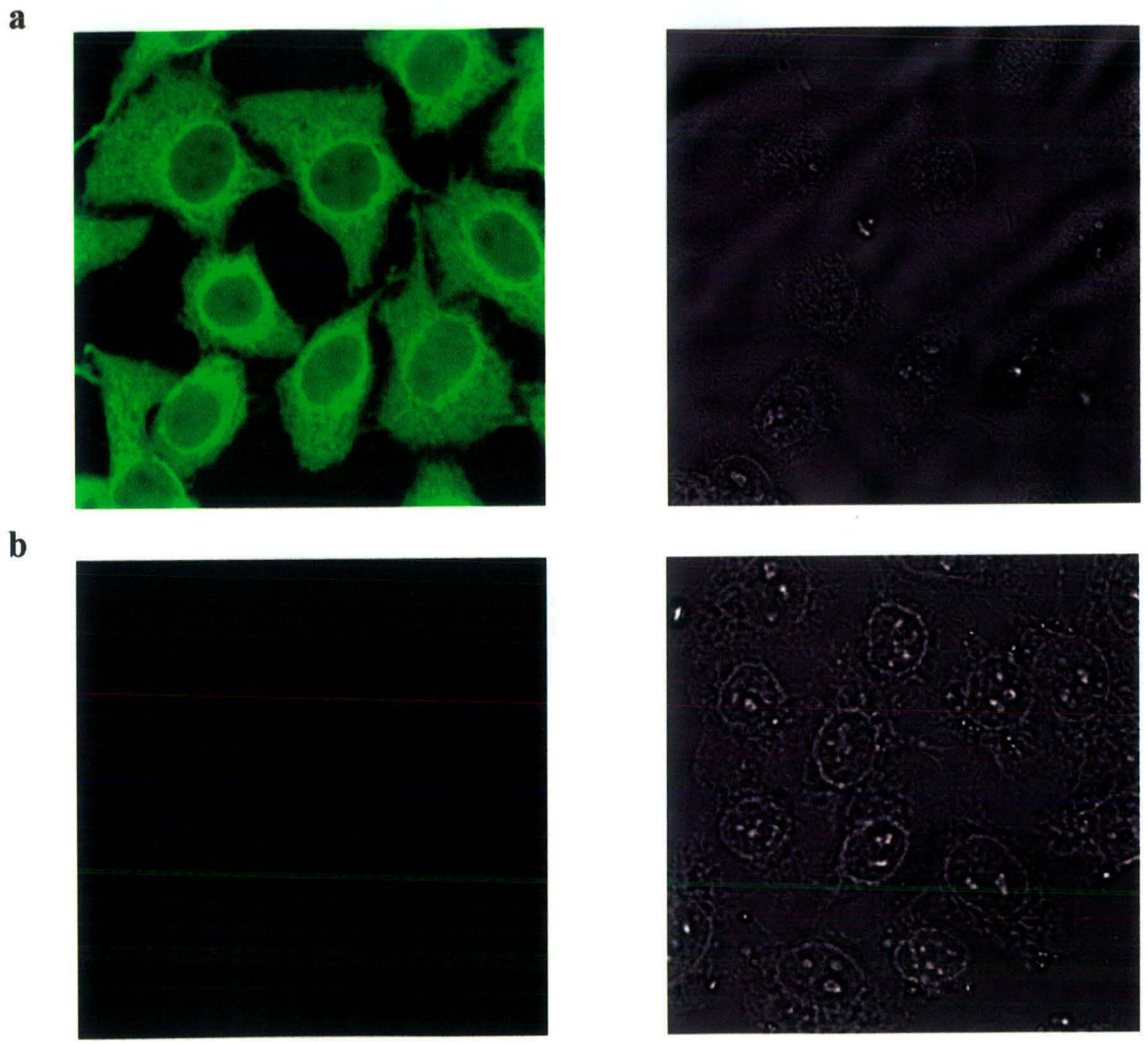


Figure 20: (**a**) Reactivity of Antibody 2C11 towards permeabilised HeLa cells. (**b**) Negative control (secondary antibody). Corresponding phase contrast images are shown on the right.

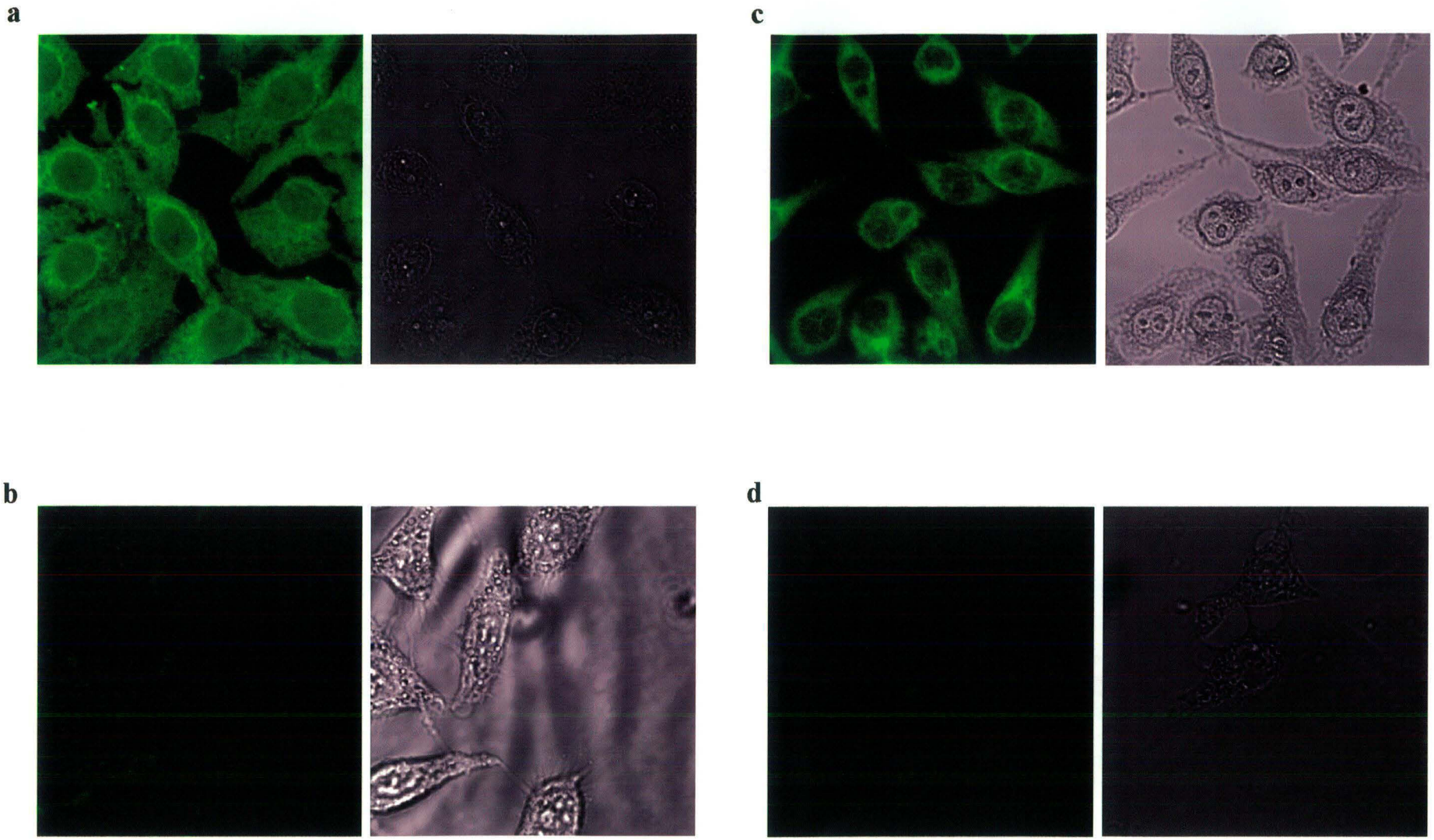


Figure 21: Reactivity of (**a, b**) Antibody 1C1 and (**c, d**) Antibody 1B5 to (**a, c**) permeabilised and (**b, d**) non-permeabilised HeLa cells. Corresponding phase contrast images are shown on the right.

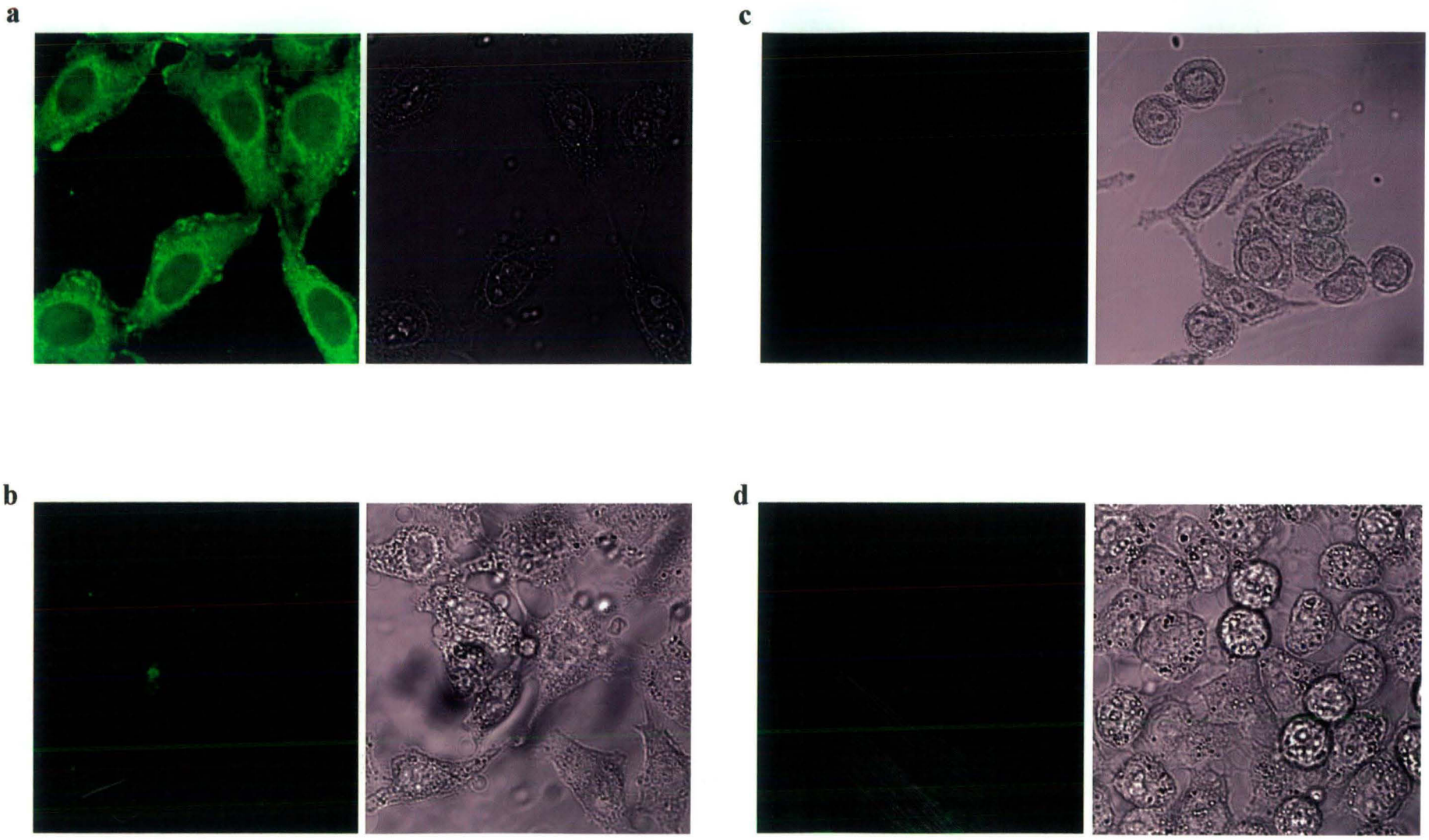


Figure 22: Reactivity of (**a, b**) Antibody 2A1 and (**c, d**) Antibody 2C1 to (**a, c**) permeabilised and (**b, d**) non-permeabilised HeLa cells. Corresponding phase contrast images are shown on the right.

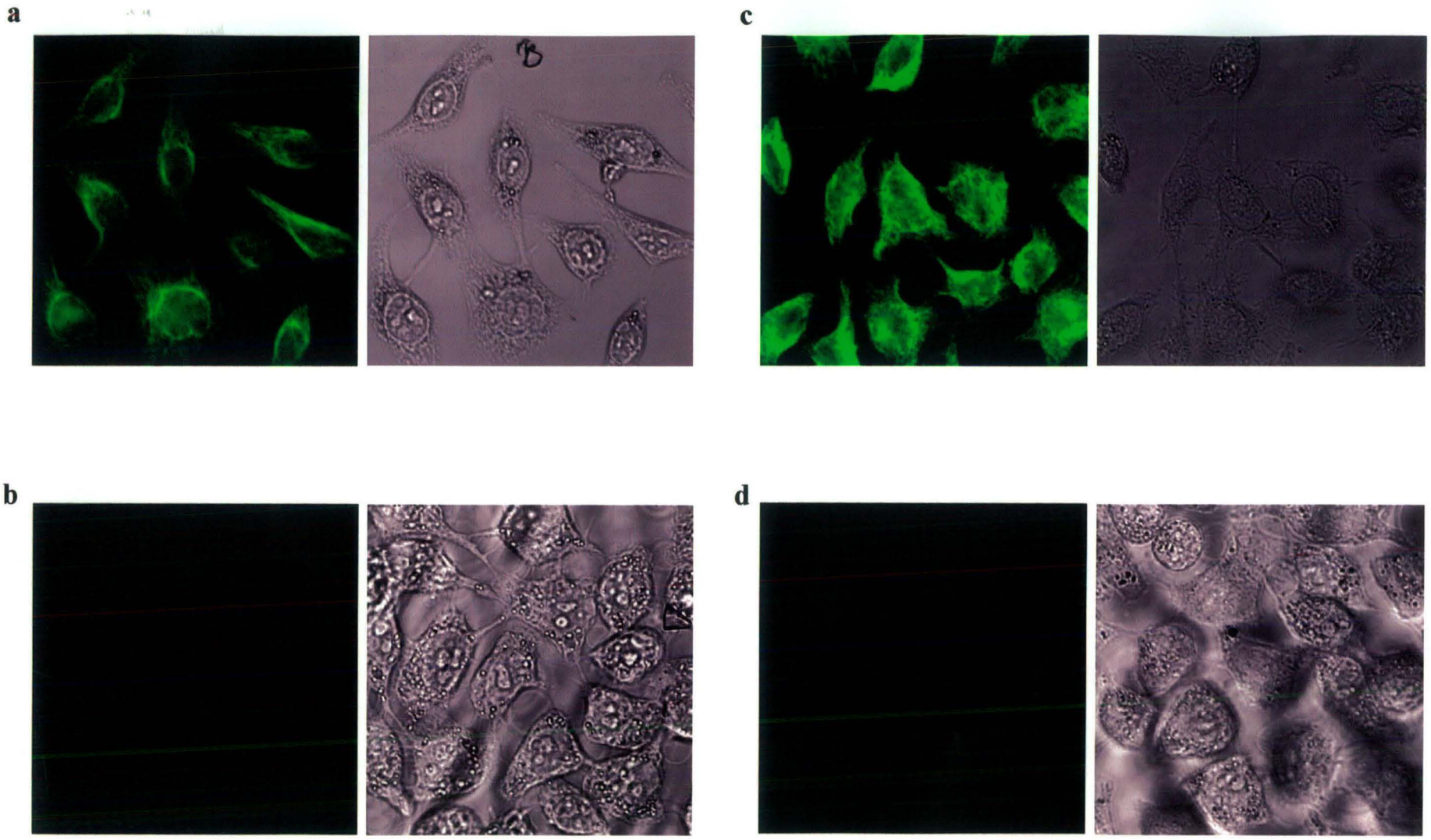


Figure 23: Reactivity of (**a, b**) Antibody 3C4 and (**c, d**) Antibody 3A1 to (**a, c**) permeabilised and (**b, d**) non-permeabilised HeLa cells. Corresponding phase contrast images are shown on the right.

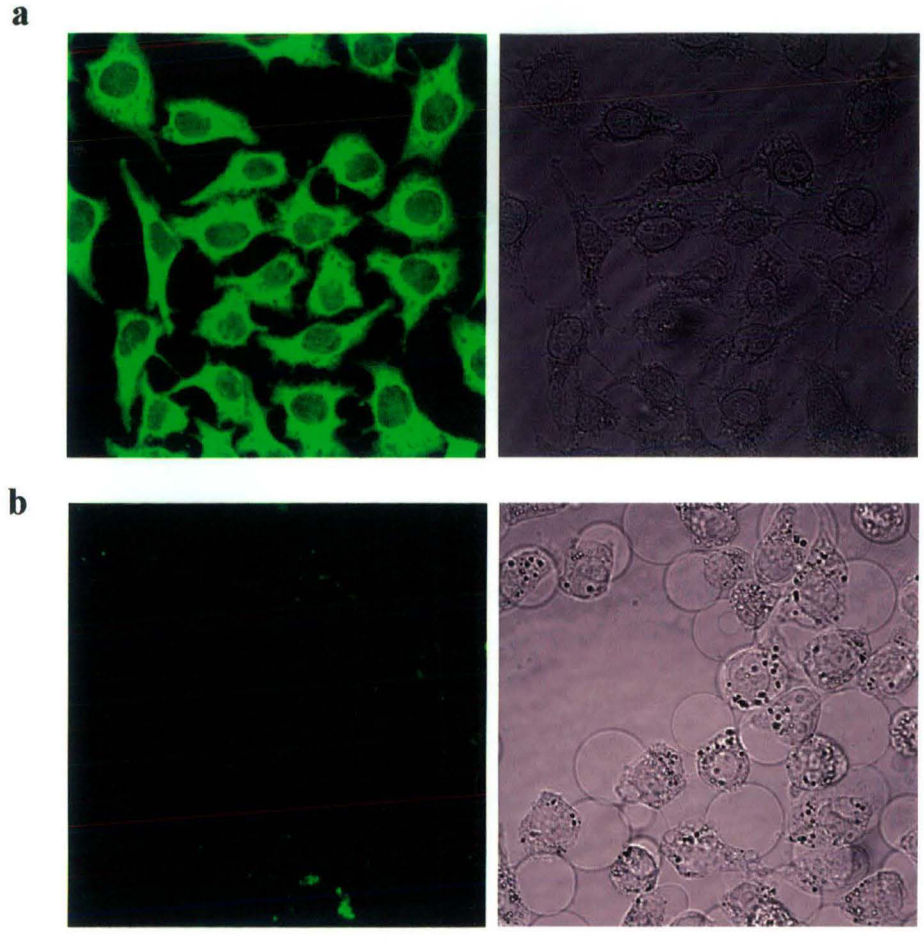


Figure 24: Reactivity of Antibody KV to (**a**) permeabilised and (**b**) non-permeabilised HeLa cells. Corresponding phase contrast images are shown on the right.

