Superantigen-like interaction of IVIG with antibody Fab fragments cloned by phage display technology

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by

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DEDICATION

To: Nora,
Ivy, and
Kim

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LIST OF ABBREVIATIONS

bp	base pairs
CD	cluster of differentiation
CDR	complementarity-determining region
cfu	colony forming units
C_H1	first constant region of antibody heavy chain
C_L	light chain consant region
cp	coat protein
D	diversity gene
DTT	dithiothreitol
EBV	Epstein-Barr Virus
ELISA	enzyme linked immunosorbent assay
Fab	$V_H/C_H1 + V_L/C_L$
Fc	crystallizable fragment of antibody molecule
Fd	$V_H + C_H1$ of the antibody molecule
FR	framework region
Hc	heavy chain
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HRP	horse radish peroxidase
Ig	immunoglobulin
ITP	idiopathic thrombocytopenic purpura
IU	international units
IVIG	intravenous immunoglobulins
J_H	heavy chain joining gene
J_L	light chain joining gene
Lc	light chain
M13	VCSM13 helper phage
PBL	peripheral blood lymphocytes
PEG	polyethylene glycol
RIBS	ribosome binding site
rpm	rounds per minute
SCID	severe combined immune deficiency
SLE	systemic lupus erythematosus
SpA	staphylococcal protein A
TNF	Tumor necrosis factor
tu	transforming units
V_H	heavy chain variable region
V_κ	kappa chain variable region
V_L	light chain variable region
V_λ	lambda chain variable region
w/v	weight by volume

1. 0 INTRODUCTION

1. 1 GENERATION OF ANTIBODIES

Mammalian B lymphocytes are formed first in the liver during fetal life but the bone marrow becomes the site of lymphopoeisis after birth and remains so throughout adult life (Rolink and Melchers, 1991; Kincade, 1987). Genes expressed in B lymphocytes encode the immunoglobulin (Ig) molecule. The monomeric immunoglobulin is composed of two identical heavy (H) and light (L) polypeptide chains. The amino-terminal domain of each polypeptide chain consists of a variable region whilst the carboxy-terminus contains the constant domain(s). Pairing together of a variable light and heavy chain region form the antigen-combining site responsible for antigen-antibody interactions. Each separate variable domain has three areas of high amino acid sequence variability (hypervariable regions) known as complementarity-determining regions (CDR) interspersed by regions of relatively constant amino acid sequence termed framework regions (FR) (Kabat *et. al.*, 1991). CDR3 is the region of greatest variability.

The immunoglobulin heavy chain variable region segment is encoded by a variable (V_H), diversity (D), and a joining (J_H) gene segments while the light chain variable region is encoded by a variable (V_L), and joining (J_L) segment. A site-specific recombination reaction known as $V_H(D)J_H$ recombination brings gene segments present in the germline together to form a functional B cell receptor gene that encodes surface immunoglobulin in a 'virgin' B cell. Virgin B cells in the primary immune repertoire expressing germline encoded antibodies of the IgM isotype migrate from the primary lymphoid organs (fetal liver, fetal and adult bone marrow) into secondary lymphoid organs - spleen, lymph nodes - where contact with antigen, further differentiation into plasma cells may result in the secretion of either IgG, IgA, or IgE isotypes. The significance of isotypes of antibodies is demonstrated in the generation of IgG antibodies which are usually somatically mutated in the variable region and have higher affinities for antigen than IgM (Potter and Capra, 1995).

1. 2 $V_H(D)J_H$ RECOMBINATION AND THE HUMAN IMMUNOGLOBULIN LOCI

Assembly of immunoglobulin genes by the $V_H(D)J_H$ recombination process machinery under the influence of recombination activating genes (RAGs) brings different gene elements of the V region together to create considerable diversity (termed combinatorial diversity) in the antibody molecule. Additional diversity can occur by random insertion or deletion of a few nucleotides at the junctions of the segments. This is termed junctional diversity. Studies involving the development of the primary immune

repetoire expressed in normal individuals, fetal B lymphocytes, and autoantibodies have led to the identification of immunoglobulin gene families. Sequence analysis of individual gene seqments, and mapping of their chromosomal locations have helped to determine which immunoglobulin gene segments are used in the development of the normal immunoglobulin repetoire (Matsuda *et. al*., 1998; Cook and Tomlinson, 1995).

1. 2. 1 The human antibody IgH locus

The human antibody variable heavy chain locus (V_H, D and J_H genes) is located on chromosome 14q32.3 (Cox *et. al*., 1982). Sequence analysis, pulse-field gel electrophoresis mapping (PFGE), deletion mapping and cloning suggest that the human V_H locus contains 51 functional V_H gene segments (Cook and Tomlinson, 1995; Tomlinson, 1997). Another study reported the existence of 44 functional V_H genes (Matsuda *et. al*., 1998). The difference in the number of functional V_H genes in these two independent studies is attributed to an insertion/deletion polymorphic region which when present results in the increase of some functional genes. The human V_H genes are grouped into 7 families (van Dijk *et. al*., 1993). Belonging to a particular Ig (V_H) family means sharing at least 80% nucleotide sequence homology while genes with less than 70% homology are members of a different family. However, a unique set of V_H segments which share high homology (72 - 82%) with V_H1 but differ at a clustered region between framework 2 (FR2) and FR3 has been placed into a different family, the V_H7 family (Schroeder *et. al*., 1990). Among the 51 known functional V_H segments, V_H3 is the largest with 22 members, V_H4 and V_H1 has 11 each, V_H2 has 3 members, V_H5 has 2, and V_H6 and V_H7 has 1 member each (Tomlinson, 1997). There are several family-specific conserved regions within the human germline V_H segments. These family-specific sequences were found in codons 9 - 30 in FR1 and 60 - 85 of FR3 (Kabat *et. al*., 1991; Tomlinson *et. al*., 1992 Matsuda *et. al*., 1993). The conservation of sequences is not limited to only the framework regions. Complementarity determining region 2 (CDR2), one of the regions showing hypervariability, also displays some amount of family-specific conservation. Codons 60 - 65 in the 3' portion of CDR2 are conserved in a family-specific way (Honjo and Matsuda, 1995). 25 functional human D gene segments are grouped into 7 families whilst 6 J_H human gene segments are known (Tomlinson, 1997).