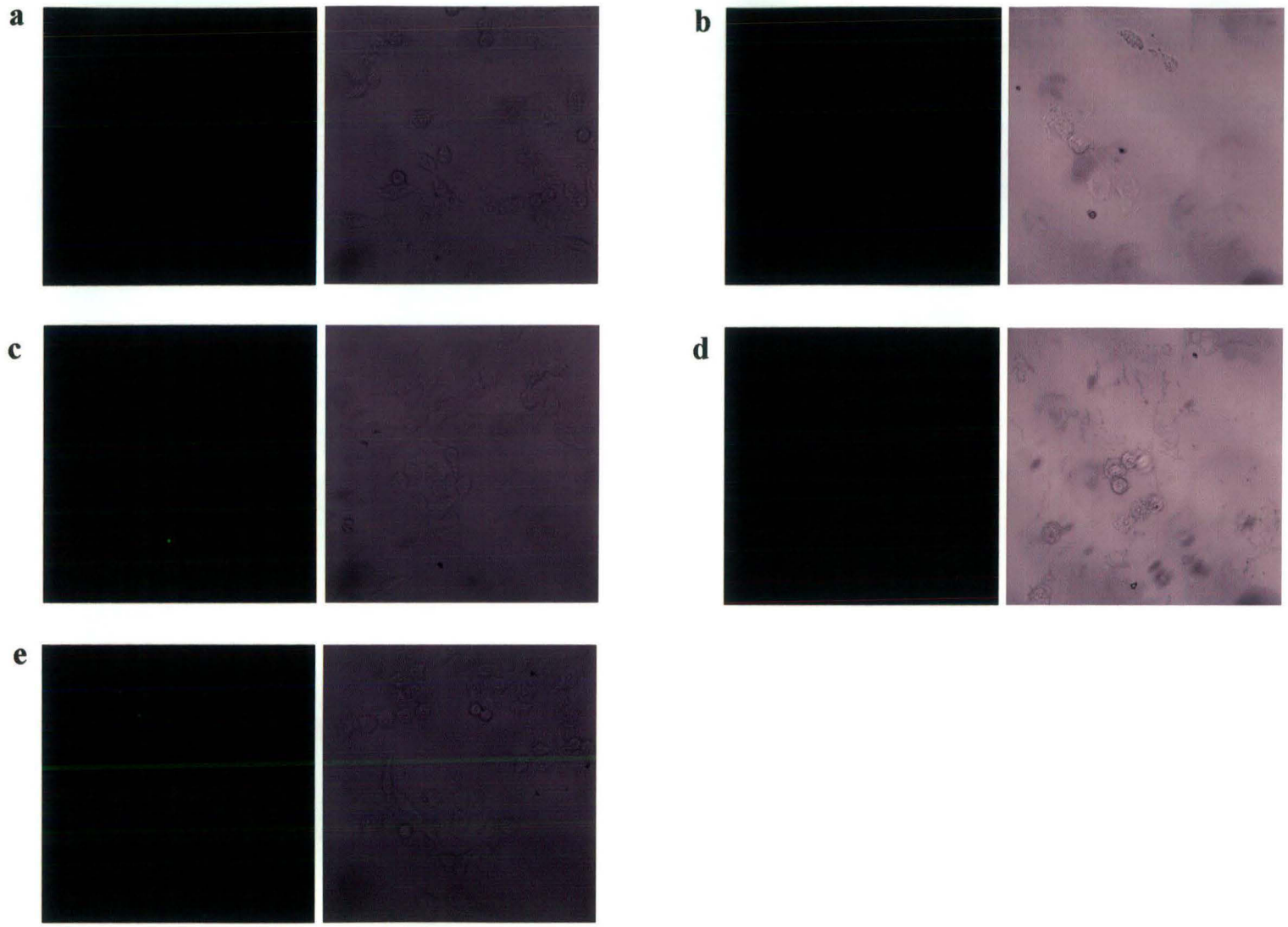


Figure 25: Reactivity of (**a, b**) commercial anti-Hb alpha, (**c, d**) commercial anti-Hb beta and (**e**) the isotype control IgMκ antibody, on permeabilised (**a, c, e**) and non-permeabilised (**b, d**) HeLa cells. Corresponding phase contrast images are shown on the right.

antibodies are different than those raised in a non-autoimmune background. An irrelevant IgMκ (Anti-TNP antibody) was employed as an isotype control; the antibody did not bind to permeabilised cells (Figure 25e), further attesting the specificity of the observations.

Reactivity to apoptotic cells

Whether the anti-Hb monoclonal antibodies could recognize apoptotic cells was then investigated, once again taking a lead from the reactivity patterns of the Hb-reactive human monoclonal antibody RN86. Jurkat cells were induced to undergo apoptosis using camptothecin as outlined in the Methods section. Two-colour flow cytometry was then carried out, using the individual antibodies and the phosphatidylserine-binding protein Annexin-V. Results are shown in Figure 26; the X-axis represents antibody binding and the Y-axis Annexin-V reactivity. For the most part, cells that bound Antibodies 1C1, 1B5, 2A1, 3C4, 3A1 and KV also bound Annexin-V, as depicted by the presence of cells in the top right quadrants and by a relative lack of cells in the bottom right quadrants. In every instance, cells binding Annexin-V and not antibody (top left quadrants) as well as cell binding neither reagent (bottom left quadrants) were apparent. Antibody 2C1 appeared to be unique in its binding characteristics; minimal recognition of apoptotic cells was observed (Figure 26d), an observation in consonance with its lack of binding to internal cellular antigens discussed above.

Observation of an apoptotic cell specificity of some anti-Hb antibodies indicated the existence of cross-reactive epitopes, thereby prompting the inverse experiment; several murine apoptotic cell specific antibodies had previously been generated in the lab from SLE-prone mice, and their recognition of mHb was assessed (Figure 27). Antibodies 2C11 (IgMκ) and 2H8 (IgMκ) exhibited significant reactivity towards mHb. Antibody 1B1 (IgG2aκ) and Antibody 2C3 (IgG2bκ) bound mHb less efficiently whereas Antibody 1B3 (IgG2aκ) was non-reactive. Antibody 1D1 (IgG3κ), which demonstrated reactivity towards healthy cells, was employed as a negative control. The postulate that cross-reactivity between Hb and apoptotic cell-associated antigen(s) exists, was thus further substantiated.

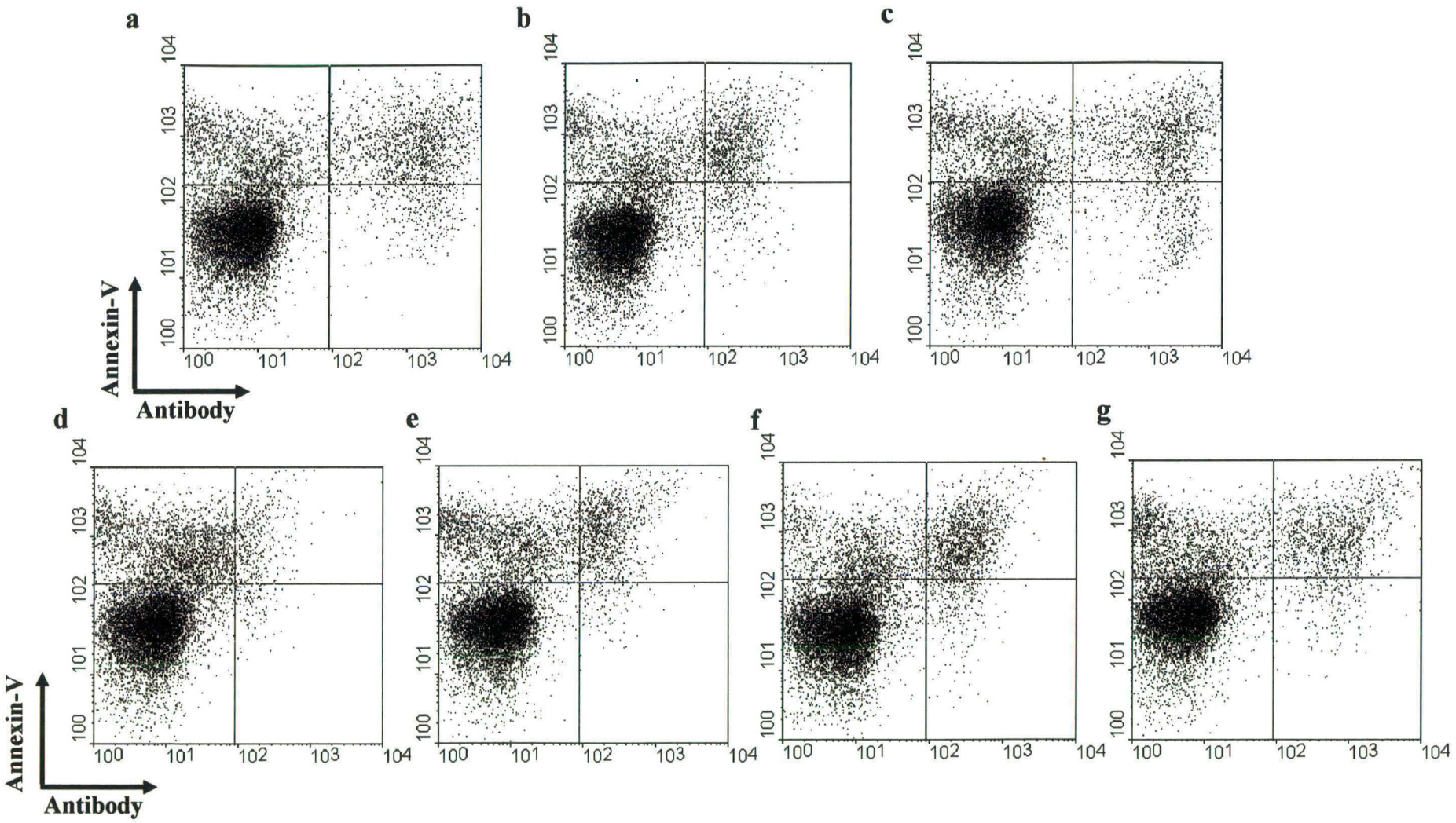


Figure 26: Two-colour flow cytometric analysis of the reactivity of the monoclonal anti-Hb antibodies to cultures containing apoptotic cells. Y axis: Annexin-V; X Axis: Antibodies: (**a**) 1C1. (**b**) 1B5. (**c**) 2A1. (**d**) 2C1. (**e**) 3C4. (**f**) 3A1. (**g**) KV.

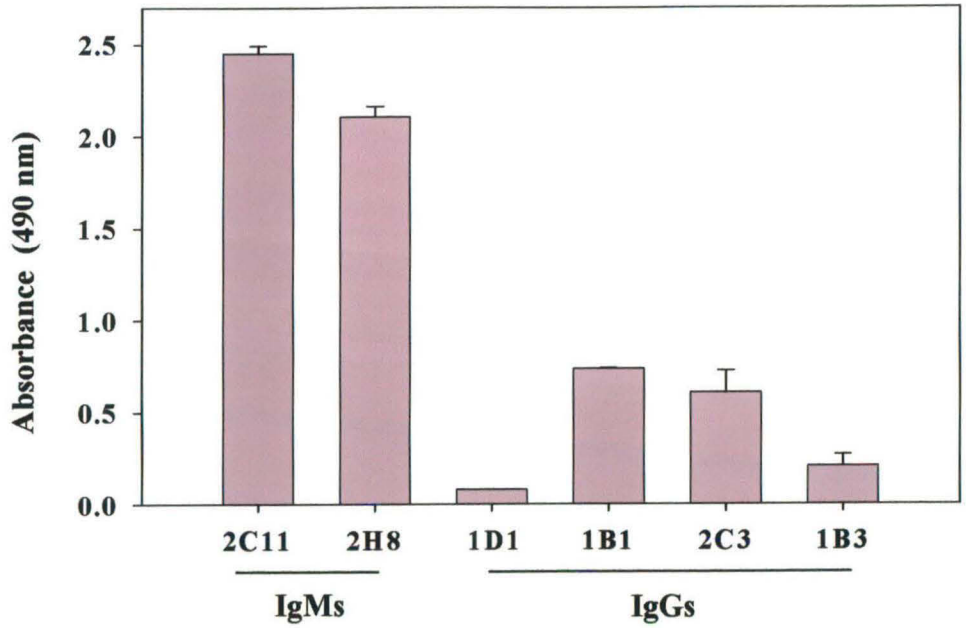


Figure 27: Reactivity of IgM and IgG apoptotic cell-specific autoantibodies to mHb. Antibody 1D1 (reactive to healthy cells) was employed as a negative control.

Reactivity to recombinant auto-antigens

Several auto-antigens are known to be externalized onto apoptotic blebs as cells die. The reactivity of the anti-Hb monoclonal antibodies to recombinant auto-antigens was therefore assessed. ELISAs were conducted using Ro60, SmB, SmD, La and the U1RNP A Protein as targets. With the possible exception of Antibody 1B5, all antibodies appeared to be cross-reactive (Figure 28a). Antibody 1C1 appeared polyreactive, reacting to Ro60, SmD and La proteins. Antibody 2A1 bound Ro60 and the U1RNP A Protein, with reactivity to the latter exceeding even that to Hb. Antibody 2C1 demonstrated the strongest reactivity to the U1RNP A Protein, while Antibody 3C4 displayed a high degree of cross-reactive binding to Ro60. Antibody 3A1 demonstrated significant reactivity towards the U1RNP A Protein and SmD. The human monoclonal antibody KV reacted most strongly with SmD. Though this data is only indicative in nature (since the panel of auto-antigens employed was not exhaustive), it revealed that some auto-antibodies with an Hb specificity can express variable degrees of cross-reactivity towards other known auto-antigens.

Reactivity to cellular antigens – Western blot

Western blots were carried out upon SP2/O cell lysate as described in the Methods section (Figure 28b). Molecular moieties bound by three antibodies (1C1, 1B5 and 2C1) were assessed. Antibodies 1C1 and 1B5 both bound multiple antigens, once again re-iterating their polyreactive nature. Recognized moieties lay in the range of 66 KDa to 100 KDa, and were distinct for the two antibodies. Antibody 2C1 was essentially non-reactive to molecules present in SP2/O cells under these conditions, further attestation to the antibody's relatively poor reactivity to non-Hb self moieties.

Given the reactivity profile of Antibody 1B5 on the one-dimensional Western blot, two dimensional Western blots were carried out as described in the Methods section. Once again, SP2/O lysate was employed. Three prominent spots were obtained for 1B5 (Figure 28e), of molecular weights consistent with those seen on the one-dimensional blot; a few other spots of significantly lower intensity were also observed. Two controls attested to the specificity of interaction: Firstly, in conditions in which only the secondary antibody

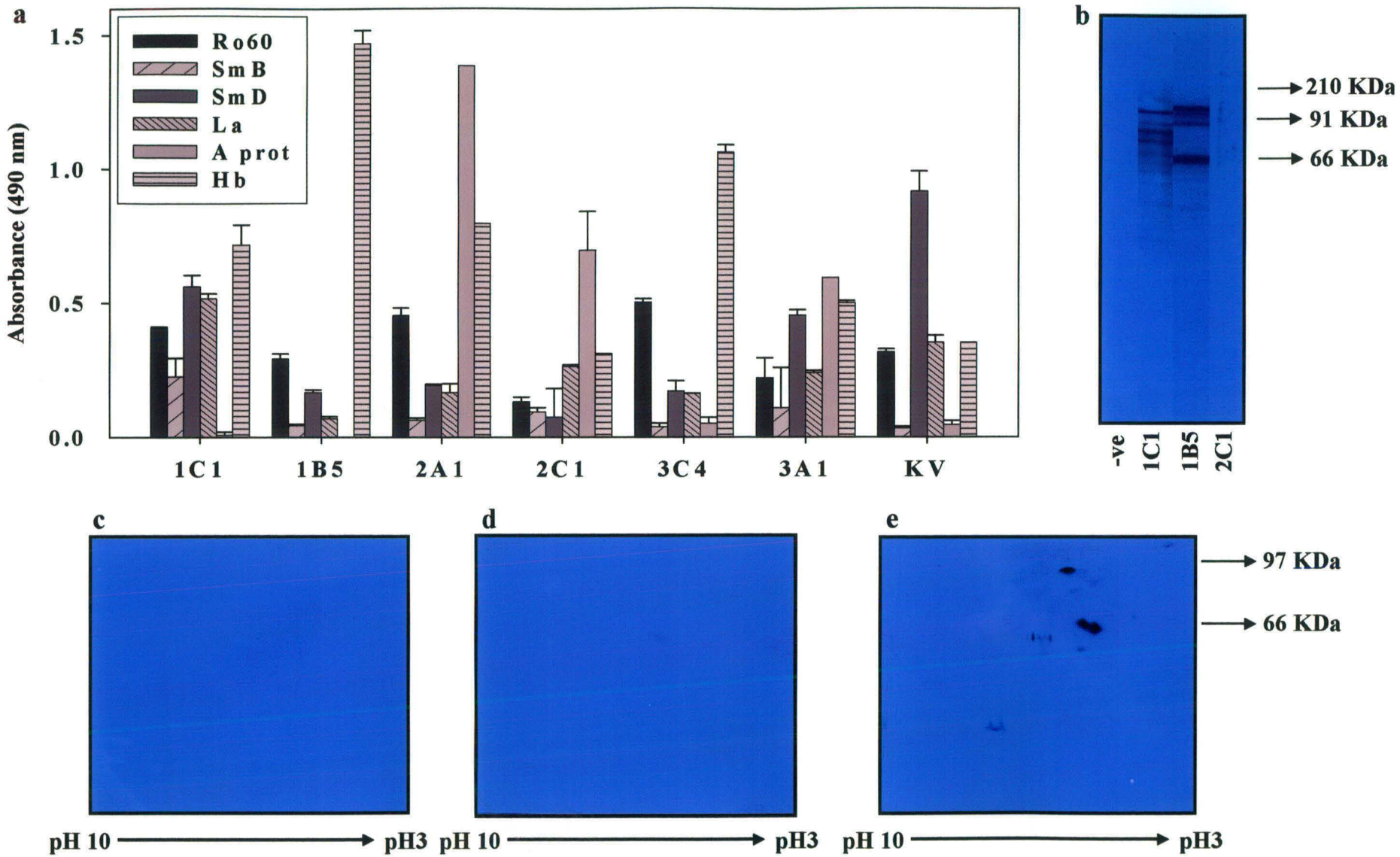


Figure 28: Antigenic reactivity of anti-Hb monoclonal antibodies. (**a**) Reactivity to a panel of recombinant auto-antigens by ELISA. (**b**) Reactivity of Antibodies 1C1, 1B5 and 2C1 to SP2/O lysate by Western blot; -ve: Secondary antibody. (**c, d, e**) Two-dimensional Western blots on SP2/O lysate. (**c**) Secondary antibody. (**d**) 2C1. (**e**) 1B5.

was employed, resulted in no reactivity (Figure 28c); secondly, when the non-reactive Antibody 2C1 was employed as an isotype control, no reactivity was observed (Figure 28e).

Immunization of young NZB/W F1 animals with Hb (Fe^{2+}) and Hb (Fe^{3+})

Since Hb appeared to be antigenic in murine lupus, in human patients of SLE, malaria and leishmaniasis, experiments were undertaken to ascertain whether the molecule was immunogenic as well. Young NZB/W F1 animals were immunized along with an aggressive adjuvant as detailed in the Methods section; Fe^{2+} mHb and Fe^{3+} mHb were independently employed, since some monoclonal antibodies had demonstrated the capability of discriminating between the two forms. Control animals received adjuvant alone.

Results are depicted in Figure 29. In each case, comparisons of reactivity were made with pre-immune serum and with antisera generated using just the adjuvant. Immunization with either ferrous (Fe^{2+}) Hb or ferric (Fe^{3+}) Hb did not result in an increased antibody response to the molecule (Figure 29a). Given the association between anti-Sm and anti-Hb responses (described above), anti-Sm tires were also assessed, and were also found insignificant (Figure 29a). Positive controls in the assays consisted of standard anti-Hb and anti-Sm antibodies. To investigate whether immunization resulted in heightened immune responses to self-antigens, FACS analysis was carried out to assess reactivity of the generated antisera to permeabilised cells; as show in Figure 29b, immunization did not result in increased reactivity. Immunofluorescence on permeabilised cells reiterated these results; antisera generated using Hb was non-reactive in this assay as well (Figure 29c-h). For both FACS and immunofluorescence, the negative control consisted of cells incubated with just the secondary antibody, and a previously established cell-reactive monoclonal antibody was employed as the primary reagent for the positive control. This data suggest that Hb was probably not the primary or initiating immunogen for anti-Hb antibody responses.