1. 2. 2 The human variable light chain locus

The human antibody variable light chain loci (V_L and J_L genes) are derived from either the kappa (κ) locus on chromosome 2p11-12 (Malcolm *et. al*., 1982) or the lambda (λ) locus on chromosome 22q11.2 (de la Chapelle *et. al*., 1983). In humans, 60% of immunoglobulin light chains are kappa while 40% are lambda. A total of 40 human V_κ

gene segments have been divided into 7 families. The human V_λ locus is divided into 10 families with a total of 38 functional genes (Williams *et. al*., 1996; Tomlinson 1997).

At the murine light chain locus, λ gene rearrangement always occurs after κ genes fail to rearrange (Retter and Nemazee, 1998). Thus if an appropriate κ gene is not assembled, rearrangement at the λ locus often follows.

1. 3 PHAGE DISPLAY TECHNOLOGY

Nearly eighty years after the discovery of antibodies by Emil Behring in 1894, Köhler and Milstein (1975) first described the production of monoclonal antibodies by hybridoma technology which has been of immense use as therapeutic immunoreagents, and in bio-medical research (Verhoeyen and Windust, 1996).

Though monoclonal antibody production offers an unlimited supply of specific rodent antibodies, the hybridoma technique has been much less successful for human monoclonal antibodies (James and Bell, 1987). A major drawback in the use of rodent antibodies in therapy of human antibodies is the problem of human antimouse antibody response which limits their use. A solution to the problem of immunogenicity of rodent monoclonal antibodies in humans would be to produce human antibodies directly from humans since it is technically much more difficult to immortalize human B cells to human tissue antigens than making (many) rodent antibodies, and also unethical to immunize human donors in human monoclonal antibody production. Recent advances in molecular biology and protein engineering has opened new vistas in biological research resulting in recombinant antibody technology that offers new immunotherapeutic opportunities. The new technology involves cloning of the human immunoglobulin gene repertoire comprising heavy chain and light chain genes, recombining them randomly to give a combinatorial antibody library, and expressing the antibodies on the surface of microorganisms.

1.3.1 Antibody production by combinatorial libraries

The display of peptides on the surface of filamentous phages (Smith, 1985), provided a significant beginning to phage antibody display technology. The first development toward combinatorial antibody libraries involved cloning the repertoire of V_H genes from an immunized mouse spleen into bacteriophage lambda (Sastry *et. al*., 1989). Shortly afterwards, Huse *et. al*. (1989), produced the first combinatorial antibody library by producing murine catalytic antibodies in lambda phage. PCR sequences of heavy (Fd; V_H + C_H1) and light chains from mouse RNA were cloned into separate lambda vectors. Vector DNA immediately downstream of the cloned mouse heavy chain DNA was digested away and similarly, vector sequences upstream of the mouse

light chain DNA were also cleaved. The two vector arms were digested at a common central restriction site using a common restriction enzyme and religated, such that a random pair of heavy and light chain was combined irrespective of their original pairing in the spleen cells, to give the first random combinatorial antibody library. Screening of the library for clones expressing functional Fabs (V_H1-C_H1 + V_L-C_L) were done by the adsorption of Fabs onto nitrocellulose filters and incubated with labeled antigen for detection of specific clones. Seperate heavy and light chain libraries were negative.

1.3.2 Production of antibody fragments by Phage Display

The screening procedure of the lambda phage system imposed limitations to the effectiveness of the technique. Screening a library of say 5 x 10^8 antibodies would require a minimum of 10,000 filter lifts (at 50,000 plaques per filter lift) making the screening extremely laborious. Besides, the antigen must be available in large quantities and be readily amenable to radioactive labeling, and should also not significantly stick to filters in the absence of antibody (Burton and Barbas, 1994). This imposes restrictions on for example, membrane-bound antigens, and hampers the effectiveness of the lambda phage system.

A new technique which was based on Smith's display of peptides on the surface of filamentous phages was utilized. Foreign DNA fragments were ligated to the minor coat protein III gene of filamentous phages, creating a `fusion phage´. The peptides were shown to bind immobilized antibodies, and led to easy screening of libraries for binding to ligands and antibodies. McCafferty *et. al*. (1990), displayed single chain variable domain fragment (scFv) antibodies on the surface of filamentous phages. ScFv consists of a heavy chain and light chain variable domains connected on the same polypeptide chain by a flexible oligopeptide linker. The use of filamentous phage genome as a cloning vehicle has some limitations. Since large inserts are known to adversely effect the infectivity of the phages, the risk of the library being populated by rapidly growing deletion mutants was high (Little *et. al*. 1997). Besides, it is difficult to prepare the double-stranded DNA necessary for cloning, hence phagemid systems were introduced as an attractive alternative to cloning directly into the phage genome (Burton and Barbas, 1994). The display of peptides on phages was applied to proteins. (Kang *et. al*., 1991a; Breitling *et. al*. 1991; Chang *et. al.*, 1991; Duchosal *et. al*., 1992) described the use of phagemid vectors in combination with helper phage rescue for the construction and rapid analysis of combinatorial antibody libraries.

Phagemids are plasmids that contain filamentous bacteriophage origin of replicaton but none of the genes required for replication, DNA packaging, and assembly of phage

particles. They are double-stranded, making cloning easier, and allow 100-fold higher efficiencies of transformation compared to phage vectors (Verhoeyen and Windust, 1996; Little *et. al*., 1995). Phagemids can be packaged into phage particles by 'rescue' with helper phages such as VCSM13 (Stratagene, La Jolla, Ca., USA) that provides all the phage proteins necessary for replication, DNA packaging and assembly of phage particles. The helper phage itself is unable to package its own genome due to a defective origin of replication (Burton and Barbas, 1994). Therefore, cells transformed with a phagemid are infected with a helper phage to provide proteins required for replication and packaging single-stranded phagemid DNA into the filamentous phage particle. The mature phage with the potential to bind antigen displays statistically one copy of Fab-cpIII fusion protein and 3-4 copies of the native cpIII protein which mediates phage infectivity (Fig. 1.0). In a most recent publication, Rondot *et. al*. (2001) reports a considerable increase in the fraction of phage particles displaying antibody fragments by the use of a newly-developed helper phage (hyperphage), and a mutated *E. coli* strain.