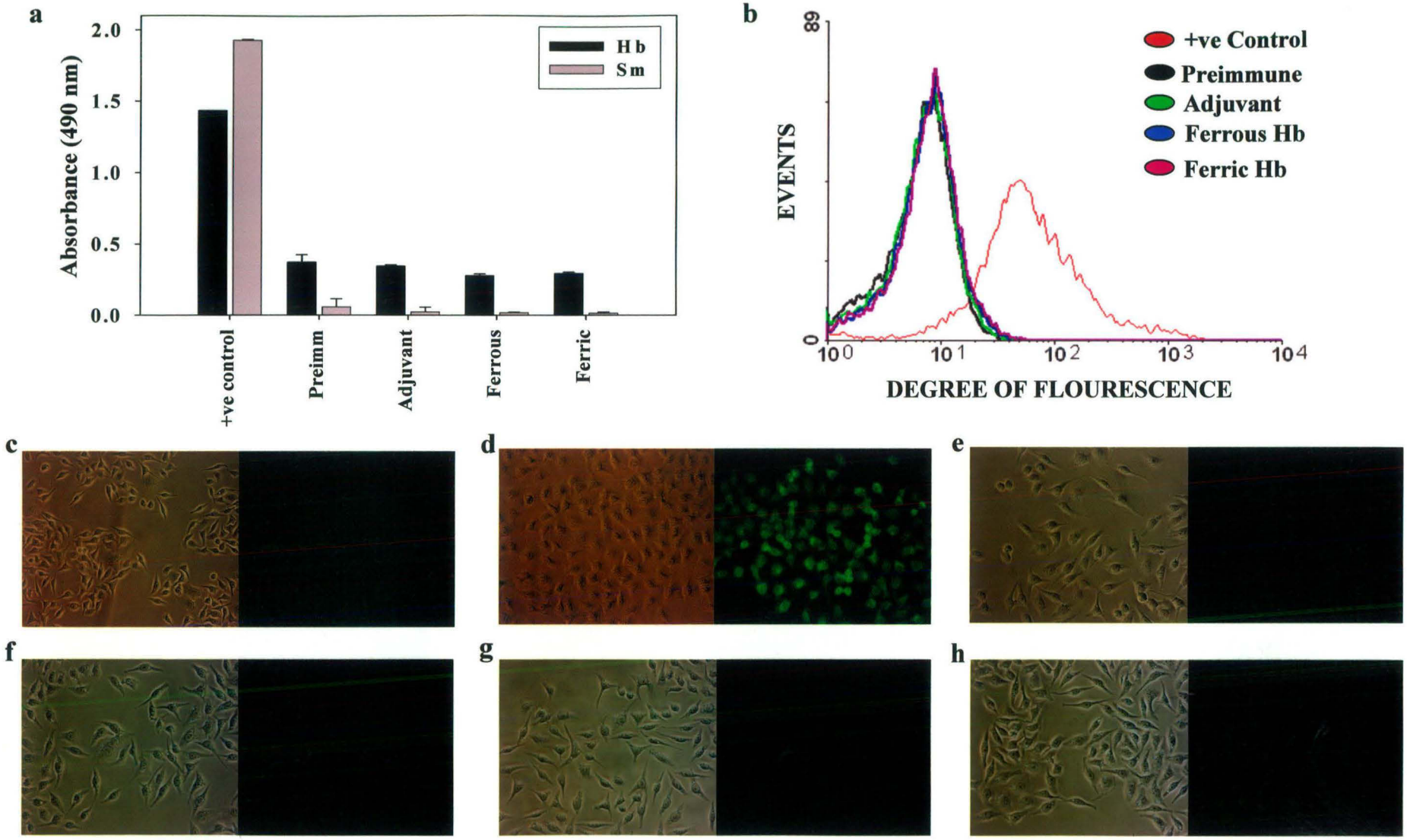


Figure 29: Lack of immunogenicity of ferrous (Fe^{2+}) and ferric (Fe^{3+}) mHb in young NZB/W F1. Reactivity of pre-immune sera, adjuvant-elicited sera and antisera to (**a**) Sm and Hb by ELISA. (**b**) Cellular antigens by FACS. (**c-h**) Immunofluoresence analysis on HeLa cells. Phase contrast images are at left in each case. (**c**) Secondary antibody. (**d**) Positive control. (**e**) Preimmune sera (**f**) Adjuvant-elicited sera (**g**) Ferrous Hb-elicited sera. (**h**) Ferric Hb-elicited sera.

Sequencing of the immunoglobulin variable region genes

Somatic mutations in the complementarity determining regions (CDR) are known to play a role in determining autoimmune antibody specificity[235]. Heavy and light chain variable region gene segments of the anti-Hb monoclonal antibodies were therefore determined, using procedures outlined in the Methods section.

Figures 30-42 describe the results; in these figures, derived sequences have been aligned with the closet germline segments. Nucleotide or amino acid identities have been indicated as dots and somatic mutations are depicted in red. Dashes in germline segments correspond to junctional additions of nucleotides or amino acids, also depicted in red. In the V-D-J (for heavy chains) or V-J (for light chains) junctional analysis, underlined bases indicate junctional deletions in germline segments.

The heavy chain sequence of Antibody 1C1 (Figure 30) demonstrated closest homology with the VH7183.a19.31 germline gene. Mutations occurred across both the framework (FWR) and CDR regions. An A to T silent mutation was observed in FWR1 region. The beginning of the CDR1 region had two replacement mutations, G to A (Serine to Asparagine) and A to G (Tyrosine to Cysteine). The FWR2 had two mutations, G to A (silent) and A to T (Lysine to Methionine). Mutations in CDR2 were G to T (Glycine to Cysteine), G to C (Serine to Threonine) and C to T (silent). The FWR3 had five replacement mutations: T to C (Phenylalanine to Leucine), A to C (Aspartic acid to Alanine), C to A (Alanine to Aspartic acid), A to G (Lysine to Arginine) and A to T (Tyrosine to Phenylalanine). There existed two replacement mutations - T to G (Tryptophan to Glycine) and A to T (Tyrosine to Phenylalanine) in CDR3. FWR4 region was free of mutations. The D region demonstrated highest homology with the DSP2.8 gene segment and the antibody used JH1. One nucleotide (A) was missing from the 3` end of the VH7183.a19.31 germline gene and six nucleotides (CCTAGT) were missing from the 5` end of the D segment. Three non-encoded bases (GGG) at the V-D junction resulted in the addition of two amino acids (Arginine and Aspartic acid). Four nucleotides (CTAC) were missing at the 5` end of the J region. The light chain of Antibody 1C1 demonstrated closest to homology to the ci12 germline sequence and Jκ1 was employed

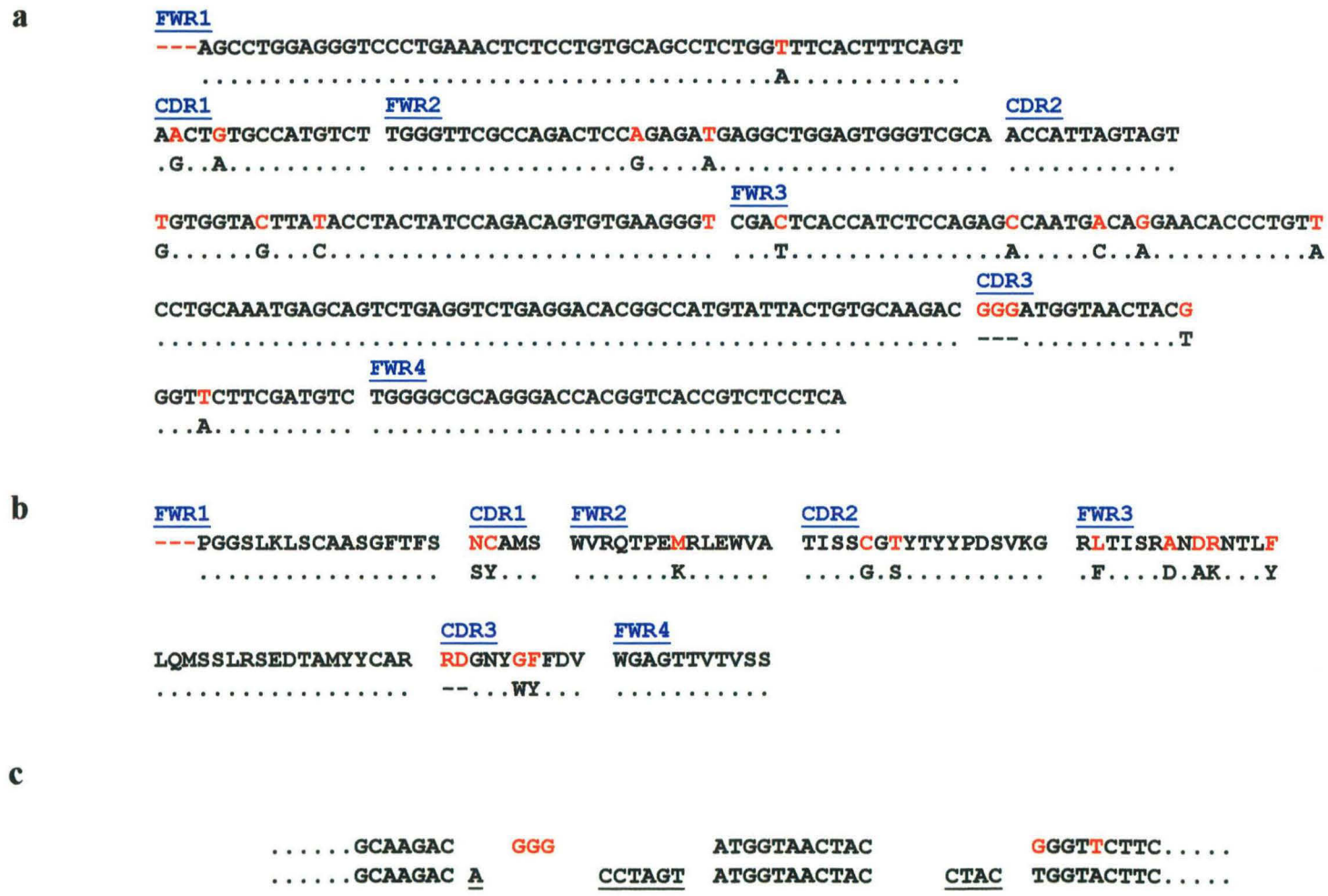


Figure 30: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 1C1 heavy chain. (**c**) V-D-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. Dashes in germline segments correspond to junctional additions of nucleotides or amino acids, also indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

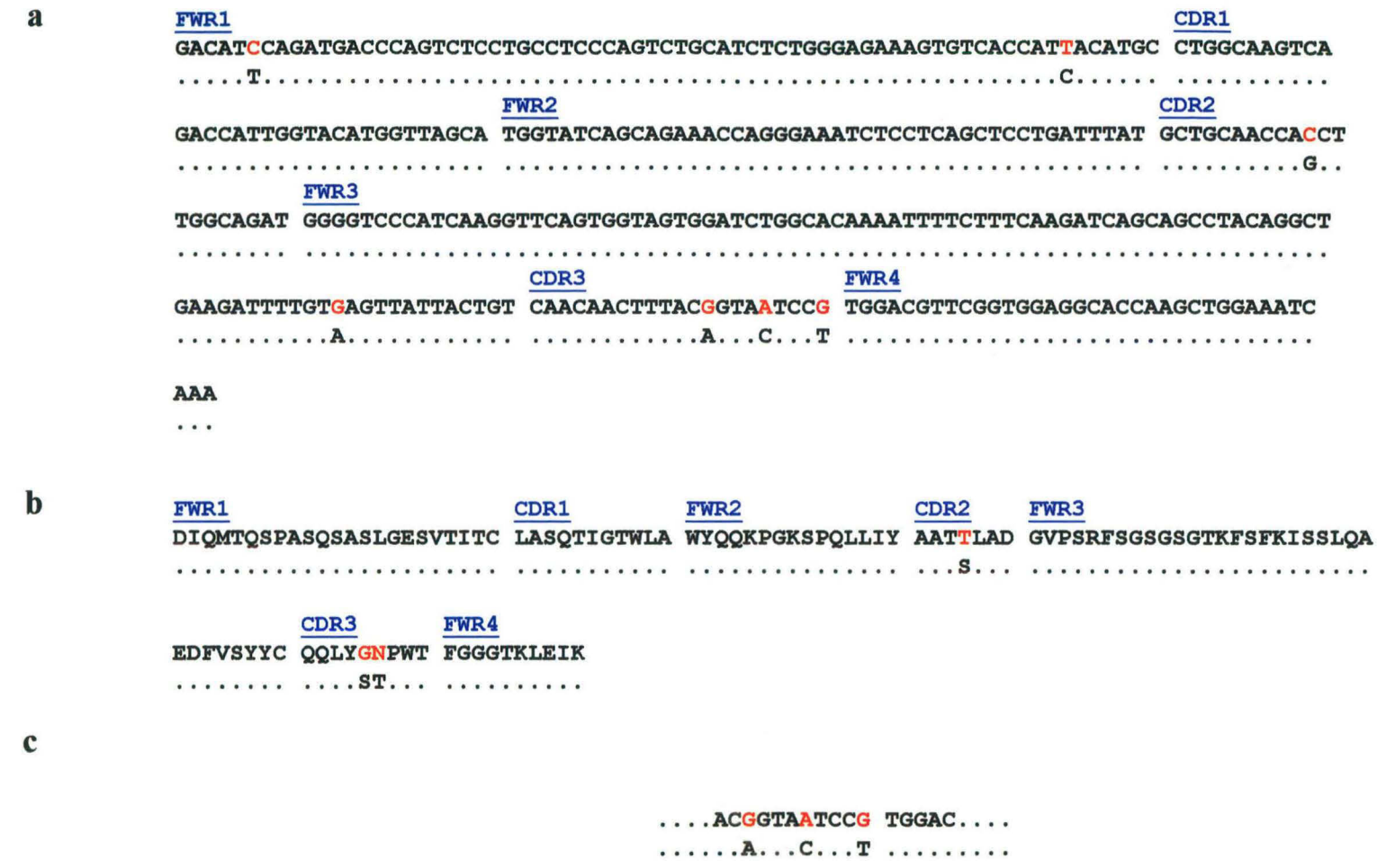


Figure 31: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 1C1 light chain. (**c**) V-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**.

(Figure 31). There were two silent mutations in FWR1, T to C and C to T. The CDR1 and FWR2 had no mutations in them. In CDR2, there was a replacement mutation of G to C (Serine to Threonine). The FWR3 had one silent mutation of A to G. CDR3 had two replacement mutations: A to G (Serine to Glycine) and C to A (Threonine to Asparagine) and one silent mutation, T to G. There was no evidence of exonuclease activity or nucleotide additions at the V-J junction.

Figure 32 shows the sequence analysis of the heavy chain of Antibody 1B5. Closest homology was obtained with the VH7183.a19.31 germline gene. A replacement mutation existed in the FWR1: G to C (Glycine to Alanine). CDR1 had one replacement mutation, G to A (Serine to Asparagine). A G to A silent mutation was seen in FWR2, and CDR2 was free of any mutations. FWR3 had one replacement mutation of A to T (Methionine to Leucine). There were no mutations in the CDR3 and FWR4 regions. The D region had the highest homology to the DSP2.2 family and the antibody employed the JH4 germ line segment. Analysis of V-D-J junction showed an extensive exonuclease activity. Two nucleotides (CA) from the 3` end of the VH7183.a19.31 germline segment had been removed and several nucleotides (TCTACTAT) from the 5` region of the D segment were missing. The 5` region of the J region also had several nucleotides (ATTACTAT) missing. The light chain employed the 8-28 germline sequence and Jκ4 gene segment (Figure 33). The light chain was free of any mutations. V-J junction analysis showed just one nucleotide (T) missing from the 3` end of the 8-28 germline segment.

Analysis of the Antibody 2A1 heavy chain variable region revealed usage of the J558.69.170 germ line gene sequence, and the DSP2.2 and JH2 segments (Figure 34). One silent mutation in FWR3 (A to G) was observed. Six nucleotides (TCTACT) were missing from the 5` end of the D region and one non-encoded nucleotide (G) was added, resulting in the addition of a non-encoded amino acid (Aspartic acid) at the V-D junction. The 5` end of the J region had three nucleotides (TAC) missing, and three non-encoded nucleotides (GGG) were added, resulting in the addition of a non-encoded base (Glycine) at the D-J junction. The light chain of Antibody 2A1 demonstrated closest homology to

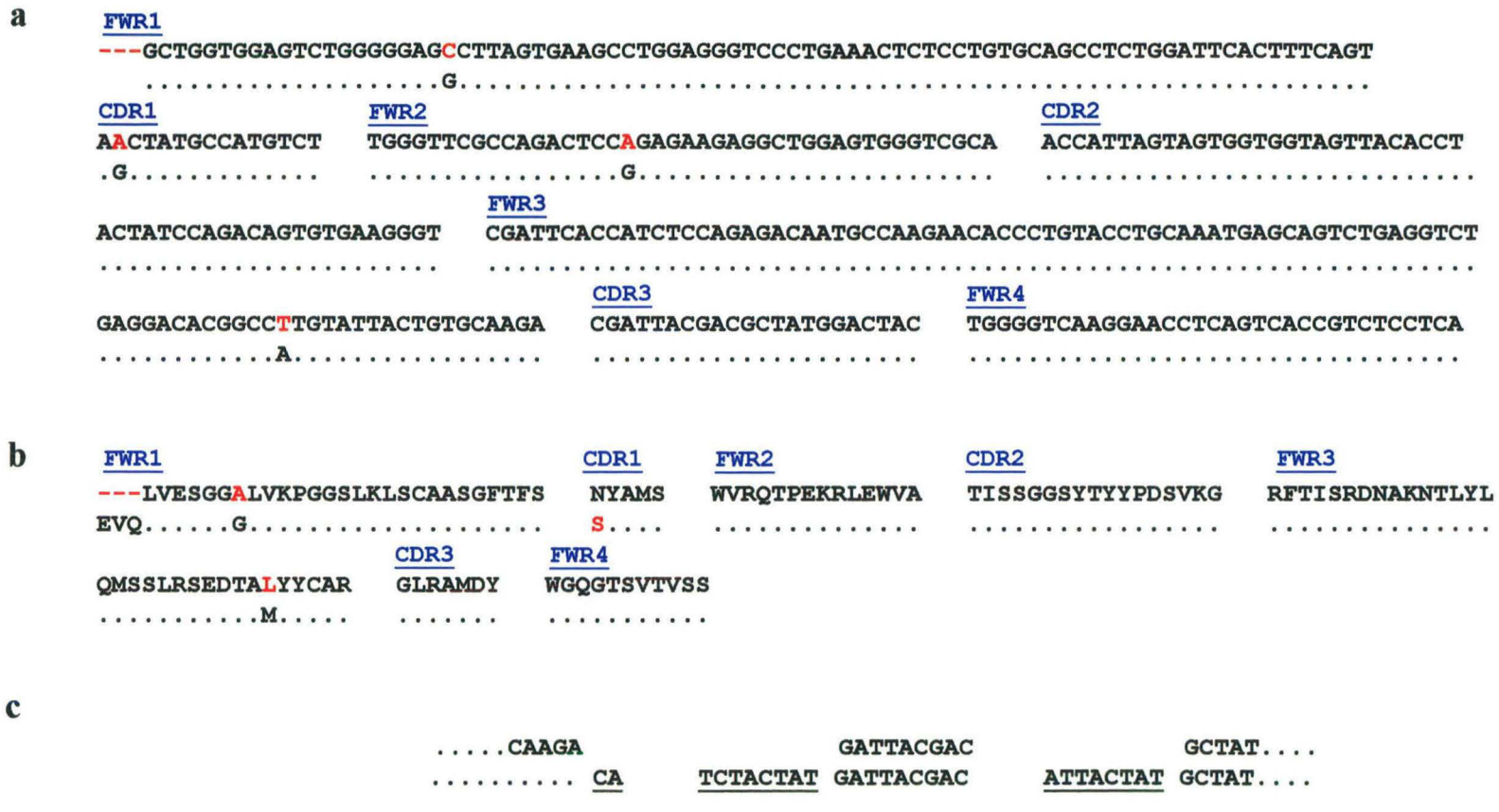


Figure 32: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 1B5 heavy chain. (**c**) V-D-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

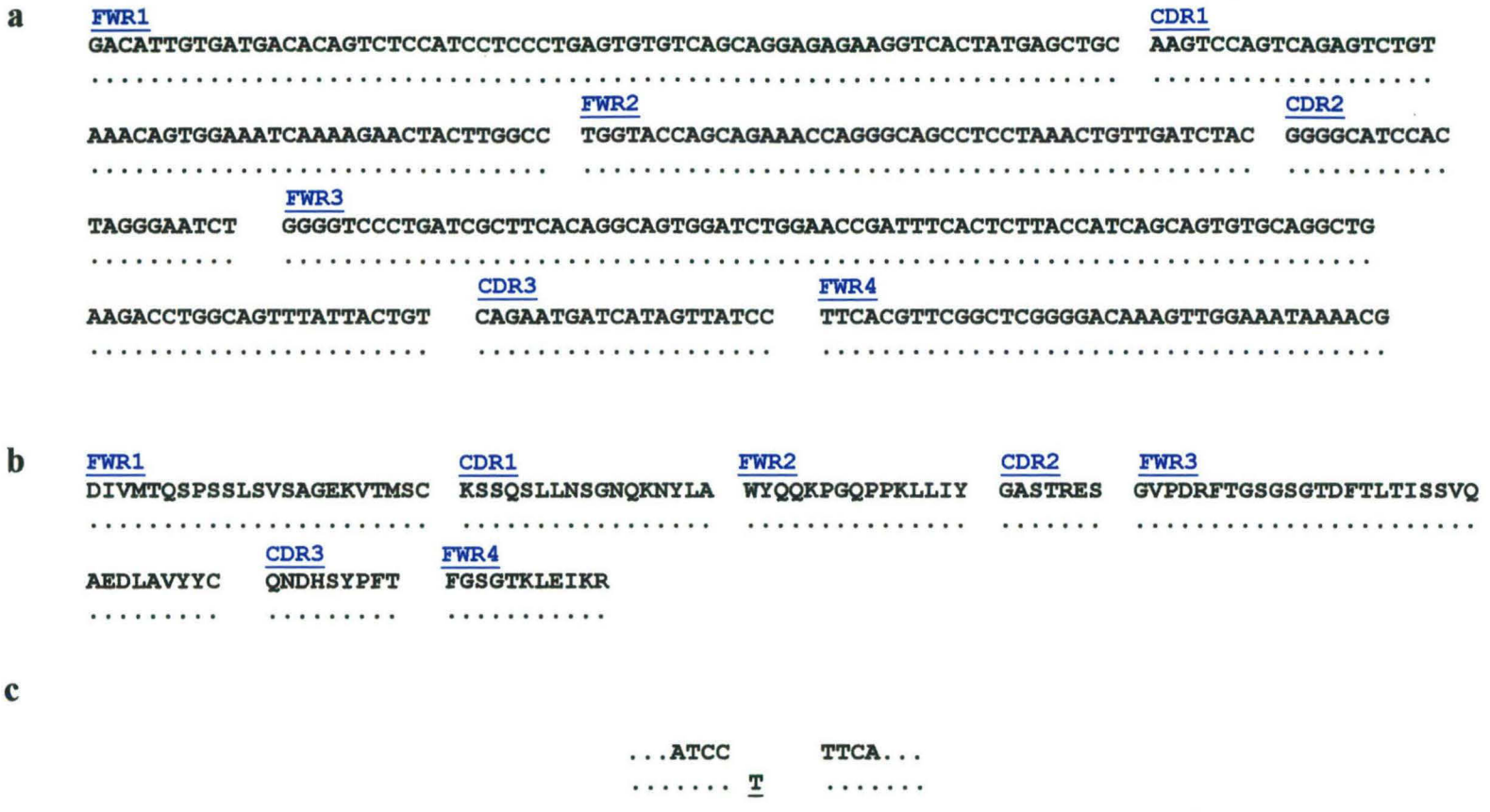


Figure 33: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 1B5 light chain. (**c**) V-J junction. Homologies with the closest germline gene sequence are indicated as dots. In (**c**), the underlined base indicates a junctional deletion in the germline segment.

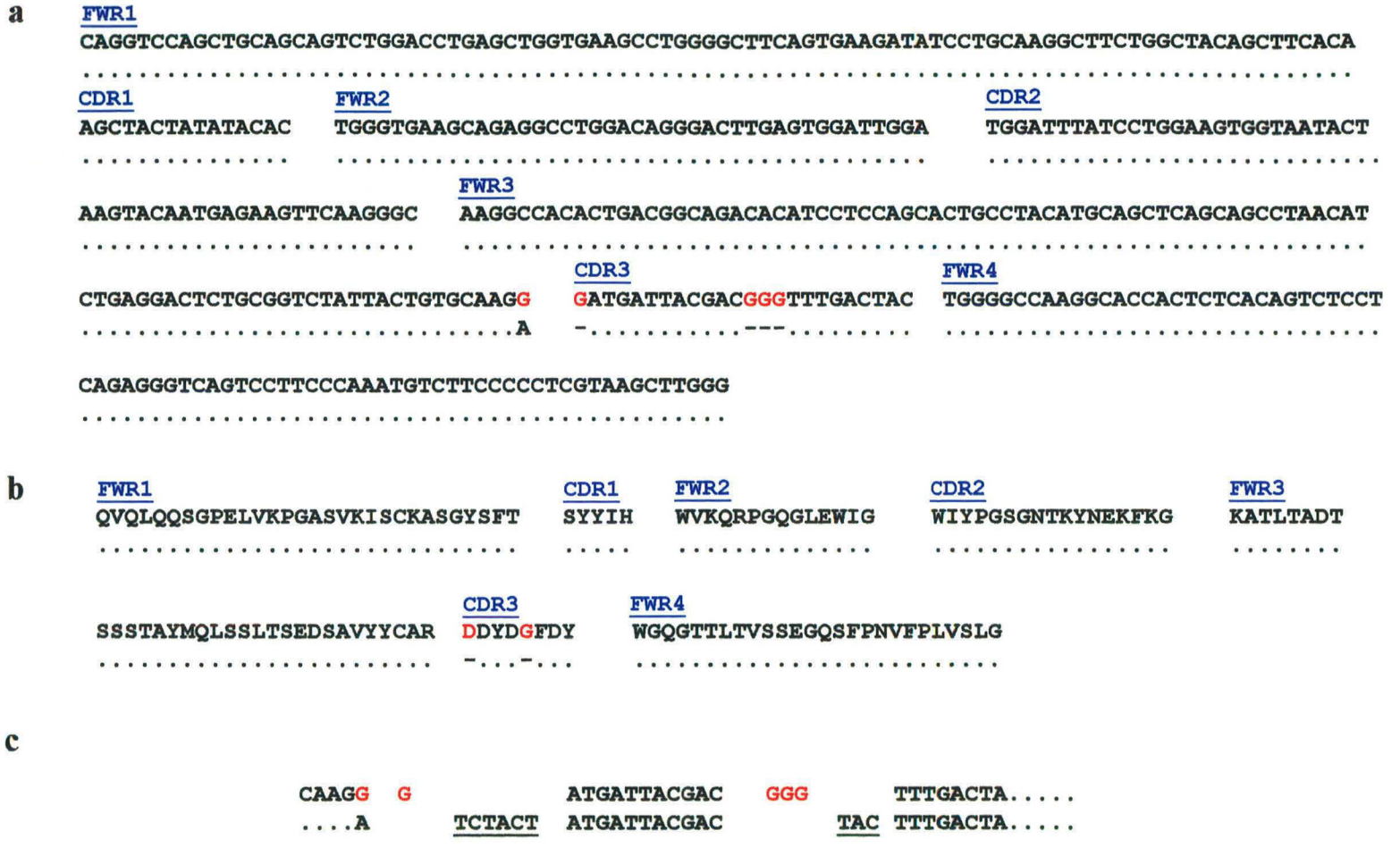


Figure 34: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 2A1 heavy chain. (**c**) V-D-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. Dashes in germline segments correspond to junctional additions of nucleotides or amino acids, also indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

8-21 germ line gene segment and used the JK2 gene segment (Figure 35). A silent mutation (T to G) was observed in the CDR3 region.

The heavy chain of Antibody 2C1 (the antibody which appeared relatively specific for Hb) used the V186.2 germline gene and the DQ52-C57BL/6 and JH1 gene segments (Figure 36). Save for a replacement somatic mutation (G to A; Alanine to Threonine) in FWR4, no mutations were seen. Two nucleotides (CT) were deleted at the 5` end of the D segment, and four non-coded bases (GGGA) were added, leading to the addition at the V-D junction of two non-encoded amino acids (Glycine and Lysine). Three nucleotides (GAC) were deleted from the 3` end of the D segment, while one non-encoded nucleotide (C) appeared to have been added, leading to the addition of a non-encoded amino acid (Alanine) at the D-J junction. Figure 37 shows the analysis result of the light chain of Antibody 2C1. Highest homology to the 23-43 germline gene was observed, and the Jκ5 segment was used. No mutations were observed, except for a silent mutation (T to G) in CDR3.

The heavy chain of Antibody 3C4 demonstrated highest homology with the J558.55.149 germline gene (Figure 38). The antibody used the DSP2.8 and JH2 gene segments. No mutations were seen, save for a single replacement mutation in CDR3 (G to C; Valine to Leucine). Analysis of V-D-J region revealed that 5` end of the D region had undergone exonuclease activity and resulted in the loss of six bases (CCTAGT). Three non-encoded bases (GGA) were added at the V-D junction, resulting in the addition of a non-encoded amino acid (Glycine). Two bases (AC) were missing from the 3` of the D region. Three bases (TAC) were removed at 5` end of the J region segment as well. The light chain sequence of Antibody 3C4 is shown in Figure 39; the variable region gene demonstrated the highest homology to the bb1 germline gene; Jκ5 was employed. A single replacement mutation (T to C; Leucine to Proline) was observed in the CDR3 region.

For Antibody 3A1, the heavy chain exhibited the highest degree of homology to the J558.47 germline gene segment; the DFL16.1 and JH1 gene segments were employed (Figure 40). No mutations were observed. Exonuclease activity deleted one base each

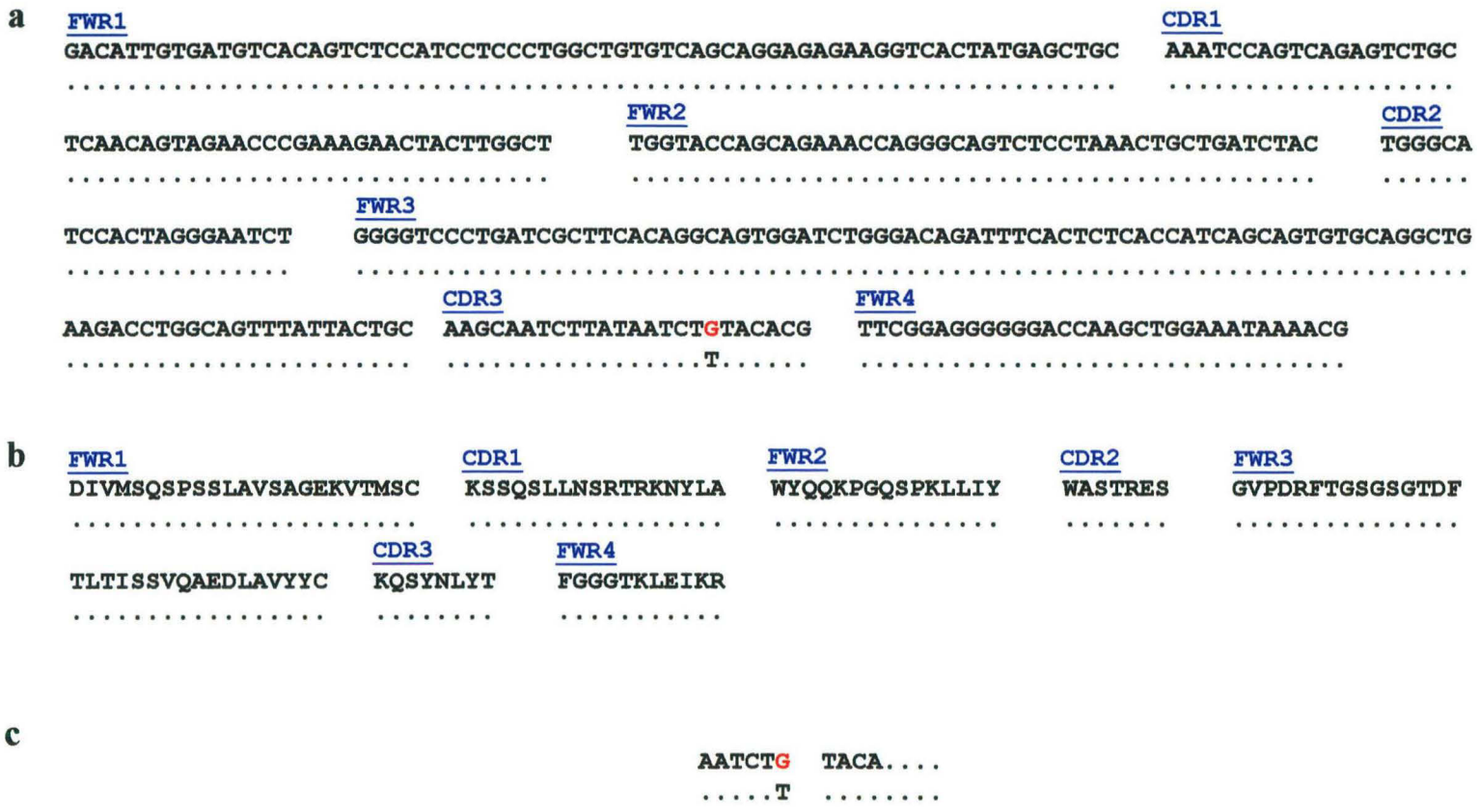


Figure 35: (a) Nucleotide and (**b**) amino acid sequence of Antibody 2A1 light chain. (**c**) V-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide mutations are indicated in **red**.

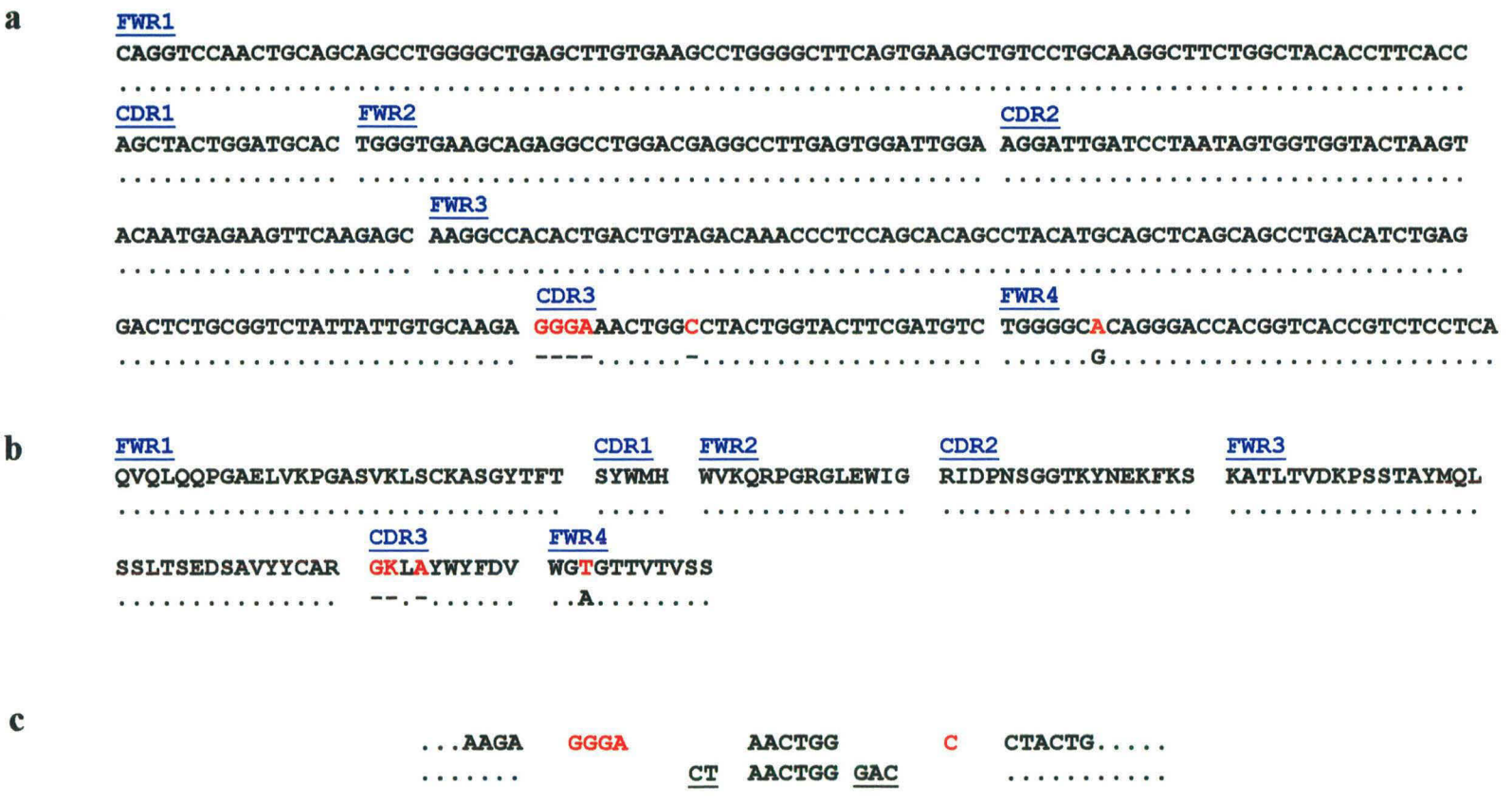


Figure 36: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 2C1 heavy chain. (**c**) V-D-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. Dashes in germline segments correspond to junctional additions of nucleotides or amino acids, also indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

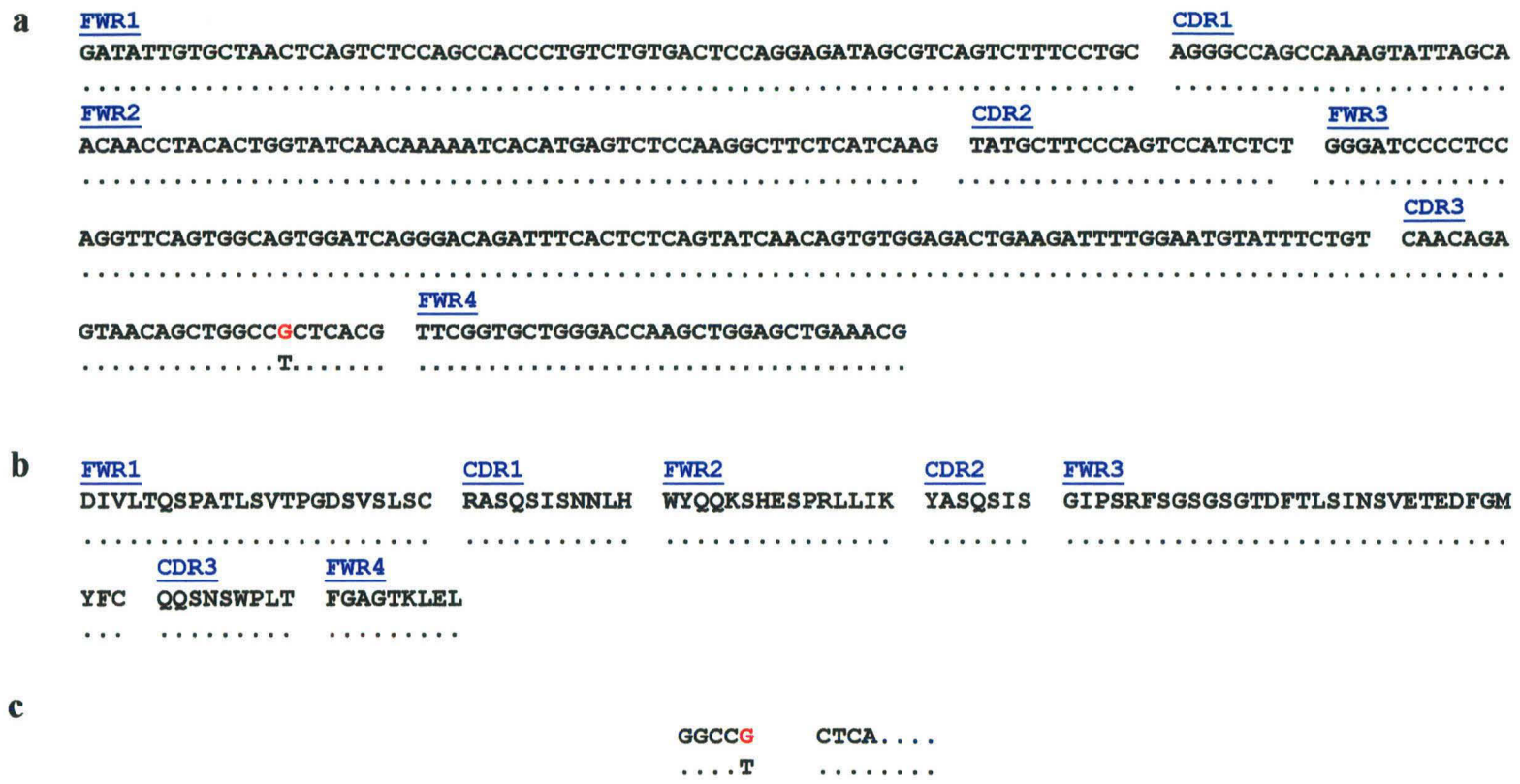


Figure 37: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 2C1 light chain. (**c**) V-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide mutations are indicated in **red**.