1.3.3 Phagemid pComb3H

The phagemid pComb3H (Fig. 1.1) used in this study is a derivative of the original pComb3 phagemid vector (Barbas *et. al*., 1991). The main features of pComb3 are that it contains both the origin of replication of the multicopy plasmid ColE1 and the origin of replication of the filamentous bacteriophage f1. The phagemid also contains the enzyme beta-lactamase gene that confers ampicillin (carbenicillin) resistance (Ap^r or Cb^r) to bacteria that harbor pComb3 DNA. All other genes required for replication and assembly of phage particles are lacking. Consequently, pComb3 needs a helper phage to provide the phage proteins necessary for replication and packaging. The helper phage VCSM13 which contains a gene coding for kanamycin resistance (Km^r) is used to superinfect bacteria that has been transformed with pComb3 DNA. VCSM13 is a male-specific phage i.e. host bacterial cells must contain an F factor that encodes the proteins forming the pili which is necessary for infectivity of the male-specific phage (Cold Springs Harbor Laboratory Course Manual, 1993).

ANTIBODY REPERTOIRE CLONING BY PHAGE DISPLAY

(*After* Fischer, 1995)

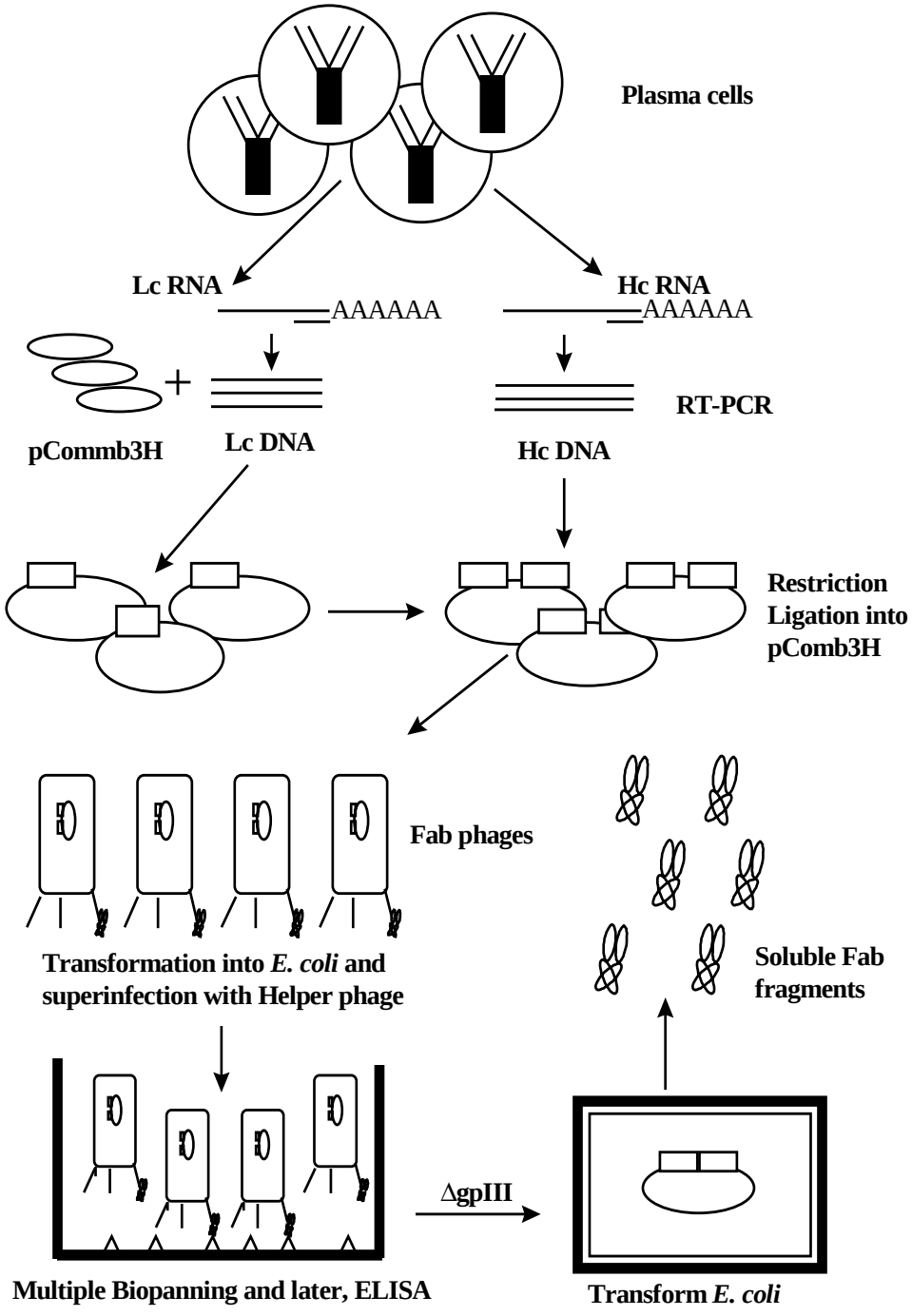

Fig. 1.0 Overview of the Phage display system with pComb3H. Amplified light and heavy chains genes are cloned into a plasmid and used to transform bacteria. Superinfection with a helper phage permits the production of phages expressing antibodies whose genetic information are carried within the phage coat. Desired antibodies are selected by successive rounds of biopanning, and subsequently ELISA on the antigen of interest. Soluble Fabs are produced upon restriction of phage coat protein gene III from the phage genome.

Phage display however suffers from potential drawbacks. Non-productive aberrant chains are often very well expressed and tend to be non-toxic to the bacterial host, whereas cells expressing functional Fab-gene III fusions may have a growth disadvantage and are selected against (Fischer *et. al*., 1999). The Fab-gene III fusion protein can also cause vector instability, creating deletions in the antibody fusion genes (Courtney *et. al*., 1995). The combinatorial construction of Fabs in the library makes it difficult to discriminate original V_H - V_L pairings from 'de novo' formed pairs, and original pairing may be lost (Fischer, 1998). The panning process which by itself is very difficult could lead to over-amplification of plastic binding clones which are inherently present in the libraries.

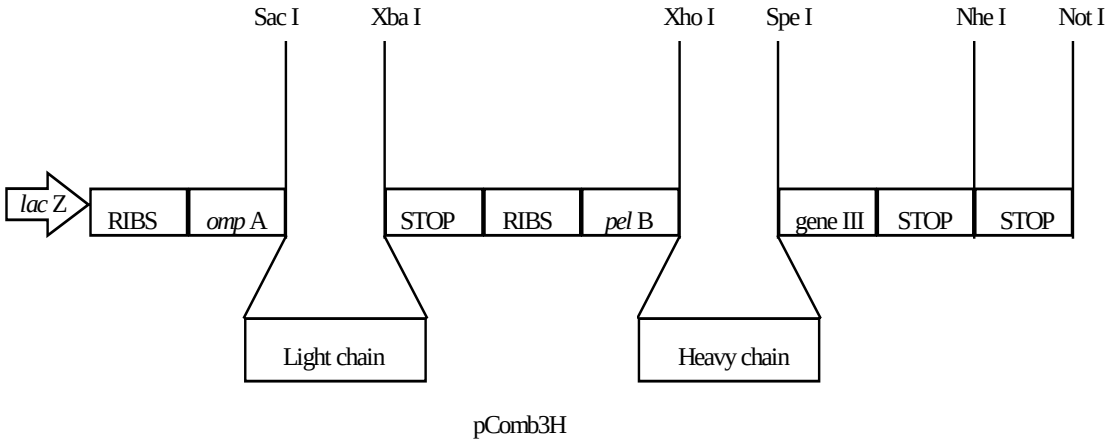

Fig. 1.1 pComb3H is a modified derivative from the original pComb3 phagemid. The *lac* Z promoter drives the synthesis of the light chain and Fd/gene III transcript. The two ribosome binding sites initiate the translation of the two separate polypeptide chains; a complete light chain (V_L - C_L), and the Fd fragment of a heavy chain (V_H - C_H1) fused to the carboxy-terminal domain of the gene III protein. Random combination of light chain and Fd fragment PCR products which have been independently amplified are cloned into the plasmid to generate a combinatorial antibody library. The leader peptides *omp* A and *pel* B target both polypeptides to the bacterial periplasm where the soluble light chain fragment and the membrane-bound Fd fragments associate via a disulfide bond. The *Sac* I and *Xba* I restriction enzyme sites are provided for cloning light chain fragments whilst Fd fragments are inserted at *Xho* I and *Spe* I restriction sites. Cleavage of pComb3H by the restriction enzyme *Spe* I (A|CTAGT) and *Nhe* I (G|CTAGT) which have identical cohesive ends that can be re-ligated results in the removal of the gene III product of M13 producing soluble Fabs. The wild type vector is provided in the form pComb3H-SS where the SS designation is for stuffer fragments. The stuffer fragments have not been well characterized in detail but they lack the restriction sites found in the vector. (After Rader and Barbas, 1997).

Phage antibodies are either produced as scFv or Fab fragments in *E. coli*, devoid of the Fc portion. For therapeutic use the Fc portion is required to yield the month-long half-life typical of antibodies (Barbas, 1995). The immobilization of antigen in plastic wells during panning results in masking or inaccessibilty of some epitopes on the antigen, compromising the conformational integrity of the antigen.

1.3.4 Recombinant antibody gene expression in *E. coli*

The wealth of knowledge accumulated from *E. coli* genetics makes it an attractive cloning vehicle for the expression of recombinant antibody fragments. Besides, growth of *E. coli* is inexpensive, cell growth is fast, and DNA transformation as well as transfection (infection) with phage is extremely efficient (Cold Spring Harbor Laboratory Course Manual, 1993). A disadvantage with using *E. coli* is that a whole antibody molecule including the Fc portion which is important for effector functions, and glycosylation which influence the complete molecule cannot be expressed in *E. coli*, since *E. coli* cannot glycosylate proteins (Verhoeyen and Windust, 1996).

1.4 SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology involving multiple organs with diverse and variable clinical manifestations. The disease is characterized by production of anti-nuclear antibodies, generation of circulating immune complexes, activation of the complement system, initiating and causing inflammation of the joints, kidney, brain, lung, skin, etc. The American Rheumatological Association have established criteria to guide medical practioners in diagnosing SLE cases (Table 1.1).

Any 4 of the 11 criteria is an indication of SLE. Patients with SLE produce autoantibodies to self-components with antinuclear antibodies being prevalent. Among these are autoantibodies to chromatin, the U1 and Sm small nuclear ribonucleoprotein (snRNP) particles, the Ro/SSA and La/SSB RNP complexes (Kotzin, 1996). U1 is a uridine-rich snRNA complexed with various polypeptides designated as 70-kD, and proteins A - G. Anti-Ro/SSA and anti-La/SSB antibodies have been implicated to have a direct pathogenic role in the development of some manifestations such as skin lesions of subacute cutaneous lupus erythematosus and congenital heart failure (Tomer *et. al.*, 1993). Anti-Ro/SSA and anti-La/SSB antibodies in SLE are 17 - 63 % and 6 - 35 % respectively. Other autoantibodies include anti-phospholipid antibodies (which complexes to β_2-glycoprotein 1) that are associated with thrombotic complications in some patients. Another subset of autoantibodies in systemic lupus erythematosus are directed to cell surface molecules. These immunopathological autoantibodies are observed in diseases such as idiopathic thrombocytopenia (platelet destruction) and

hemolytic anemia (red blood cells destruction). Anti-dsDNA antibodies which are the hallmark of systemic lupus erythematosus (Stollar, 1989) and which serves as a major diagnostic marker for the disease appear to play a prominent role in the immune complex glomerulonephritis.

Table 1.0 **The 1982 revised criteria for SLE (Tan *et. al*., 1982).**

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous-raised patches with adherent keratotic scaling and follicular plugging; athropic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation.
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5. Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a. Pleuritis - convincing history of pleuritic pain, or rub heard by physician, or evidence of pleural effusion, or b. Pericarditis - documented by electrocardiogram, rub, or evidence of pericardial effusion
7. Renal disorder	a. Persistent proteinuria greater than 0,5 g per day, or greater than 3+ if quantitation is not performed, or b. Cellular casts, red cell, hemoglobin, granular, tubular, or mixed.
8. Neurologic disorder	a. Seizures, in the absence of offending drugs or known metabolic derangements, e.g. uremia, ketoacidosis, or electrolytic imbalance, or b. Psychosis, in the absence of offending
9. Hematologic disorder	a. Hemolytic anemia with reticulocytosis, or b. Leukopenia, less than 4 x 10^6/ml two or more occasions, c. Lymphopenia, less than 1.5 x 10^6/ml, d. Thrombocytopenia, less than 100 x 10^6/ml in the absence of offending drugs.
10. Immunologic disorder	a. Positive LE cell preparation, or b. Anti-native DNA antibody in abnormal titer, or c. Anti-Sm present, or d. False-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by negative Treponema specific test
11. Antinuclear antibody	An abnormal titer of ANA by IF or an equivalent assay at any point in time and in the absence of drugs associated with drug-induced lupus

Anti-dsDNA antibodies which rarely occurs in other conditions are virtually diagnostic of SLE. The combination of high levels of anti-DNA antibodies with how levels of C3 is virtually 100 % diagnostic of SLE in patients who are suspected to have the disease on clinical grounds. Most anti-dsDNA antibodies recognize the sugar phosphate backbone of DNA, while others are base specific or bind to unique three-dimensional structures on DNA. The pathological consequence of anti-DNA antibodies is their role in kidney damage. Immune complexes of DNA-anti-DNA are deposited in the kidney glomeruli, or sometimes they form in situ in the kidney. An alternate hypothesis have it that anti-dsDNA antibodies are able to cross-react with glomerular structures that are not DNA in origin (Kotzin, 1996). Local complement activation induces glomerulonephritis with a detectable consumption of serum complement (Balow, 1991). It is important however to note that the presence of high levels of abnormal serum anti-dsDNA titer does not correlate with any particular manifestation of SLE except nephritis. Thus, it is not possible to predict from serological results that a given patient will develop renal disease, for example (Tomer *et. al*., 1993).