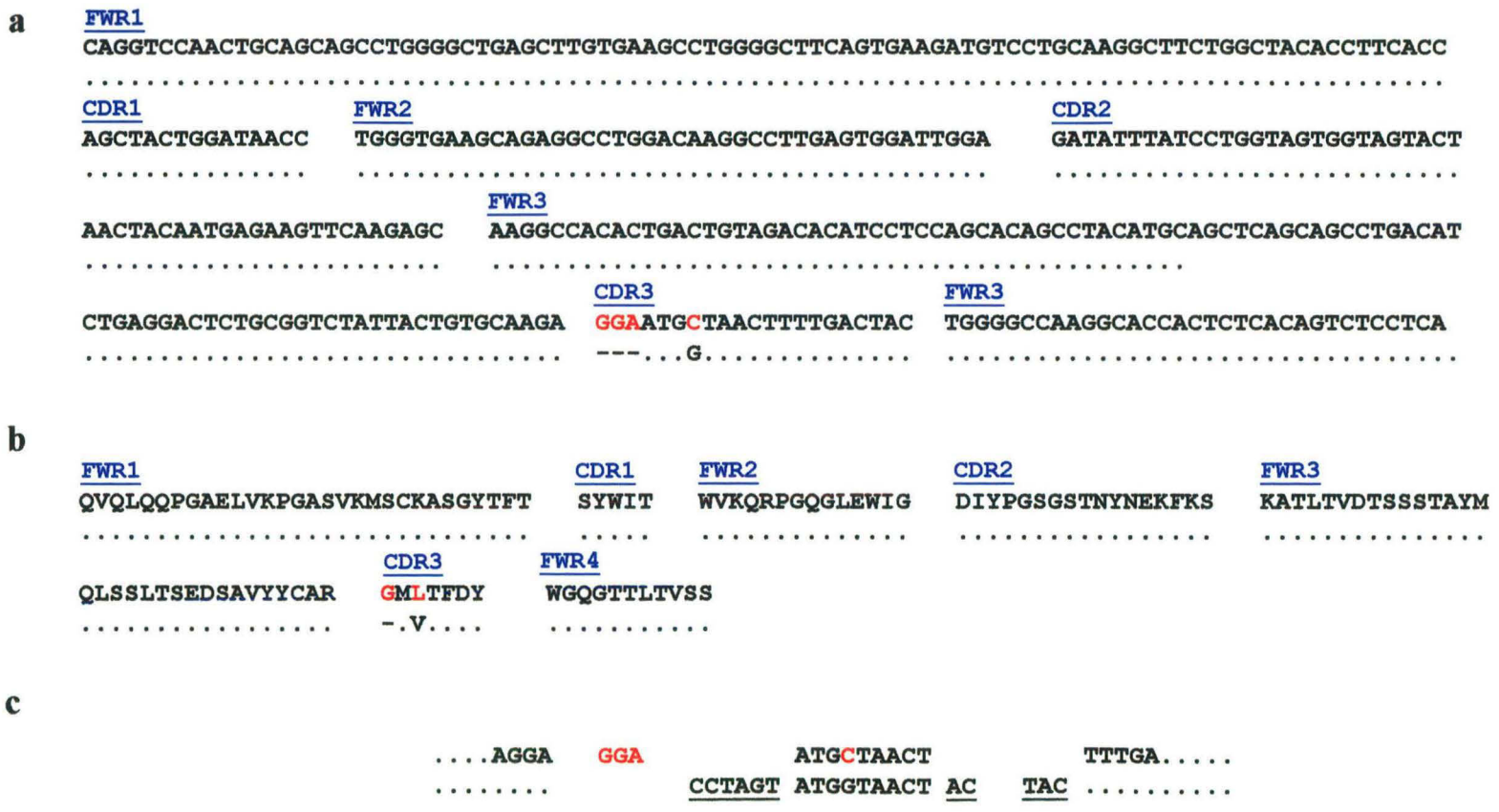


Figure 38: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 3C4 heavy chain. (**c**) V-D-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. Dashes in germline segments correspond to junctional additions of nucleotides or amino acids, also indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

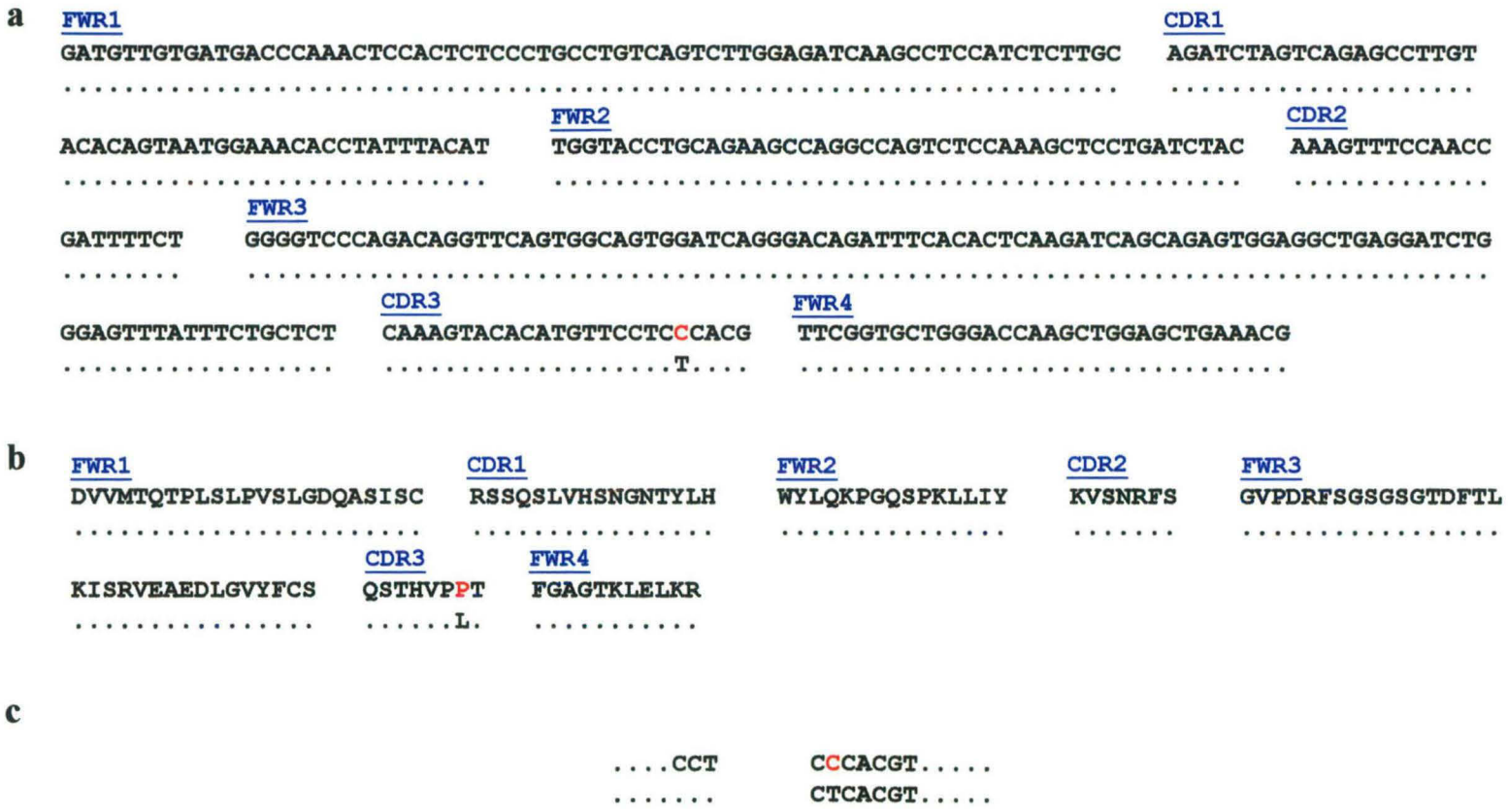


Figure 39: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 3C4 light chain. (**c**) V-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**.

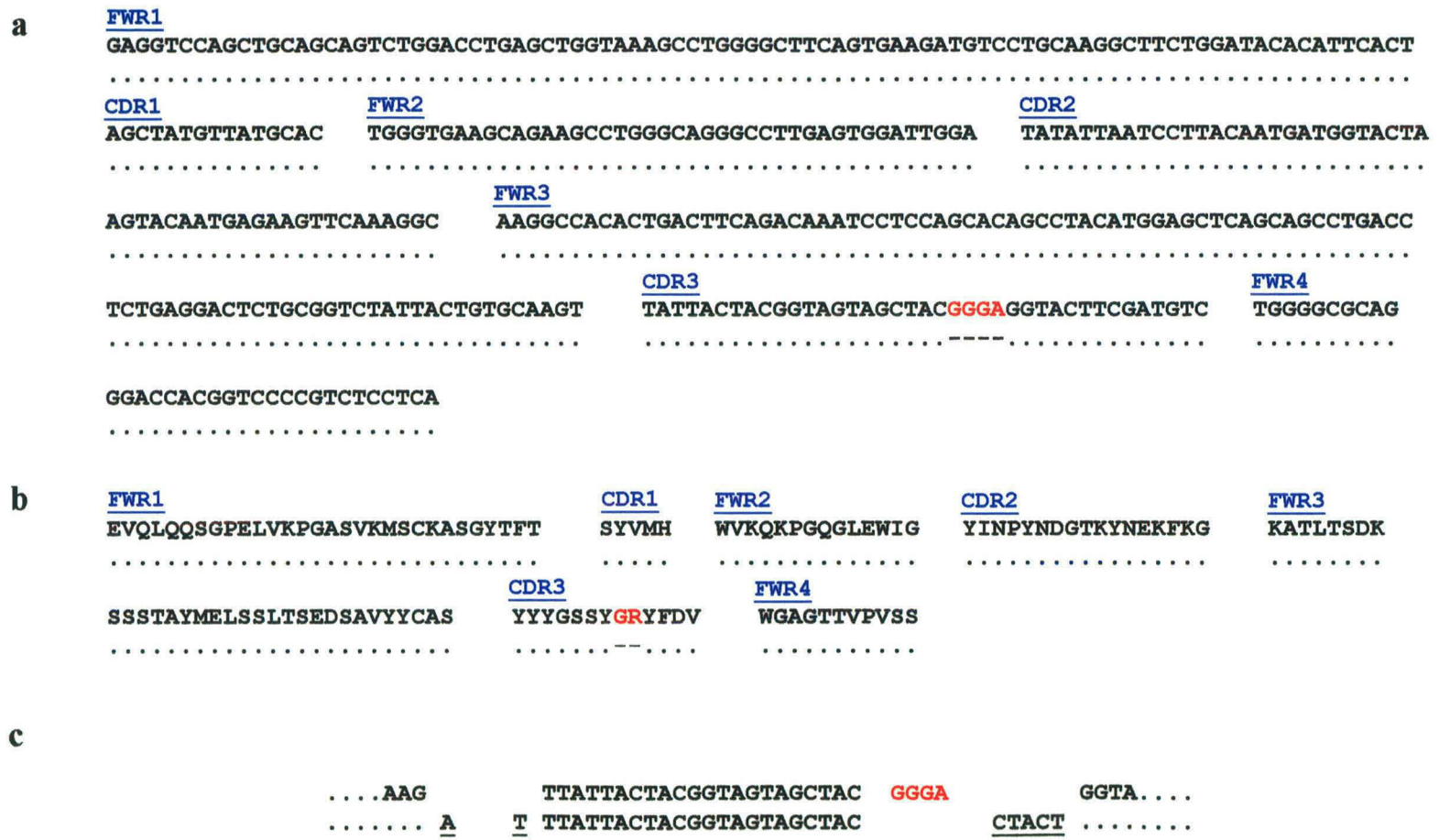


Figure 40: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 3A1 heavy chain. (**c**) V-D-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. Dashes in germline segments correspond to junctional additions of nucleotides or amino acids, also indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

from the 3` end of the J558.47 gene (A) and the 5` end of the D segment (T). In addition, 5 bases (CTACT) were deleted from the 5` end of J segment. Four non-encoded nucleotides (GGGA) were added between the D and J, which contributed to the addition of two non-encoded amino acids (Glycine and Arginine). Data for the light chain of Antibody 3A1 is shown in Figure 41. Several mutations were seen, spread across the sequence, which demonstrated the highest homology to the 21-12 germline gene and used the Jκ2 gene segment. FWR1 contained a replacement mutation (C to A; Serine to Tyrosine). CDR1 did not have any somatic mutation. In the FWR2, there were two replacement mutations (T to A; Tyrosine to Asparagine and A to G; Lysine to Arginine). The CDR2 also had one replacement mutation, C to T (Alanine to Valine). In the FWR3, an A had been mutated to T, causing replacement of Asparagine to Isoleucine. CDR3 contained a G to T mutation, causing an amino acid replacement (Serine to Isoleucine). FWR4 had no mutations. In the V-J junction, exonuclease activity at the 3` end of the V region resulted in the loss of 5 nucleotides TCCTC.

The heavy chain of the human monoclonal anti-Hb antibody demonstrated the closest homology with the IGVH3-11*03 germline gene (Figure 42). The D region demonstrated highest homology to IGHD-10*02, it was extensively modified as described below, limiting confidence in this classification. The J region belonged to IGHJH4*02. In the FWR1, there were two mutations, one replacement (T to G; Leucine to Valine) and other silent (G to A). A silent mutation (C to T) was observed in FWR3. Thirteen bases (GTATTACTATGTT) appeared to be missing from the 5` end of the D region, and six non-encoded bases (GCTTCC) were added at the V-D junction, leading to the addition of two non-encoded amino acids (Alanine and Serine). Twelve bases (AGTTATTATAAC) were missing from the 3` end of the D region, and four (ACTA) from the 5` end of the J region. In addition, nine non-encoded bases (TGCAGGTT) were added at the D-J junction, as a result of which, three non-encoded amino acids (Alanine, Glycine, Phenylalanine) were added. The light chain of KV could not be sequenced due to the problems in cloning procedures.

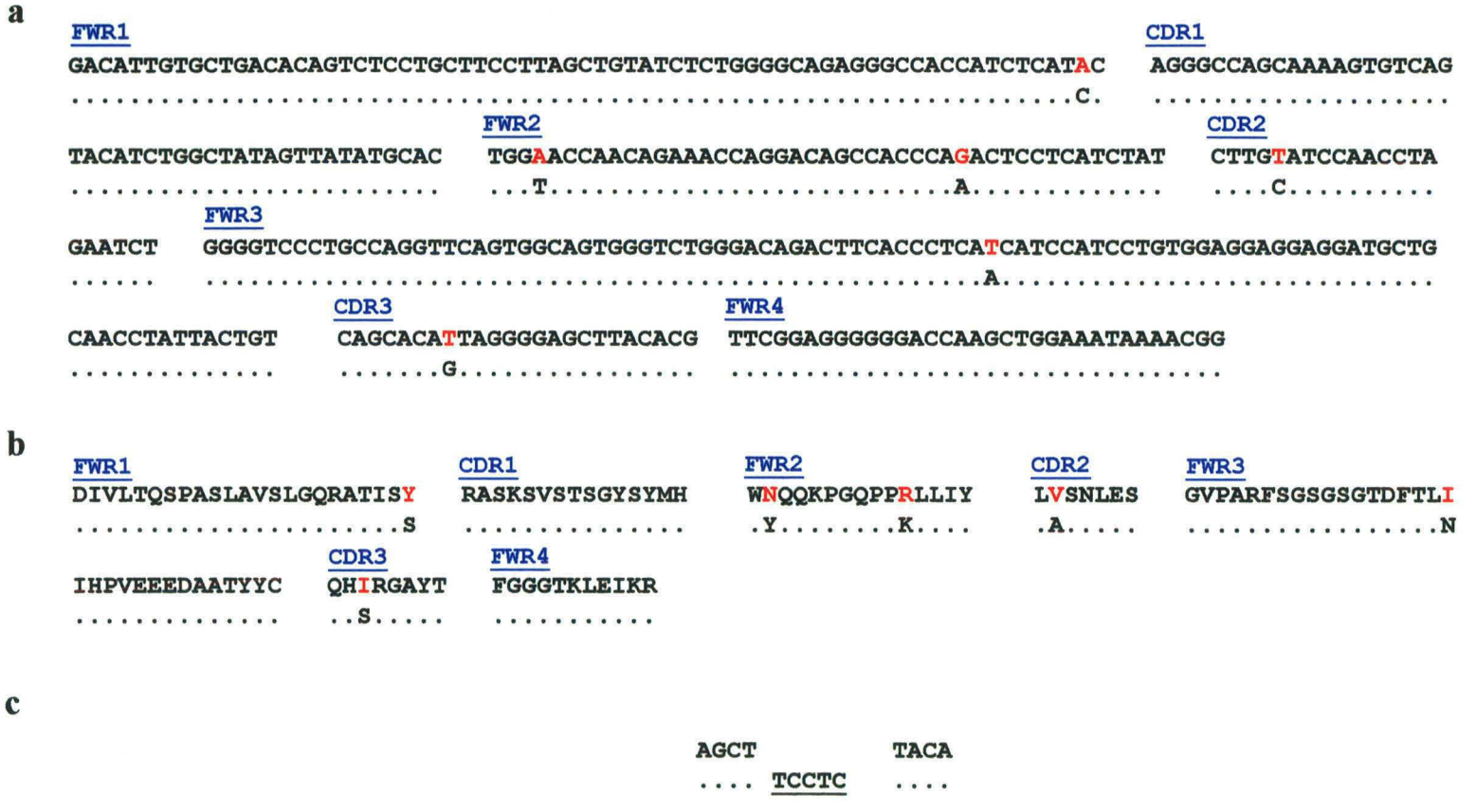


Figure 41: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody 3A1 light chain. (**c**) V-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

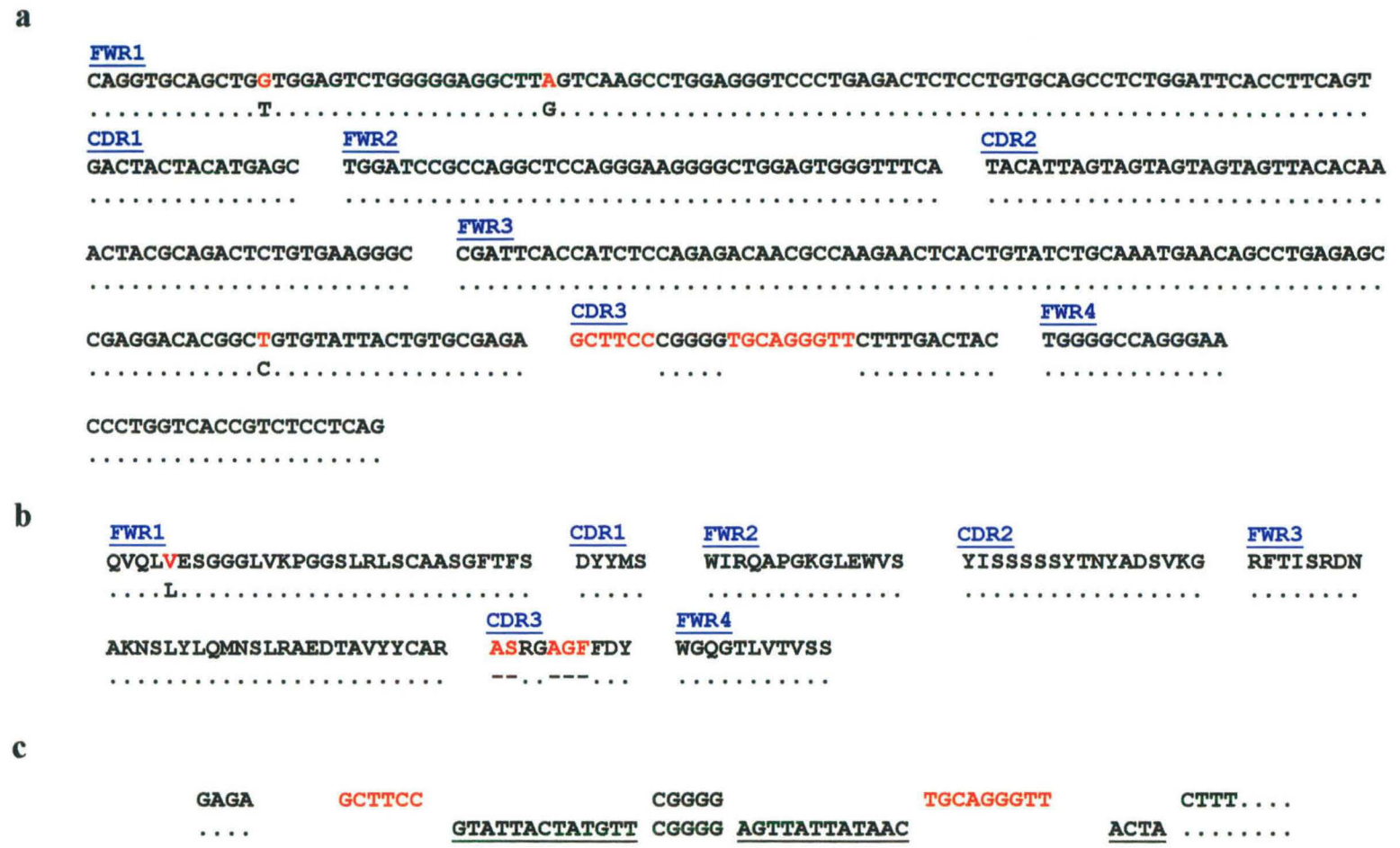


Figure 42: (**a**) Nucleotide and (**b**) amino acid sequence of Antibody KV heavy chain. (**c**) V-D-J junction. Homologies with the closest germline gene sequence are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. Dashes in germline segments correspond to junctional additions of nucleotides or amino acids, also indicated in **red**. In (**c**), underlined bases indicate junctional deletions in germline segments.

Biological consequences of the anti-Hb antibody response

Effects on cytokine secretion

Free Hb causes cellular toxicity[207] and induces the production of inflammatory cytokines from monocytes and endothelial cells[236,237]. TNF-α, IL-6 and IL-8 are the major cytokines produced. The effect of the anti-Hb monoclonal antibodies on Hb-induced cytokine secretion was investigated, as described in the Methods section (Figure 43). Sub-optimal doses of Hb caused minimal increase in the secretion of TNF-α. In most cases, addition of the antibodies alone did not result in secretion of the cytokine, with Antibody 2A1 being the exception; a slight increase was observed, although the difference with the negative control (medium alone) was found not significant. Co-incubation of Hb and the Antibody 2A1 with cells caused a significant increase in the secretion of TNF-α, an effect not seen with the other anti-Hb antibodies, or with the two isotype controls employed in the experiment (Figure 43a). Increases were observed over conditions where medium alone was employed (p=1.54919E-4), where Hb alone was added (p=5.56842E-4), as well as where the antibody alone was added (p=0.00884). Antibody 2A1 thus appeared to be acting in synergy with Hb. Similar results were seen when levels of IL-6 were measured in the supernatants (Figure 43b). In this case too, sub-optimal doses of Hb or the antibodies by themselves did not cause significant increases in the secretion of the cytokine. Antibody 2A1 was capable of significantly increasing the secretion of IL-6 over the medium control (p=0.00143). The combination of Hb and Antibody 2A1 caused a significant increase over conditions where medium alone (p=0.01045), Hb alone (p=0.01267) and the antibody alone (p=0.0133) were employed, and synergy once more was apparent. None of the other antibodies or isotype controls caused this effect. Incubation of Hb with cells caused a significant increase (p=0.00451) in the secretion of IL-8, as did incubation with Antibody 2A1 alone (p=0.00489) (Figure 43c). None of the other anti-Hb antibodies or isotype controls caused such an increase. Co-incubation of Hb and Antibody 2A1 caused further increase in the secretion of IL-8 with respect to medium (p=9.34797E-4), Hb alone (p=0.00835) and Antibody 2A1 alone (p=2.20336E-4), effects not seen with the other anti-Hb antibodies or isotype controls.

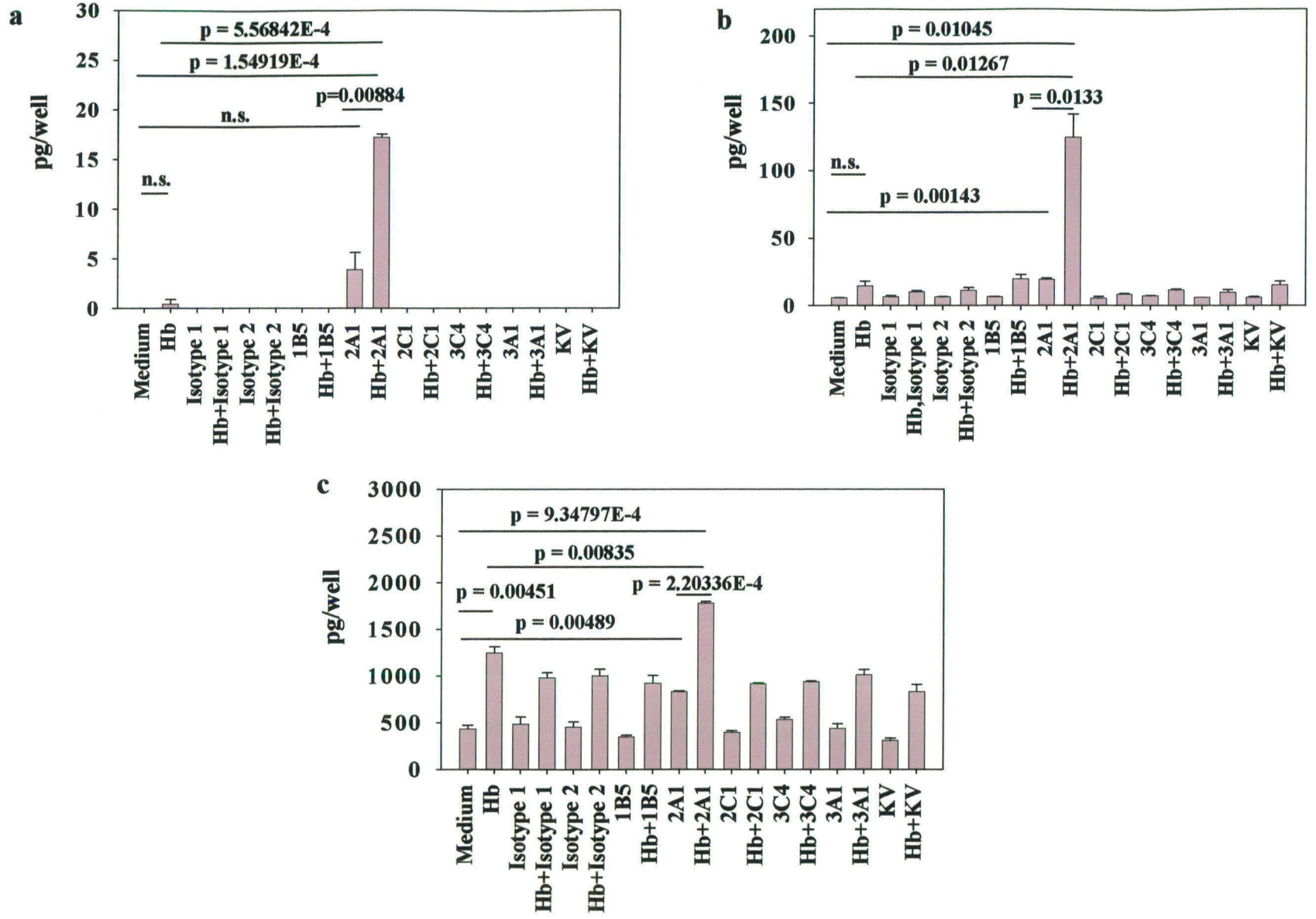


Figure 43: (**a**) TNF-α (**b**) IL-6 and (**c**) IL-8 secretion by Hb, by individual anti-Hb auto-antibodies and by Hb plus the antibodies. Isotype 1 and Isotype 2 refer to antibodies of the IgMκ isotype of irrelevant specificity, taken as negative controls.

Endothelial cells first come into contact with free Hb upon its release from RBC and are known to suffer significant damage[206]. These cells secrete chemotactic factors (mainly IL-8 and VCAM) in response to Hb that cause migration of immune cells to the site of injury. Human Brain Microvascular Endothelial Cells (HBMEC) were incubated with Hb with and without Antibody 2A1 (or an isotype control antibody). Supernatants were transferred to fresh 24-well plates, after which transmigration experiments using THP-1 cells were carried out as described in the Methods section (Figure 44). Incubation of endothelial cells with suboptimal doses of Hb did not elicit factors in the supernatant which could cause the chemotaxis of monocytes; similarly, Antibody 2A1, or the antibody employed as the isotype control, did not enhance transmigration compared to conditions where just the medium was employed. The combination of Antibody 2A1 and Hb significantly enhanced migration over the medium control ($p=0.01402$) and over conditions where just Hb ($p=0.01309$) or just the antibody ($p=0.02889$) were employed. Significant differences ($p=0.000917$) were also seen in comparison with conditions where Hb and the isotype control antibody were employed.

It can be inferred from these results that some anti-Hb antibodies, in combination with Hb, appear to be capable of inducing the synergistic release of inflammatory cytokines (and possibly other moieties) which in turn may play a role in inducing the migration of monocytic cells.

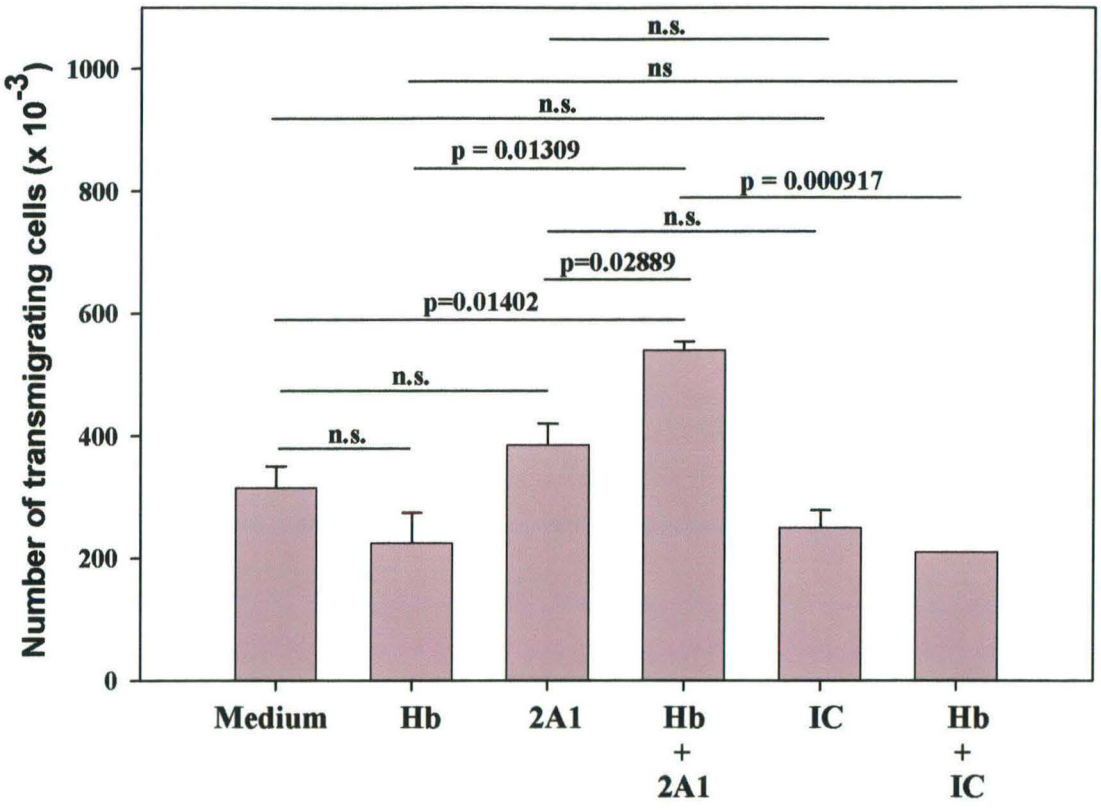


Figure 44: Transmigration of THP-1 cells towards supernatant obtained from HBMEC cells after incubation under the specified conditions. IC: Isotype control; an antibody of the IgMκ isotype of irrelevant specificity, employed as negative controls. n.s.: Not significant.

Discussion

DISCUSSION

Defects in the immune system can have serious pathological consequences. For example, aberrant recognition of self-molecules can result in autoimmune responses. It is now believed that autoimmunity *per se* may not be very infrequent; however, when autoimmune T cells and auto-antibodies secreted by B cells adversely organ function, autoimmune diseases occur.

SLE is a prototypic systemic autoimmune disease. As the name suggests, multiple organs may become targets of the autoimmune response. Diagnosis is based on presence of four out of eleven clinical criteria (enumerated by the American College of Rheumatology), either simultaneously or during any period of observation. Most organ damage in SLE is believed to be antibody-mediated, though T cells play a critical role in the generation of auto-antibody responses[93]. Auto-antibodies against some antigens can be observed as early as nine years before the onset of clinical symptoms[22]. Although auto-antibodies against more than a hundred auto-antigens have been described, most may be epi-phenomena, and may not necessarily affect end-organ function. A few auto-immune specificities have been found to be associated with specific pathologies, however; anti-dsDNA antibodies have been linked to chronic renal failure[110,111], anti-Ro and anti-La antibodies with neonatal lupus[25,27,29], antibodies to the ribosomal P protein with the neuro-psychiatric manifestation often seen in lupus[32], and anti-phospholipid (targeting cardiolipin, phosphatidylserine and other lipids) and anti-RBC antibodies (against Rh antigens, Band 3 and other antigens) with the lysis of RBC. As a result of autoimmune hemolysis, and also of non-autoimmune hemolysis in diseases such as malaria and leishmaniasis, Hb is released into circulation. Hb scavenging systems, such as haptoglobin (the Hb-binding protein) and hemopexin (the heme-binding protein) may be quickly overwhelmed[192,201,202,203]. Free heme and/or released iron can exert a variety of toxic effects.

Some evidence of aberrant T cell reactivity to the Hb does exist. The NZB animals develop spontaneous auto-antibodies to their own RBCs by 6 months of age, leading to autoimmune hemolytic anemia. When autologous RBC lysate was added to splenocytes, heightened proliferative responses were observed. Incubation of the splenocytes from these animals with the autologous purified Hb resulted in the preferential proliferation of $CD4^+$ T cells; unlike in the non-

autoimmune strain BALB/c animals, Hb-induced proliferation could not be inhibited by Hp[221]. Anti-Hb reactivity was also apparent in a human patient who had undergone multiple transfusions[220]. Using microarray analysis of auto-antibodies found in cord blood, it was found that both maternal and fetal auto-antibodies recognized alpha and beta subunits of Hb[238]. Such observations indicated that Hb can indeed act as auto-antigen, and such reactivity may manifest in individuals predisposed towards autoimmunity.

Auto-reactivity to other heme-containing proteins has also been observed. Grave's Disease and Hashimoto's Thyroiditis are organ-specific autoimmune diseases in which the thyroid gland is the target of auto-reactive antibodies and T cells. Auto-antibodies (mainly of the IgG isotype, but also IgA and IgE) and auto-reactive T cells target thyroid peroxidase (TPO)[239], a membrane bound heme-containing protein involved in the synthesis of thyroid hormones[240].

Lupus is characterized by aberrant apoptosis; excessive apoptosis has been observed in peripheral cells, as have defects in the uptake of apoptotic cells[71,72,73,74]. RN86, a human monoclonal which specifically targets apoptotic cells (Figures 1a, 1b) was generated from B cells derived from an SLE patient[67]. Further analysis revealed that, upon Western blots on RBC lysate, the antibody bound to a protein of molecular mass of around 64 KDa (Figure 1c). As more than 90% protein of RBCs is Hb[222,224], which in its quaternary conformation is of equivalent molecular mass, ELISA on commercial hHb was performed; strong reactivity of RN86 to hHb was demonstrable. This study aimed to explore the extent, characteristics and implications of Hb auto-reactivity.

For further analyses, sera were obtained from lupus patients visiting the rheumatology clinic at the All India Institute of Medical Sciences, New Delhi. Only patients in the midst of a disease "flare" (as defined by clinical criteria such as the SLEDAI) were chosen, in the hope of obtaining sera expressing auto-antibody titres. Sera were also obtained from equivalent number of healthy individuals, to be employed as negative controls. Western blots and ELISA on human Hb demonstrated the presence of anti-hHb antibodies in a certain percentage of lupus patients (Figure 2). Control sera did not demonstrate the presence of such antibodies, revealing reactivity to be related to the on-going disease process.

A panel of SLE Sera was obtained from the Center for Disease Control and Prevention (CDC) at Atlanta. These sera were previously characterized for the presence of different auto-antibodies. Interestingly, only sera designated to contain antibodies to the Smith (Sm) antigen demonstrated the additional presence of antibodies reactive towards hHb, both on ELISA and on Western blot analysis (Figure 3); different batches of sera obtained from the CDC over a three-year period demonstrated the same pattern of reactivity, further strengthening the authenticity and validity of these observations. Though present in only about 30% of SLE patients, anti-Sm antibodies are specific to the disease and their presence is therefore included as a diagnostic criteria[241,242]. Unexplained associations between different auto-antibody reactivities have been reported; for example, investigators have found an association between anti-Sm reactivity and reactivity to the ribosomal P protein[30]. To assess if observation of the association between anti-Sm and anti-hHb auto-reactivity could be further extended, simultaneous ELISAs were conducted upon sera from Indian SLE patients, using Sm and Hb as targets (Figure 4a, b). Of the three patients demonstrating anti-hHb antibodies in their sera, two harbored anti-Sm antibodies as well; control sera did not contain significant titres of antibodies to either molecule. These observations indicated that Sm reactivity could be a sufficient but not a necessary criterion for anti-hHb reactivity. Most auto-antibodies that target Sm have been shown to be of the IgG isotype[231,243]. The present analysis indicated that anti-hHb reactivity could be mediated by both IgG and IgM isotypes, with the relative dominance of the isotypes varying between patients (Figure 4c).

To see if anti-hHb reactivity was a phenomenon that extended beyond classical autoimmune disease, sera obtained from patients of malaria (both *Plasmodium vivax* and *Plasmodium falciparum*) and leishmaniasis were analyzed; these diseases, like lupus, are characterized by extensive hemolysis[244-246]. In addition, enhanced apoptosis is observed[225] and the presence of auto-reactive antibodies has been demonstrated[247,248]. Interestingly, a significant number of patients harbored antibodies reactive to hHb in serum (Figure 5). As indicated above, antibodies to Sm are conventionally believed to occur only in lupus. Some reports, however, have demonstrated the presence of such antibodies in patients suffering from malaria and leishmaniasis as well[248,245]; no such reactivity was observed in this study, further strengthening previous findings that an anti-Sm antibody response is not essential for the appearance of anti-hHb antibodies. In patients of both leishmaniasis[228] and malaria[248], auto-reactive antibodies of both the IgG and IgM isotype

demonstrate an increase. Anti-hHb reactivity in leishmania patients, however, was mainly IgG-mediated. While patients harboring *P. vivax* demonstrated a similar preferential increase in IgG anti-hHb titres, those infected with *P. falciparum* showed equivalent increases in IgG and IgM anti-hHb reactivity. The reason for these differences, as well as the biological significance of the observation, is at present unknown. The data indicated that anti-hHb autoimmune antibody responses may also occur in non-autoimmune pathologies characterized by extensive hemolysis. It would be of interest to determine whether they are present in diseases in which significant hemolysis is not observed.