1.5 IDIOPATHIC THROMBOCYTOPENIC PURPURA

Platelets differentiate from megakaryocytes which are very large polyploid bone marrow cells. Upon migration from the bone marrow space, one-third are sequestered in the spleen, while the remaining two-thirds circulate for 7-10 days. The normal blood platelet count varies between 150,000 - 450,000/μl and since only a small fraction are consumed in the process of hemostasis, most platelets circulate until they become senescent and are removed by phagocytic cells.

The normal function of platelets is their adherence and aggregation to cover damaged areas. Thus when the endothelium is damaged, subendothelial layers come into direct contact with the blood, and platelets adhere and aggregate to cover up the damaged areas (Deckmyn and De Reys, 1995; Macchi *et. al*., 1997). In the subendothelium, platelets bind directly through their collagen receptors (e.g. gpIa/IIa, gpVI), or indirectly through the adhesion protein - von Willebrand factor (vWF), forming a bridge between the subendothelium and the vWF receptor on the platelets. vWF binds to platelets through contact with glycoprotein Ib (gpIb). The multimeric vWF interacts with gpIb on other platelets resulting in agglutination. Aggregated platelets become activated, produce and secrete or facilitate the production of additional platelet-activating substances such as adenosine diphosphate (ADP), serotonin, thromboxane A2, thrombin, and gpIIb/gpIIIa which help in the recruitment of additional platelets. Platelet to platelet contact is maintained when gpIIb/IIIa on adjacent platelets are bridged by fibrinogen during platelet aggregation. Deficiency of gpIIb/IIIa, or antibodies interfering with their function, particularly antibodies blocking the final step

of platelet aggregation i.e. inhibiting fibrinogen to bridge adjacent gpIIb/IIIa, have profound effects in humans; it results in severe bleeding. Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease caused by circulating antibodies that react with target antigens (the glycoproteins gp IIb-IIIa, gp Ib-IX) on platelet membranes, with the consequence that the platelets are recognized and eliminated by the host's immune system (Woods *et. al*., 1984; Kiefel *et. al*., 1991). The anti-glycoprotein antibodies function as opsonins and accelerate platelet clearance by phagocytic cells. The resultant thrombocytopenia (depletion of platelets) induces a purpura (hemorrhage discharge into skin, mucous membranes, internal organs and other tissues) when the platelet count reaches a critical level, usually < 30,000/μl (Karpatkin, 1997). Platelets interact with Fc parts of antibodies via the FcγII receptor, cross-linking of which can lead to platelet aggregation. Other mechanisms involve Fc independent platelet inactivation and complement-mediated platelet activation. Autoantibodies in idiopathic thrombocytopenic purpura (ITP) can occur in combination with another disease or not, and can be accompanied by thrombocytopenia or without. Anti-platelet antibodies are involved in hematological complications in patients with systemic lupus erythematosus displaying circulating auto-antibodies to platelets, red blood cells, lymphocytes, and nuclear and cytoplasmic cellular components.

Treatment of idiopathic thrombocytopenic purpura is directed towards the inactivation or removal of a major site of platelet destruction and anti-platelet antibody production namely, the spleen. Administration of corticosteroids prevents sequestration of antibody-coated platelets by the spleen. There arises decreased Fc γ-receptor expression on macrophages thus inhibiting phagocytosis of platelets. In addition, the amount of platelet-associated IgG decreases, thus corticosteroids also impair antibody production and/or binding to platelets. Splenectomy which is only done seldomly, removes the potential site of destruction of damaged platelets as well as the source of anti-platelet antibody production. Since a third of platelet mass is sequestered in the spleen, splenectomy results in increased platelet count of about 30%. However, post-splenectomy thrombosis is a benign self-limited condition that does not require specific therapy. High dose IVIG (1 g/kg in 2 days) is given as emergency treatment in ITP patients with platelet count below 10,000/μl. IVIG induces increased platelet counts and the mechanism of action is believed to be via blockade of the reticuloendothelial system and, the presence of anti-idiotype antibodies in the IVIG preparation (Bussell and Hilgartner, 1986).

1.6 INTRAVENOUS IMMUNOGLOBULINS (IVIG)

Intravenous immunoglobulins (IVIG) are pooled normal polyspecific immunoglobulin G (IgG) obtained from serum of several thousand healthy donors. IVIG contains primarily unmodified IgG which has functionally intact Fc-dependent effector functions and only trace amounts of IgA, IgM, soluble CD4, CD8 and HLA molecules (Blasczyk *et. al.*, 1993). Since IVIG is prepared from large donor pools (typically more than 10,000), they represent a broad spectrum of antibodies reflecting the natural exposures and immunizations of the adult populations from which the donors were drawn (Wolf and Eibl, 1996). IVIG therapy is mostly used in patients suffering from primary immunodeficiency syndromes. IVIG is also administered in anti-inflammatory diseases and several autoimmune diseases such as idiopathic thrombocytopenia (Imbach *et. al.*, 1985) autoimmune hemolytic anemia, dermatomyositis, refractory systemic lupus erythematosus, myasthenia gravis, Kawasaki disease (Fischer *et. al.*, 1996; Wolf and Eibl, 1996).