NZB/W F1 mice have been extensively employed in the study of lupus and serve as a good model of human disease, since the kinetics of disease onset and subsequent pathologies appear similar to that seen in human lupus[89]. Even though hHb and mHb share a high degree of homology, it was essential to use mHb as a target to ascertain whether or not auto-reactivity to the molecule existed in NZB/NZW F1 animals. Hb from NZB/NZW F1 was purified to homogeneity and characterized by mass spectrometry, HPLC and N-terminal sequencing. Anti-mHb titres exhibited an age-dependent increase, in general consonance with the appearance of anti-self and anti-nuclear reactivity[95,249], thus demonstrating a relationship with disease onset (Figure 10a-c). As previously reported in MRL$^{lpr/lpr}$ mice[30], anti-Sm antibody titres also demonstrated an age-related increase, though a slightly extended lag period in their appearance (in comparison with the appearance of anti-mHb antibodies) was observed (Figure 10d). Titres to individual Hb subunits were also ascertained, in an effort to establish whether a particular subunit was preferentially targeted. Though the beta subunit appeared to be more antigenic (Figure 11a, b), the auto-antibody response to the individual subunits was lower than to the whole molecule, possibly indicating the partial dependence of reactivity upon Hb conformation.

In autoimmune diseases, the sequestration of auto-antibodies or T cells in various organs is associated with specific pathologies. For example, anti-dsDNA can be detected in the kidneys and may be linked to the onset of nephritis[14]. In addition, such antibodies have been shown to mediate neuronal death in the CNS, resulting in memory loss[15,16]. Similarly, transplacental transport of anti-Ro and anti-La antibodies, followed by their binding to fetal cardiac tissue, has been linked to neonatal lupus[25,27,29]. The migration of activated $CD8^+$ T cells across the blood brain barrier has

been associated with symptoms associated with multiple sclerosis[250]. Hb is known to mediate damage to kidneys[214] and CNS[215,216,217]. The involvement of the lungs in SLE is also a relatively common phenomenon[36] and this organ is also the venue of Hb-mediated gaseous exchange. Anti-mHb deposition in organs frequently targeted in lupus was therefore studied as a function of age, in both autoimmune and non-autoimmune prone animals (Figure 11c). mHb-reactive antibodies could be eluted (by incubation in a low pH buffer) from the kidneys, lungs and brains of NZB/W F1 animals, and titres appeared to demonstrate age-dependence, with variations in kinetics; while in the lungs, there appeared to be a steady increase of anti-mHb reactivity, titres in the kidneys and the brain registered a significant rise only after ten months of age. Nevertheless, the appearance and presence of anti-mHb in different organs broadly correlated with the onset of clinical symptoms. Similar low pH organ eluates from old BALB/c mice did not demonstrate the presence of antibodies reactive to mHb. The specificity of such reactivity to the autoimmune strain, as well as the kinetics of appearance of reactivity as animals age, point to possible clinical relevance.

To investigate anti-Hb autoimmune antibody responses in further detail, monoclonal antibodies were generated. B cells isolated from SLE patients (in the midst of a disease "flare") were transformed with Epstein Barr Virus as part of another study. Cells secreting antibodies reactive to hHb were "fused" with an appropriate partner and the human monoclonal antibody KV2C8 was generated. Six murine monoclonal anti-mHb antibodies were generated using spleen cells sourced from aging NZB/W F1 mice. Five murine monoclonal antibodies were of the IgMκ istoype, while the sixth was of IgAκ isotype.

As discussed above, extensive hemolysis characteristic of lupus and other diseases leads to the release of free Hb. Serum levels of the Hb-binding protein Hp fall to low levels in diseases characterized by extensive hemolysis, thereby affecting Hb clearance. In an oxidative environment such as that seen in lupus and in other diseased states, it is postulated that the iron in the heme moiety is converted from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) form, resulting in the formation of metHb. Indeed, presence of oxidatively modified Hb in CSF has been demonstrated under conditions of oxidative stress after hemorrhage[251]. MetHb can elicit a number of biological effects; for example, it causes a dose- and time-dependent increase in the activation of endothelial cells[252]. Free heme may be released as well, causing a number of toxic effects[192,214]. To assess whether the

anti-Hb monoclonal antibodies could distinguish between the ferrous and ferric forms of Hb, metHb was generated upon incubation of Hb with H_2O_2; the fact that H_2O_2 is known to exist at increased concentrations *in vivo* in SLE[210], made these experiments additionally relevant. Both hHb and mHb were employed. ELISA assays revealed that Antibodies 1C1, 3C4, 3A1 and KV bound the ferrous and ferric forms of Hb to almost the same extent. The data indicated these antibodies bound motifs or epitopes not modified or affected due to the oxidation process. In contrast, Antibodies 1B5, 2A1 and 2C1 demonstrated significantly enhanced recognition of ferric Hb. Patterns of reactivity on murine and human Hb were almost the same for all antibodies, validating the results (Figure 15a). This is an observation of significance and implies that a disease-directed change in a self-protein is giving rise to structural neo-epitopes recognized by disease-related monoclonal auto-antibodies.

Cross-reactivity of the monoclonal antibodies with other heme-containing proteins (cytochrome c and myoglobin) and with heme was then assessed (Figure 15b). Myoglobin was specifically chosen as it exhibits high structural similarity with Hb subunits[191]. Antibody 2C1 appeared to be the most specific to Hb, demonstrating minimal binding to the other moieties; interestingly, it was also one of the antibodies which bound Fe^{3+} Hb better than Fe^{2+} Hb. Antibody 3A1 demonstrated reactivity towards Hb and heme; the other antibodies exhibited varying degrees of cross-reactivity towards the other moieties, as described in detail in the Results section. Therefore the data demonstrated that, even in this limited analysis of cross-reactivity, while one antibody bound Hb with high specificity, others were more broadly reactive.

Free Hb is normally bound by Hp which aids in its clearance and prevents the Hb-mediated toxic events[195]. It has been shown that binding of Hb to Hp stabilizes the former. Hp shares structural similarities with IgG and so can be considered a "natural antibody" to Hb. In a previous study, antibodies to Hb did not exhibit the stabilizing effect as did Hp, and bound Hb on a site distinct from Hp[253]. In the present study, experiments were carried out to ascertain whether the binding of Hp to Hb could prevent the binding of the monoclonal antibodies to Hb. Given the cross-reactive nature of some of the antibodies, it was first important to ascertain whether they demonstrated binding towards Hp as well; with the experimental design employed, such reactivity would have vitiated the results, making the data un-interpretable. None of the monoclonal antibodies bound

Hp. Subsequent competition assays revealed that for all antibodies, Hb-Hp association did not affect Hb-Antibody interaction, possibly indicating that the binding sites of Hp and the anti-Hb auto-antibodies on Hb do not overlap. No conclusive results could be obtained for the human monoclonal antibody KV due to unexpectedly high binding of the anti-human Ig reagent with Hp. Similarly, commercial, polyclonal antibodies directed against the alpha and beta subunits of Hb unexpectedly demonstrated significant reactivity towards Hp, precluding interpretation of the data (Figure 16b).

Experiments were then conducted to assess whether the antibodies demonstrated preferential binding towards either the alpha or the beta subunit of Hb (Figure 16c). In spite of high degree of structural similarities between the two subunits, most antibodies could indeed distinguish between the two. Antibodies 1C1, 2A1, 3C4, 3A1 and KV recognized beta subunit better than the alpha subunit. While Antibody 1B5 could not distinguish between the two subunits, Antibody 2C1 was the only antibody which bound the alpha subunit to a greater extent. It was of interest to note that Antibody 2C1 also appeared to be the most specific in the assays discussed above.

Upon intra-molecular epitope mapping using contiguous, non-overlapping peptides representing the alpha and beta subunits of Hb as targets in ELISA, differing results were obtained for the different antibodies (Figure 17, Figure 18). While some antibodies were essentially non-reactive (Antibodies 3A1 and KV), another was predominantly reactive towards one peptide (Antibody 2C1, the relatively Hb-specific monoclonal antibody), while another (Antibody 2A1) preferentially bound two peptides. Even though Antibody 1B5 appeared to exhibit poly-reactive behavior, binding preferences for certain peptides were apparent. When the binding specificities of all antibodies were considered, the peptide 100-119 of alpha subunit and 100-119 of the beta subunit appeared to be recognized by most antibodies. These regions have been found to be immunodominant in previous published reports as well[218,219,254]. Competition analysis between different reactive peptides, as has been done for polyreactive human monoclonal antibodies reactive to Ro60[255], would further validate these findings. Non-reactivity of Antibodies 3A1 and KV towards the peptides may be due to the fact that the conformational epitopes they recognize do not find adequate representation in the peptides employed, or due to the inadvertent destruction of linear epitopes caused by current peptide design. The non-reactivity of commercial, polyclonal

sera against the alpha and beta peptides, however, is surprising. It would be interesting to see if the immunization of animals (both non-autoimmune prone, and those prone to develop autoimmunity) with these peptides (as opposed to the entire Hb) results in the generation of autoimmune responses, as was observed upon immunization of SmD peptides[112]. If indeed such responses occur, postulates of crypticity and molecular mimicry could be put forth.

As previously indicated, this study was initiated on the observation that an apoptotic cell-specific human monoclonal antibody exhibited frank cross-reactivity to hHb. By applying converse logic, the reactivity of the established anti-Hb monoclonal antibodies towards cellular proteins was assessed. Upon FACS analysis, none of the antibodies, including those that appeared to be poly-reactive on previous analysis, bound the surface of healthy, non-permeabilised cells. These results ruled out non-specific "stickiness" being responsible for reactivity. In contrast, all antibodies (with the possible exception of Antibody 2C1) demonstrated significant reactivity towards cells that had been previously permeabilised (Figure 19). This data implied that, in several instances, antibodies that bound Hb also had the capacity to bind cellular antigen(s), leading to the postulate that at least some anti-Hb antibody specificities may arise due to cross-reactivity to such antigen(s), or vice-versa.

Confocal analysis (Figures 20-25) confirmed these results; while none of the antibodies bound non-permeabilised cells, most demonstrated avid intracellular reactivity, binding cytoplasmic and/or peri-nuclear moieties. Antibody 2A1 appeared to also bind antigen(s) sequestered near the cell membrane, while antibody 3A1 appeared to additionally bind nuclear antigen(s). In contrast with these results, commercial polyclonal antibodies against the alpha and beta subunits of Hb, employed here as controls, did not bind to either non-permeabilised or permeabilised cells, indicating differences between some auto-immune and non-autoimmune anti-Hb responses. Interestingly, in consonance with FACS results, Antibody 2C1 also did not appear to recognize cellular antigens even upon permeabilised cells, once again distinguishing it from the other antibodies.

During apoptosis, blebs containing a number of auto-antigens such as Ro, DNA and La are extruded from the cell surface[256], and apoptotic cells are thought to constitute an antigenic insult for the development of lupus in genetically susceptible individuals[131,257]. In furtherance of the hypothesis that cross-reactivity with cellular antigens, as well as the process of apoptosis, may be responsible in part for anti-Hb responses, reactivity of the monoclonal antibodies upon apoptotic cells was assessed using two-colour flow cytometry, employing the antibodies as well as the phosphatidylserine binding protein Annexin-V (Figure 26). In consonance with their reactivity patterns on permeabilised cells, Antibodies 1C1, 1B5, 2A1, 3C4, 3A1 and KV preferentially recognized cells that also bound Annexin-V, which demarcated them as being apoptotic. Although there existed cells that bound Annexin-V but not antibodies, cells binding only the antibodies and not Annexin-V were relatively rare.

Antibody 2C1 demonstrated minimal binding to apoptotic cells, as evident from the lack of a significant number of cells binding both the antibody and Annexin-V; these results once again demonstrated the antibody's unique properties. To re-iterate: Antibody 2C1, unlike the other antibodies, did not cross-react with either heme, or the heme-containing proteins myoglobin and cytochrome c; it was the only antibody that preferentially bound the alpha subunit of Hb; it was the only antibody that uniquely bound a single peptide from the alpha subunit (peptide 110-119); and it was the only antibody to demonstrate poor recognition of cellular antigens, as well as of antigens extruded upon cells undergoing apoptosis.

As part of another study, a panel of murine monoclonal antibodies specifically targeting apoptotic cells had been established. To further probe the link between apoptosis and anti-Hb reactivity, the ability of these antibodies to bind Hb was assessed; significant binding was observed with several antibodies, with antibodies of the IgM isotype demonstrating higher activity (Figure 27). Previous work with the human and murine apoptotic-cell specific antibodies had established their polyreactive nature; irrespective of isotype or the presence of somatic mutations in the complementarity determining regions (Reference 67 and unpublished observations). The anti-Hb monoclonal antibodies revealed a similar polyreactive nature when reactivity was assessed upon a panel of recombinant auto-antigens, with individual differences apparent (Figure 28). These reactivities, on selected auto-antigens were only indicative in nature; more comprehensive analysis

would be required to delineate the extent and the nature of the non-Hb cross-reactive epitopes within cells. A beginning has been made in this regard; Western blots using three representative antibodies (Antibodies 1C1, 1B5 and 2C1) to probe reactivity upon SP2/O lysates further revealed intermolecular cross-reactivity of Antibodies 1C1 and 1B5; the nature of the reactivity appeared to be distinct however. Antibody 2C1 did not bind any antigens in the lysate, consistent with previous observations. On two-dimensional Western blotting using Antibody 1B5, three major spots were obtained, which await characterization.

Analysis thus far had demonstrated beyond doubt that Hb was antigenic, in both murine and human disease states. Since antigenicity does not necessarily imply immunogenicity, it was important to determine whether Hb was immunogenic in a murine strain genetically pre-disposed to systemic autoimmunity. Both Fe^{2+} mHb and F^{+3} mHb were employed to immunize 2-month old (pre-autoimmune) NZB/W F1 mice, since previous data had shown that some monoclonal antibodies derived from these animals displayed the capability of discriminating between the two forms. In addition, an aggressive adjuvant was employed to maximize the chances to break tolerance. Immunization with neither Fe^{2+} mHb nor F^{+3} mHb generated early autoimmunity to Hb, Sm (assayed because of data demonstrating a possible association between the two reactivities) or other intracellular antigens, indicating Hb might not be an initiating auto-antigen. Indeed, in a non-autoimmune scenario, Hb is known to be a poor immunogen[258,259] and is capable of generating immune responses only when it is internally cross-linked[259] or bound to Hp[260].

Somatic mutations are known to accumulate in antibody variable regions as immune responses mature. Analysis of V_H and V_L sequences has demonstrated that, by and large, IgG antibodies tend to carry more mutations than IgM antibodies, although exceptions do occur. The CDRs tend to contain more replacement mutations (which result in a change in amino acids) than silent mutations (which result in no such change)[8].

Several reports exist describing the presence of somatic mutations in auto-antibodies specific for different auto-antigens[261]. For example, mutations to the amino acid arginine in the CDR3 has been shown to be important for anti-dsDNA and anti-Sm reactivity in both murine and human antibodies[262,263,235]. "Reverse" mutations, or mutations to amino acids such as glycine, lead to a

loss of reactivity to DNA, and addition of arginine residues at specific points cause upto a fifty-fold increase in anti-DNA reactivity. In addition to somatic mutations, the presence of arginine in the CDR3 may also result from to N-base addition, or because of unusual reading frames arising due to the D region. Residues surrounding the interacting amino acids have been shown to influence reactivity; it was found that when aspartic acid residue was incorporated close to arginine residue, there was stabilization of charges, reducing DNA binding[17].

Auto-antibody responses in lupus have been further characterized, and additional defects enumerated. In the MRL/lpr animals, a decrease in the kappa light chain rearrangement and RAG expression is observed[264]. Antibodies derived from SLE patients demonstrate a decreased CDR3 length[53]. Several studies have shown a skew in the gene families employed by auto-antibodies, an observation that may be reflective of antigenic specificity. Reports suggest that the majority of the IgM auto-antibodies have V_H regions belonging to the J558 family, while IgA antibodies tend to employ 7183 family. Biased use of the J chain has also been observed, in both heavy and light chains; most IgMs use the J_H4 gene family and IgAs the J_H1 family. The J_κ family usage is skewed towards $J_\kappa1$ and $J_\kappa5$ for IgMs and $J_\kappa4$ for IgA auto-antibodies[262,263]. The human data suggests a bias towards V_H3 and V_H 4-34 gene family usage[265].

The heavy and light chain variable regions of the anti-Hb monoclonal antibodies were sequenced subsequent to PCR amplification using specific primers. Sequences were analyzed for family usage, as well as for the presence of somatic mutations. The analysis has been summarized in the Tables 1a and 1b. Antibodies 1C1 (IgAκ) and 1B5 (IgMκ) both used the 7183 heavy chain Ig gene family, demonstrating the closest homology with the same germline gene (VH7183.a19.31); as indicated above, the family is known to be employed by other auto-antibodies. The other murine anti-Hb antibodies employed the J558 gene family[262], albeit demonstrating closest homologies with different germline genes. As J558 corresponds the V_H1 family and 7183 to the V_H5 family, these two gene families of the heavy chain appeared to be over-represented in anti-Hb responses. While use of the D segment varied widely, three murine antibodies (Antibodies 1C1, 2C1 and 3A1) employed the J_H1, two the J_H2 (Antibodies 2A1, 3C4) one (Antibody 1B5) the J_H4 (1B5) gene segment. Varied use of the light chain variable genes was observed, as was the employment of the J segment; one antibody (Antibody 1C1) used as $J_\kappa1$, two (Antibodies 2A1 and 3A1) $J_\kappa2$,

	GENE SEGMENTS	REPLACEMENT/ SILENT MUTATIONS IN CDR	REPLACEMENT/ SILENT MUTATIONS IN FWR	MUTATIONS PER UNIT LENGTH IN CDR	MUTATIONS PER UNIT LENGTH IN FWR	MUTATIONS PER UNIT LENGTH IN CDR/FWR
1C1 HEAVY CHAIN	VH7183.a19.31 DSP2.8 JH1	3	3	0.188	0.081	2.32
1C1 LIGHT CHAIN	ci12, Jκ1	3	0	0.1111	0	∞
1B5 HEAVY CHAIN	VH7183.a19.31 DSP2.2 JH4	∞	2	0.0344	0.0238	1.69
1B5 LIGHT CHAIN	8-28, Jκ4	0	0	0	0	0
2A1 HEAVY CHAIN	J558.69.170 DSP2.2 JH2	0	0	0	0	0
2A1 LIGHT CHAIN	8-21, Jκ 2	0	0	0	0	0
2C1 HEAVY CHAIN	V186.2 DQ52-C57BL/6 JH1	0	∞	0	0.012	0
2C1 LIGHT CHAIN	23-43, Jκ5	0	0	0	0	0

Table 1a: Anti-Hb monoclonal antibody germline gene segments (listed as V, D, J regions for the heavy chains and V, J regions for the light chains) and mutation analysis. ∞ indicates division by zero.

	GENE SEGMENTS	REPLACEMENT/ SILENT MUTATIONS IN FWR	REPLACEMENT/ SILENT MUTATIONS IN CDR	MUTATIONS PER UNIT LENGTH IN CDR	MUTATIONS PER UNIT LENGTH IN FWR	MUTATIONS PER UNIT LENGTH IN CDR/FWR
3C4 HEAVY CHAIN	J558.55.149, DSP2.8 JH2	∞	0	0034	0	∞
3C4 LIGHT CHAIN	bb1, Jκ5	∞	0	0.032	0	∞
3A1 HEAVY CHAIN	J558.47 DFL16-1 JH1	0	0	0	0	0
3A1 LIGHT CHAIN	21-12, Jκ2	∞	∞	0.067	0.049	1.36
KV HEAVY CHAIN	IGHV3-11*03 IGHD3-10*02 IGHJH*02	0	0.5	0	0.012	0

Table 1b: Anti-Hb monoclonal antibody germline gene segments (listed as V, D, J regions for the heavy chains and V, J regions for the light chains) and mutation analysis. ∞ indicates division by zero.

one (Antibody 1B5) $J_\kappa 4$ and two (Antibodies 2C1 and 3C4) $J_\kappa 5$. The length of the CDR3 in the heavy chain varied from antibody to antibody, and demonstrated no apparent correlation with antibody specificity or degree of cross-reactivity, as has previously been reported[53]; while in Antibodies 1B5, 2A1 and 3C4, it was shortened to 7, 8 and 7 residues respectively due to exonuclease activity, other antibodies (including Antibody 2C1, which appeared relatively more specific to Hb, as discussed above) demonstrated a longer length. In other antibodies, no significant deviations were observed from the germ line sequence length.