The proposed mechanism of action of IVIG in autoimmunity and systemic inflammatory diseases include: the blockade of Fc-gamma (Fc-γ) receptors on phagocytic cells, interference with activated complement, modulation of cytokine production and release, modulation of T and B lymphocyte functions, suppression of autoantibody production, and selection or shaping of immune repertoires (Mouthon *et. al.*, 1996). IVIG has been observed to block and saturate Fc-gamma receptors on cells of the reticuloendothelial system namely, macrophages and monocytes, as well as neutrophils, and T and B cells. Administration of IVIG to ITP patients resulted in decreased clustering of IgG-coated thrombocytes (Kimberly *et. al.*, 1984) and an increased platelet count (Clarkson *et. al.*, 1986). These observations are attributed to the blockade of Fc-γ receptors-mediated clearance of autoantibody-coated thrombocytes. IVIG has been shown *in vivo* to interfere with activated complement by binding to the C3b and C4b components and diverting them from interacting with target cells during complement activation (Basta *et. al.*, 1991). Basta and Dalakas (1994) reasoned the observed decrease in complement deposition in small vessels of dermatomyositis patients treated with IVIG was most likely due to formation of complexes between IVIG and activated complement components such as C3b. The anti-inflammatory effects of IVIG in vivo have been observed in the way IVIG interferes with cytokine production in B and T cells. In vitro studies on cultured monocytes have revealed that IVIG behaves as an anti-inflammatory agent as it triggers the production and extracellular release of interleukin-1 (IL-1) receptor antagonist (IL-1ra), a naturally occurring inhibitor of IL-1 activity. IL-1 is a pro-inflammatory cytokine produced by monocytes and macrophages in response to lipopolysaccharide (LPS), or Fc-γ receptor cross-linking by surface-bound IgG, soluble polymeric IgG or IgG-containing immune

complexes (Wolf and Eibl, 1996). In vivo administration of IVIG to Kawasaki disease patients led to a decreased production of IL-1 (Leung *et. al.,* 1989) as well as in rabbits injected with LPS mixed with IVIG (Arend *et. al*., 1991). It is believed the beneficial effect of IVIG in modulating decreased amounts of pro-inflammatory monokines is that it suppresses production of TNF-α and IL-1 by elevating intracellular levels of cyclic adenosine 3', 5'-monophosphate (cAMP) following its interaction with Fc-γ receptors on monocytes (Shimozato *et. al*., 1991).

Some immunomodulatory effect of IVIG is due to its reactivity with functional molecules on lymphocytes. IVIG reacts with CD5 and CD4 molecules on lymphocytes (Hurez *et. al*., 1994; Vassilev *et. al.,* 1993). $CD5^+$ (B-1) subpopulation of B cells are believed to be a predominant source of autoantibody producing cells (Casali and Notkins, 1989), thus the presence of anti-CD5 antibodies in IVIG may be of potential relevance for therapeutic modulation of autoimmunity. IVIG has also been shown to react with the CD4 molecule on T and B cells (Lam *et. al*., 1993). Anti-CD4 antibodies purified from IVIG have been found to inhibit in vitro infection of $CD4^+$ T cells by HIV-1 (Hurez *et. al*., 1994). IVIG contains antibodies capable of reacting with the V-region of other antibodies (anti-idiotypic antibodies) and B cell antigen receptors. The presence of anti-idiotypic antibodies in IVIG is demonstrated by specific retention of autoantibodies on affinity columns of IVIG coupled to sepharose. These anti-idiotypic antibodies in IVIG have the ability to inhibit the activity of autoantibodies or downmodulate their production (Wolf and Eibl, 1996). IVIG also contains IgG fractions against certain Vβ chains of the T cell receptor lineage (Marchalonis *et. al*., 1992) and have the capability to regulate the function of pathogenic autoreactive T cells. The suppressive effects of IVIG have been observed in its ability to inhibit the proliferation of in vitro activated B and T lymphocytes (Wolf and Eibl, 1996). IVIG was observed to have suppressed the production of IgM by EBV-transformed B lymphoblastoid cells (Kondo *et. al*., 1994), and also inhibited B cells stimulated by pokeweed mitogen from producing antibodies (Kondo *et. al*., 1991). In addition, the presence of other immunologically active proteins (other than immunoglobulins) in IVIG preparations may contribute to its immunomodulatory effects. These include HLA class I and II antigens and soluble CD4 and CD8 molecules. These molecules are thought to play a role in cell-cell interactions during immune response and may contribute to immunomodulation of IVIG (Wolf and Eibl, 1996). Other antibody activities found in IVIG preparations (due to its large antibody repertoire) which could influence its mechanism of action include antibodies to alloantigens such as blood group antigens (Gordon *et. al*., 1980), HLA antigens, and rheumatoid-factor-like isotype specific antibodies (Quinti *et. al*., 1987).

1.7 THE IDIOTYPIC-ANTIIDIOTYPIC NETWORK IN AUTOIMMUNITY

Idiotypic networks are thought to regulate humoral and possibly cell-mediated immunity. First proposed by Jerne (1974), the theory hypothesizes that the variable regions of antibodies or B cell receptors (idiotypes) can induce the formation of a second set of antibodies or B cell receptors (anti-idiotypes) with specificities against the first set. Some of the anti-idiotypic antibodies may recognize the antigen-combining site or paratope on the primary antibody in which case they appear as the 'internal image' of the original inducing epitope on the antigen. These antibodies are termed Ab2β. Other anti-idiotypes may recognize epitopes lying outside the paratope on Ab1 (the primary antibody). These subsets of anti-idiotypes are referred to as Ab2α. The network theory predicts that the idiotypes on Ab2 antibodies can also elicit Ab3 or anti-antiidiotopic antibodies whose idiotopes mimic that of Ab1, thereby limiting the network as decreasing levels of antibody are produced in each round of activation (Kuby, 1997). The network theory further predicts that within an individual, the interconnection between complementary idiotypes and anti-idiotypic structures on antibody molecules, as well as B and T lymphocytes leads to an immunoequilibria state (Potter and Capra, 1995). This state could potentially be disrupted through mimicry of self-antigen by auto-antiidiotypic antibody resulting in a regulatory breakdown, the outcome of which could be autoimmune disease. Theories of the idiotypic network controlling autoimmunity are about observations that certain anti-idiotype antibodies administered perinatally can lead to prolonged inhibition of the corresponding (autoimmune) idiotypes later in life, presumably by blocking immunoglobulin receptors on immature B cells which are then deleted (Kearney and Vakil, 1986). However, certain anti-idiotype antibodies given neonatally enhance idiotype-positive B cell responses even in the absence of T cell help (Leffel, 1997).

1. 8 AIMS OF THIS STUDY

The objective of this study is the use of the phage display technology to investigate:

1. the molecular analysis of Fabs bound by IVIG in a patient with SLE,
2. compare these Fabs with similar ones from patients with AITP cloned in our laboratory,
3. understand the mechanism(s) by which IVIG interact with Fabs.

2. 0 MATERIALS AND METHODS

Unless otherwise credited with the manufacturers of reagents and equipment, commonly used buffers including TE, TAE, PBS, TBS, and bacterial culture media like LB, SB, and SOC, as well as LB plates with the appropriate antibiotics were all prepared as recommended by Sambrook *et. al*., 1989.

2. 1 PATIENT

A girl (SH), aged 8 years and 5 months was transferred to our hospital because of acute myocarditis with clinical signs of cardiac failure. Two days earlier, upon her admission to another hospital, she had presented with ascites, peripheral and pulmonary edema, and a weight gain of 4 kg due to progressive heart failure. Also present were an elevated antistpreptolysin O titer of 800 IU/ml (which increased to maximum of 1,170 IU/ml), erythema marginatum, fever, hematuria, and proteinuria. Since she met 2 of the major Jones criteria (cardiac failure most likely due to preceding carditis and erythema marginatum) and at least 1 of the minor Jones criteria - fever (Jones 1944), and since she had no other specific symptoms, she was treated for rheumatic fever. Specific virologic examinations to rule out viral carditis did not provide significant evidence of an acute infection. The patient was seropositive for several antinuclear antibodies (ANA) including, anti-Ro/SSA, anti-LA/SSB, anti-Sm, antihistone, and anti-Scl 70 (Topoisomerase I), anti-ribosome. Anti-dsDNA antibodies were positive only once by ELISA at a borderline level of 6.3 IU/ml (cutoff 5.97 IU/ml). Repetitive DNA ELISAs and the immunofluorescence test for *Crithidia luciliae* were always negative. After undergoing IVIG, high-dose penicillin and aspirin therapy, our patient was discharged for further outpatient care. Her antistreptolyson titer had dropped to 507 IU/ml during therapy. Several days prior to a scheduled admission for heart catheterization (since cardiac function was still borderline), the patient had to be readmitted for fever, headache and vomiting. Upon examination, stomatitis, macrohematuria, a sore throat, and cervical lymphadenopathy were present. Heart biopsy upon catheterization did not show signs of viral infection or vasculitis. At this point, however, renal biopsy showed mesangial lupus nephritis (World Health Organization grade II). In the course of clinical follow up, ANA remained positive (Osei *et. al.*, 2000).

2. 2 ISOLATION OF LYMPHOCYTES FROM PERIPHERAL BLOOD LYMPHOCYTES

Isolation of lymphocytes from peripheral blood lymphocytes (PBLs) was carried out by using Ficoll-Paque reagent (Pharmacia, Freiburg, Germany). Ficoll-Paque, a sodium diatrizoate solution, is a low viscousity medium which offers rapid isolation and high quality lymphocytes. Diluted anticoagulant-treated blood is carefully layered on the Ficoll-Paque solution and centrifuged for a short period of time. Differential migration

of the components of blood during centrifugation results in the formation of layers containing different cell types; erythrocytes and granulocytes sediment to the bottom, and lower density lymphocytes are sandwiched between an upper layer plasma and the Ficoll-Paque reagent. The lymphocytes are then recovered from the interface and washed with PBS to remove platelets, Ficoll-Paque and plasma that might be present. The upper layer plasma which is essentially free of cells may be saved for future use.

20 ml peripheral blood was obtained from the systemic erythematosus and rheumatic fever patient for phage display library construction. 90 ml Ficoll-Paque was pipetted into two 50 ml centrifuge tubes and 10 ml vol/vol PBS:blood was carefully and slowly layered on top of each with the tube held at a slanting position of about 50 degrees. The mixture was centrifuged for 30 minutes at 1,500 rpm in a Beckman GS-6 Centrifuge (Beckman Instruments Inc, Palo Alto, Ca, USA) with the brakes turned off. Using a Pasteur pipette, the upper layer plasma was carefully aspirated, aliquoted into 1.5 ml microfuge tubes and stored at -80 ^{o}C. The lymphocytes were aspirated, pooled into new 50 ml tube, topped to 20 ml with PBS and homogenized by gentle swirling, and ca. 30 µl was saved for lymphocyte count on a hemocytometer (HBG, Germany) using Jenamed2 flourescence microscope (Carl Zeiss Jena, Germany). The lymphocytes were centrifuged for 10 minutes at 1,200 rpm with the brakes at 'low' position, and the pellet was used for total RNA isolation.

2. 3 <u>RNA ISOLATION FROM LYMPHOCYTES</u>

Isolation of total RNA from lymphocytes was carried out by using TRI reagent (MOLECULAR RESEARCH CENTER, INC, Cincinnati, OH, USA) according to the manufacturer´s protocol. TRI reagent contains phenol and guanidine thiocynate in a monophase solution. The RNA isolation steps basically include homogenizing or lysing the sample in TRI reagent, phase seperation from the aqueous phase by adding chloroform and centrifuging, RNA precipitation from the aqueous phase by additon of isopropanol, RNA wash using ethanol, and RNA pellet resuspended in appropriate buffer.

The lymphocytes (ca. 10^6-10^7 cells/ml) were homogenized in 1 ml TRI reagent and incubated at room temperature for 5 minutes. 200 µl chloroform (Merck GmbH, Darmstadt, Germany) was added, vigorously shaken for 15 seconds and incubated at room temperature for a further15 minutes, and centrifuged at 12,000 xg (ca. 11,500 rpm) for 15 minutes at 4 ^{o}C. The aqueous phase was carefully pipetted into a new microfuge tube, mixed with 500 µl isopropanol (Merck, Germany), incubated at room temperature for 10 minutes, and centrifuged at 12,000 xg for 10 minutes at 4 ^{o}C. The pellet was washed with 1 ml of 75% ethanol (Merck, Germany), centrifuged as above,

air-dried briefly for about 5 minutes and resuspended in 50 µl TE buffer. 10 µl of the RNA was analysed on 1% agarose (Gibco BRL, USA) gel electrophoresed at 200 V for 15 minutes. The rest of the RNA was stored at -80 ^{o}C for synthesis of cDNA.

2. 4 SYNTHESIS OF Fd, λ AND κ cDNA BY REVERSE TRANSCRIPTION

Total RNA was used for the synthesis of first strand cDNA using 3' primers that were subsequently used in the PCR. The 3' primers specifically associate to the 3' end of the light chain (CL) constant region or the CH1/hinge (Heavy chain) allowing for incorporation of the interchain cystein codon of each chain in the product (Persson *et. al*. 1991; Kang *et. al*., 1991b).