As indicated above, mutations to arginine, or non-encoded junctional additions of the amino acid, have been associated with anti-dsDNA reactivity[17]. In the present study, mutation or non-encoded addition to the amino acid glycine in the CDR3 occurred in six heavy chains (Antibodies 1C1, 2A1, 2C1, 3C4, 3A1 and KV) and one light chain (Antibody 1C1). In addition, two sequences (the heavy chain of Antibody 1B5 and the light chain of Antibody 3A1) contained a germline-encoded glycine residue in CDR3. Thus, a total of nine out of thirteen sequences analyzed contained the amino acid glycine in CDR3. While mathematically this may appear a significantly high number, actual proof of the influence of the residue on anti-Hb specificity would have to await mutation analysis.

Two analyses were carried out to study the relative numbers of somatic mutations in the CDR regions and FWR. Firstly, attempts were made to calculate the ratio of the replacement to silent mutations in the CDR and FWR regions. Random mutation processes produce replacement to silent mutation ratios of 2.9 or lower[266]. Replacement mutations per unit length (amino acid) were also independently calculated for the CDR and FWR regions, and a ratio of these values determined[267]; a value greater than one would indicate that somatic mutations were indeed preferentially directed to the CDR regions.

For heavy chain of Antibody 1C1, replacement (R) to silent (S) mutation ratios for the FWR and CDR regions were 3 and 3 respectively, indicating their non-random nature. Further analysis revealed that the CDR regions carried 2.32 times more replacement mutations per unit length than the FWR regions. For the light chain, the R/S ratio was 3 for the CDR, whereas the FWR was non-mutated. Analysis of the heavy chain of Antibody 1B5 revealed a slight increase in the propensity

of R mutations to be directed to the CDR, with a value of 1.69 being obtained for the ratio of R mutations per unit length in the CDR versus FWR. The light chain of Antibody 3A1 too showed a similar propensity, with the ratio being 1.36. The enumeration of the mutations conducted above is conservative as far as its relevance to anti-Hb specificity is concerned due to two reasons; firstly, in several instances, analysis was hampered by the denominator in the calculations being zero, indicated by the symbol "∞" in Tables 1a and 1b. Secondly, non-encoded amino acids added at the junctions, which in all probability would influence antibody specificity, were not included in the analysis.

Upon release from RBC, free Hb first comes into contact with leukocytes and endothelial cells[236]. These cells can alter the behavior of other cell types by causing the secretion of IL-6, IL-8 and TNF-α in response to Hb, and the amount of cytokine secreted increases with increasing dose and time of incubation[237]. Inflammatory cascades set up as a result can have serious implications; clinical trials in patients who received Hb as a blood substitute had to be terminated because of associated fatalities[237].

Inflammatory cascades have been implicated in lupus pathogenesis. High levels of serum TNF-α have been reported in patients[186], resulting in increased levels of pro-inflammatory cytokines such as IL-1, IL-6 and IL-8[148]. Anti-IL-6 therapy has beneficial effects in lupus mouse models[161]. Higher numbers of unstimulated cells secreting IL-6, IL-8 and TNF-α have been observed in SLE patients than in controls[171], and high concentrations of these cytokines correlate with increased disease activity. These cytokines have been implicated in the pathology of NPSLE as well; levels of IL-6 and IL-8 were found to be higher in the CSF of SLE patients[165].

Whether anti-Hb antibodies could influence Hb-induced secretion of inflammatory cytokines was then investigated. Experiments were carried out at sub-optimal doses of Hb, in order to better elucidate antibody-mediated effects. Antibodies were also individually assayed for their ability to influence cytokine secretion. Interestingly, Antibody 2A1 exhibited the capabilty to induce the secretion of cytokines. The other anti-Hb antibodies, as well as antibodies of irrrevant specificity employed as isotype controls, did not exhibit such reactivity. Co-incubation of cells with Antibody 2A1 and Hb resulted in significant synergistic effects on cytokine secretion which, once again, was

not observed with any of the other antibodies (Figure 43). The reasons of these effect are curently unclear, but may be related to the antibody's unique binding characterisitics. Antibody 2A1 was the only one to demonstrate dominant reactivity towards peptide 110-119 of the Hb alpha subunit as well as peptide 110-119 of Hb beta subunit, to demonsrate punctate binding to regions close to or at the plasma membrane, and to exhibit a high degree of cross-reactive recognition of the A Protein. Anti-dsDNA antibodies too have been shown to demonstrate such effects; incubation of human mononuclear cells with murine anti-DNA antibodies caused secretion of IL-1β, IL-6, IL-8 and TNF-α[268]; cross-reactive binding to cell surface molecules such as acidic Rib P proteins present on the kidney measngial cells[269,270] may intiate such events or these effects might be seen after endocytosis of the antibodies via binding to the membranous brush border myosin[271]. These reports suggest transport of auto-antibodies across the cell membrane into the cytoplasm and the nucleus where they may be involved in altering cellular functions. Althought literature also suggests existence of an Fcα/μ receptor (that binds IgM Fc) in the thymus, spleen, liver, kidney, testis, placenta, small and large intestines and on the B cells and macrophages [272], such a receptor is unlikey to be responssible for the observed effects due to the demostrated specificity of recognition.

Endothelial cells lining the vasculature are particularly sensitive to Hb-mediated damage as they are in direct and continuous contact with cell-free Hb[273]. Damage is specifically Hb-mediated; other heme-containing proteins like myoglobin and cyt c do not demonstrate such effects[213]. Endothelial cells respond to the presence of Hb by secreting cytokines such as IL-8, a chemo-attractant in a dose and time dependent manner[252]. The cytokines further activate the endothelial cells and cause monocyte adherance to the endothelium[237,274], an essential step for migration of cells into tissue. Activation of endothelial cells by TNF-α causes significant attachment of mononcytes via adhesion molecules ICAM (Intercellular Adhesion Molecule), VCAM-1 (Vascular-Cell Adhesion Molecule), MCP-1 (Monocyte Chemoattractant Protein-1) and E-selectin[275,276]. Other reports suggest higher levels of soluble VCAM-1, ICAM-1, E-selectin in the cerebrospinal fluid (CSF) of patients who had subarachnoid hemorrhage, possibly linking free Hb with elevated levels of these adhesion molecules[277]. Increased levels of soluble VCAM-1 have also been found in lupus patients, and levels correlate with disease activity and severity[278].

Supernatant obtained upon incubation of endothelial cells with Antibody 2A1 did not enhance the transmigration of monocytes as compared to medium and the isotype control antibody. On the other hand, supernatant obtained from endothelial cell cultures incubated with Antibody 2A1 together with Hb significantly enhanced the migration of monocytic cells (Figure 44). Although the precise cytokines and/or soluble adhesion factors responsible for these effects remain to be identified, the data supplement previous results, while shedding additional light on the biological roles Hb-reactive antibodies might play in the patho-physiology of diseases characterized by presence of free Hb.

Summary and Conclusions

SUMMARY AND CONCLUSIONS

A human monoclonal antibody specifically targeting apoptotic cells demonstrated cross-reactive recognition of human hemoglobin (hHb).

Antibodies present in a certain percentage of SLE sera demonstrated high reactivity to hHb in ELISA and upon Western blots. Studies using auto-reactive sera obtained from the CDC (Center for Disease Control and Prevention, Atlanta) from SLE patients demonstrated that only sera containing antibodies to Sm antigens contained antibodies which bound hHb. Subsequent analysis revealed that an anti-Sm antibody response was probably sufficient but not necessary for an anti-hHb antibody response.

Anti-hHb reactivity in human SLE patients could be mediated by both IgG and IgM antibody isotypes. Antibodies in a certain percentage of sera from malaria and leishmaniasis patients were also reactive to hHb (but not to Sm), indicating that aberrant immune recognition of hHb was present in other clinical conditions associated with RBC lysis, enhanced apoptosis and auto-antibody production. The anti-hHb reactivity in these sera was mainly mediated by the IgG isotype; in patients harboring *P. falciparum* however, anti-hHb antibodies of the IgG and IgM isotypes were equally represented.

Mouse Hb (mHb) was purified on a CM-52 ion exchange column; protein purity was assessed by SDS-PAGE and HPLC, and its identity confirmed by N-terminal sequencing and mass spectrometry. Old, autoimmune-prone NZB/W F1 mice demonstrated higher titres of antibodies to mHb than did young animals; the kinetics of appearance of anti-mHb reactivity paralleled the appearance of anti-nuclear antibody reactivity, a hallmark of lupus. Enhanced antibody reactivity to the alpha and beta subunits of hHb was also observed as animals aged. Antibodies to the Sm protein demonstrated a similar age-related increase, albeit with an extended lag-period compared with anti-mHb responses. Low-pH eluates obtained from the lungs, brain and kidneys from NZB/W F1 demonstrated age-dependent increases in antibodies reactive to mHb, while eluates from tissues derived from old BALB/c animals did not contain

antibodies which bound mHb, potentially implicating anti-Hb auto-antibody responses in end-organ pathology.

A certain percentage of supernatants of 456 EBV transformed B cell lines derived from SLE patients demonstrated the presence of antibodies reactive to hHb. A human monoclonal antibody KV (IgM_{λ}) reactive towards hHb was established upon fusing cells with the heteromyeloma K_6H_6/B_5. Spleen cells of aging NZB/W F1 mice were fused with the B-cell myeloma SP2/O. Six monoclonal antibodies (1B5, 2A1, 2C1, 3C4, 3D1 [all IgM_k], and 1C1 [IgA_k]) were established. While some antibodies were equally reactive towards ferrous (Fe^{2+}) Hb and ferric (Fe^{3+}) Hb, others had the capability of distinguishing between these two forms. Antibody 2C1 appeared to be relatively Hb specific, whereas other antibodies demonstrated varying degrees of cross-reactivity towards heme and other heme-containing proteins cytochrome c and myoglobin.

Competition studies revealed that Hp and the monoclonal antibodies bound distinct sites on Hb. Most antibodies demonstrated preferential reactivity towards either Hb-alpha or Hb-beta, while Antibody 1B5 bound them both equally. Studies using synthetic peptides spanning alpha and beta Hb helped elucidate epitopes recognized by some anti-Hb monoclonal antibodies; Antibody 2C1 predominantly bound to a single peptide, while Antibody 2A1 bound two non-homologous peptides, one each on the alpha and beta subunits of Hb, demonstrating intra-molecular cross reactivity. Other antibodies were either poorly reactive, or demonstrated a more poly-reactive binding pattern.

Though antibodies were non-reactive towards cell surface moieties, they (with the exception of Antibody 2C1) demonstrated cross-reactive recognition of predominantly cytoplasmic antigen(s) on FACS and confocal microscopy. Reactive antibodies also bound epitopes exposed on the surface of cells undergoing apoptosis. The cross-reactivity of the anti-Hb monoclonal antibodies with non-Hb self antigens was further elucidated by ELISA on selected recombinant auto-antigens; Ro60, La, SmD, SmB and the U1RNP A protein were differentially recognized. The IgA Antibody 1C1 reacted to different moieties than did the

IgMs upon Western bolt analysis on cellular lysates. Further elucidation of cross-reactive moieties would help elucidate the etiology of anti-Hb antibody responses.

Immunization of pre-diseased, autoimmune prone mice with either ferrous of ferric mHb did not result in the enhanced generation of anti-mHb or other anti-self antibody responses, indicting that mHb was probably not the primary immunogen in the generation of anti-mHb responses.

The light and heavy chain variable region genes of the anti-Hb monoclonal antibodies were sequenced. Comparisons with closest germline sequences revealed that, while some antibodies appeared to be essentially germline encoded, others carried somatic mutations. Nine of thirteen CDR3s were found to contain a Glycine residue, either germline encoded, or arising as a result of somatic mutation or a non-encoded addition at the junctional regions.

Antibody 2A1 stimulated the release of TNF-α, IL-6 and IL-8 from THP-1 (human monocyte) cells; significant synergy in cytokine production was observed when cells were co-incubated with the antibody and Hb. Such synergistic effects were also observed upon incubation of endothelial cells with the combination of antibody and Hb; supernatants arising from such cultures were significantly more efficient in inducing the transmigration of monocytes than antibody or Hb alone. The results reveal that anti-Hb responses may be involved in the amplification of inflammatory cascades in diseases of immune dysfunction.

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Appendix

APPENDIX

10X PBS

Na_2HPO_4	14.4 gm
KH_2PO_4	2.4 gm
KCl	2.0 gm
NaCl	88.0 gm
Final volume (in dist. water)	1000.0 ml

Phosphate Buffer (10mM, pH 6.5)

KH_2PO_4 (1M)	15 ml
Total volume (in dist. water)	1500 ml

pH 6.5 (using 10 mM K_2HPO_4)

Phosphate Buffer (15mM, pH 8.5)

K_2HPO_4 (1M)	2.25 ml
Total volume (in dist. water)	150 ml

pH 8.5 (using 15 mM KH_2PO_4)

Freezing Medium – Human Cells

FCS	20.0 %
DMSO	10.0 %
RPMI Medium	70 %

Freezing Medium – Murine Cells

DMSO	10.0 %
FCS	90.0 %

ELISA

Carbonate Buffer:

Na_2CO_3	1.501 gm
$NaHCO_3$	2.930 gm
Total volume (in dist. water)	1000 ml

pH 9.2

Washing Buffer (PBST):

Tween 20	0.05 % v/v in 1X PBS

Blocking Buffer:

BSA	5 % w/v in 1X PBS

Primary and Secondary Antibody Dilution Buffer:

BSA	5% w/v in PBST

Citrate Buffer:

Citric acid	4.67 gm
Na_2HPO_4	10.00 gm
Total volume (in dist. water)	500 ml

pH 5.5 (with glacial acetic acid)

OPD Substrate Solution:

OPD	10 mg (in 10 ml citrate buffer)
H_2O_2	10 µl

Stop Solution:

H_2SO_4 (in dist. water)	2N

FACS

FACS Buffer:

BSA	1.0 %
NaN_3	0.2 %
Total volume (in 1X PBS)	500 ml

Permeabilization Buffer:

Triton-X 100	0.001% in methanol

Annexin Binding Buffer (10X):

HEPES	2.380 gm
NaCl	8.180 gm
$CaCl_2$	0.277 gm
Total volume (in water)	100 ml

Fixative (2%):

Paraformaldehyde	1 gm
Total volume (in 1X PBS)	50 ml

Heat to 70^0C for dissolution.

SDS-PAGE

30 % *Acrylamide Solution:*

Acrylamide	28.8 gm
Bisacrylamide	1.2 gm
Total volume (in dist. water)	100 ml

10% SDS Solution:

SDS	10 gm
Total volume (in dist. water)	100 ml

6X Sample Buffer:

0.5 M Tris HCl pH 6.8	0.29 M
Glycerol	25.00 %
SDS	8.30 %
β-Mercaptoethanol	12.50 %
Bromophenol Blue	1.2 mg
Total volume (in dist. water)	10.0 ml

15% Resolving Gel Solution:

30% Acrylamide	5.00 ml
1.5 M tris pH 8.8	2.50 ml
Dist. water	2.40 ml
10% SDS	0.10 ml
10% APS	0.05 ml
TEMED	8.0 µl

12% Resolving Gel Solution:

30% Acrylamide	4.00 ml
1.5 M tris pH 8.8	2.50 ml
Dist. water	3.30 ml
10% SDS	0.07 ml
10% APS	0.10 ml
TEMED	8.0 µl

10% Resolving Gel Solution:

30% Acrylamide	3.30 ml
1.5 M tris pH 8.8	2.50 ml
Dist. water	4.00 ml
10% SDS	0.10 ml
10% APS	0.08 ml
TEMED	8.0 µl

5% Stacking Gel Solution:

30% Acrylamide	0.70 ml
0.5 M tris pH 6.8	1.25 ml
Dist. water	3.00 ml
10% SDS	0.05 ml
10% APS	0.04 ml
TEMED	6.0 µl

Running Buffer (5X):

Tris	15 gm
Glycine	72 gm
SDS	5 gm
Total volume (in dist. water)	1000 ml

Coomassie Blue Staining Solution:

Methanol	90 ml
Coomassie Brilliant Blue	0.1 gm
Stirred for 1 hr	
Glacial acetic acid	20 ml
Total volume (in dist. water)	200 ml

Destaining Solution:

Methanol	40 ml
Glacial acetic acid	14 ml
Total volume (in dist. water)	200 ml

Silver Staining

Fixative:

Methanol	25.00 ml
Glacial acetic acid	12.00 ml
Formic acid	0.05 ml
Total volume (in dist. water)	100 ml

Sodium Thiosulphate:

Stock:

$Na_2S_2O_3$	0.2 gm
Total volume (in dist. water)	2.5 ml

Diluted:

$Na_2S_2O_3$ (stock solution)	75 µl
Total volume (in dist. water)	50 ml

Silver Nitrate:

Stock:

$AgNO_3$	0.2 gm
Total volume (in dist. water)	2.5 ml

Diluted:

$AgNO_3$ (stock solution)	1.00 ml
HCHO	0.04 ml
Total volume (in dist. water)	50.00 ml

Developer:

Na_2CO_3	0.3 gm
$Na_2S_2O_3$ (stock solution)	8.3 µl
HCHO	80.0 µl
Total volume (in dist. water)	100.0 ml

Stop Solution:

Glacial acetic acid	1 % in dist. water

Western Blotting

Lysis Buffer:

Tris HCl (50 mM, pH 7.4)	6.050 gm
KCl (25 mM)	1.863 gm
$MgCl_2$ (5 mM, hydrated)	1.016 gm
EDTA (1 mM)	0.372 gm
NaN_3	0.020 %
Protease inhibitor (added just before lysis)	20µl/ml buffer
Total volume (in dist. water)	1000 ml

Transfer Buffer:

Glycine	14.4 gm
Tris	3.0 gm
Methanol	200.0 ml
Total volume (in dist. water)	1000 ml

Ponceau 'S' Solution:

Ponceau S	0.5 %
Glacial acetic acid	1.0 %

Blocking Buffer:

BSA	5 % (in 1X PBS)

Primary And Secondary Antibody Dilution Buffer:

BSA	5 % (in 0.05% PBST)

Two Dimensional Isoelectric Focussing

M2 Lysis Buffer:

Tris HCl, pH 7.4	50.0 mM
Glycerol	10.0 % v/v
Triton-X 100	1.0 % v/v
EDTA	0.5 mM
EGTA	0.5 mM
NaCl	150.0 mM
Protease inhibitor	20 µl/ml

The volume was made up with dist. water

2-D Sample Buffer:

Urea	21.0 gm
Thiourea	8.0 gm
CHAPS	1.0 gm
DTT	0.4 gm
Total volume (in dist. water)	50.0 ml

Equilibration Buffer:

Tris HCl (1.5 M, pH 8.8)	50 mM
Urea	6 M
Glycerol	30 %
SDS	2 %
Bromophenol Blue	0.002 %

The volume was made up with dist. Water

PCR

All the reagents were from Promega. Primers were from Novagen.

cDNA	2.5 µl
25mM $MgCl_2$	2.5 µl
10X buffer	2.0 µl
10mM dNTP mix	2.5 µl
5` primer	0.5 µl (5 pM)
3` primer	0.5 µl (5 pM)
Taq polymerase	0.3 µl (5 U).

Final volume was made up to 25 µl with water

Tris Acetate EDTA Buffer (50X):

Tris acetate	40 mM
EDTA	1 mM

Bacterial Culture

Luria Bertani (LB) Broth:

Tryptone	10 gm
Yeast extract	5 gm
NaCl	5 gm
Total volume (in dist. water)	1000 ml

LB Agar Plates:

Tryptone	10 gm
Yeast extract	5 gm
NaCl	5 gm
Agar	1 % w/v
Total volume (in dist. water)	1000 ml

To make LB ampicillin plates, 100 µG ampicillin was added per mL of LB agar solution.

IPTG/X-gal Stock Solution:

IPTG	1.25 gm
X-gal	1.00 gm
Total volume (in DMF)	25 ml

LB Agar with IPTG/X-gal:

IPTG/X-gal stock solution	1.0 ml
Total volume (in LB agar)	1000 ml