10 µl of total RNA was added to 2 µl of either heavy (Fd; V_H + C_L1), or light chain (κ and λ) 3' constant region primer in a 1.5 ml microfuge tube, 8 ml of sterile water was added, and incubated at 70 ^{o}C for 10 minutes, chilled for 5 minutes on ice and centrifuged for 5 seconds. 7 µl sterilized water, 10 µl 5x reverse transcriptase buffer (Gibco BRL, Life Technologies, USA), 5 µl DTT (Gibco BRL, USA), 2 µl RNAase Inhibitor (Promega, Madison, WI, USA), 3 µl dNTP-mix (Promega, USA), were added, mixed and incubated at 42 ^{o}C for 2 minutes, and 3 µl Superscript reverse transcriptase (Gibco BRL, USA), was added and incubated at 42 ^{o}C for 50 minutes. The reaction was centrifuged for about 10 seconds, incubated at 70 ^{o}C for 15 minutes, briefly centrifuged for about 10 seconds and incubated for 10 minutes on ice. 1 µl RNAase H (Promega, USA) was added, and incubated at 37 ^{o}C for 20 minutes. The resulting cDNA was shortly centrifuged and stored at -20 ^{o}C for PCR.

2. 5 RT-PCR AMPLIFICATION OF Fd, λ, AND κ CHAINS

Each PCR was run in a 0.5 ml microfuge tube in a total volume of 100 µl and contained 75.2 µl sterile water, 10 µl of 10x PCR buffer (without Mg^{2+}-ions; Promega, USA), 5 µl (1.25 mM) $MgCl_2$ (Promega, USA), 0.8 µl dNTP-mix (final concentration 200 µM), 3 µl of either 3' constant region primer namely, cG1z, cK1d, cL2, 3 µl of the appropriate 5' variable region primer, 1 µl (5 Units) *Taq* DNA polymerase (Promega, USA), a drop of mineral oil (Sigma Chemical Co., St. Louis, MO, USA), and 2 µl of the corresponding 3' constant region primer cDNA (template). For either positive or negative control reactions, the following 5' variable region primers were used: conGa (V_H chains), conKa (kappa chains) and conL1 (lambda chains), [Table 1,1]. In negative control reactions however, no cDNA were added, rather water was added. Finally, the reaction was centrifuged for about 30 seconds at 12,000 rpm and thermocycling was carried out in a HYBAID Omnigene DNA Thermocycler (MWG-BioTech GmbH, Germany), using the following reaction conditions; initial denaturation: x1 (94 ^{o}C) for 3

minutes, cycling: x40 with primer annealing - 52 ^{o}C for 1 minute, extension - 74 ^{o}C for 1 minute, denaturation - 93 ^{o}C for 30 seconds, and final extention - 74 ^{o}C for 10 minutes. At the end of the thermocycling, 10 µl of the PCR reaction was run on a 2% agarose gel using ΦX 174/Hae III marker (Gibco BRL, Life Technologies, USA). A band of ca. 680 bp for V_H and L_H fragments, 330 bp for the positive controls, and no amplication product(s) in the negative controls were indicative of a successful PCR.

2. 5. 1 Purification and Quantification of PCR products

PCR DNA was eluted and purified from agarose gel using S & S Biotrap (Schleicher & Schuell GmbH, Dassel, Germany). The Biotrap is an electro-seperation system for elution and purification of charged macromolecules e.g. proteins as well as nucleic acid fragments from 14 to 150,000 base pairs without losses due to denaturation.

Except the PCR product controls using conGa (Fd), and conKa/conL1 (light chain) that were discarded, the rest of the PCR products were pooled as follows; all Fd products were combined together and, all light chain products (κ and λ) also combined together. Each was extracted with 1:1 vol./vol. of Phenol:Chloroform (Roti-Phenol, Carl Roth, Germany). The PCR DNA was run on 2% agarose gel at 150 V for 1-1/2 hours. The heavy and light chain PCR products were excised out of the agarose gel and electroeluted from the gel slice at 200 V for 1-1/2 hours using the S & S Biotrap, extracted with 1:1 vol./vol. Phenol:chloroform, and each resuspended in 50 µl TE buffer. 1 µl of the DNA was analysed on a 2% agarose gel and the concentration of the light and heavy chain PCR products were determined to be 0.491 µg/µl and 0.598 µg/µl respectively.

2. 5. 2 Restriction of PCR products

Preparation of antibody light and heavy chain inserts for cloning was carried out by enzymatic double restriction using Sac I (40 U/µl) and Xba I (40 U/µl) which utilize buffer A for light chain DNA and, Xho I (40 U/µl) and Spe I (40 U/µl) which utilize buffer H for heavy chain DNA (Bohringer Mannheim GmbH, Germany).

10 µg light chain PCR DNA (0.491 µg/µl) was restricted using 1.5x excess units of the light chain restriction enzymes i.e. 57 U Sac I/µg DNA and 112.5 U Xba I/µg DNA. The reaction was incubated at 37 ^{o}C for 3h, ethanol precipitated, electroeluted as above, resuspended in TE buffer, and quantitated. Similarly, 10 µg heavy chain PCR DNA (0.598 µg/µl) was restricted using 1.5x excess units of the heavy chain restriction enzymes i.e.109.5 U Xho I/µg DNA and 25.8 U Spe I/µg DNA, at 37 ^{o}C for 3h, ethanol precipitated, electroeluted, resuspended in TE buffer, and the amount measured.

PCR PRIMERS

Table 1.1 Human variable and constant region PCR primers used for phage antibody library construction (Kang *et. al*., 1991b).

Human Heavy Chain Variable Domain 5' Primers

V_H1a	5′ - CAG GTG CAG CTC GAG CAG TCT GGG - 3′	(24 mer)
V_H1f	5′ - CAG GTG CAG CTG CTC GAG TCT GGG - 3′	(24 mer)
V_H2f	5′ - CAG GTG CAG CTA CTC GAG TCG GG - 3′	(23 mer)
V_H3a	5′ - GAG GTG CAG CTC GAG GAG TCT GGG - 3′	(24 mer)
V_H3f	5′ - GAG GTG CAG CTG CTC GAG TCT GGG - 3′	(24 mer)
V_H4f	5′ - CAG GTG CAG CTG CTC GAG TCG GG - 3′	(23 mer)
V_H4g	5′ - CAG GTG CAG CTA CTC GAG TGG GG - 3′	(23 mer)
V_H6a	5′ - CAG GTA CAG CTC GAG CAG TCA GG - 3′	(23 mer)

Human Heavy Chain constant domain 3' Primer

IgG1	CG1z	5′- GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG - 3′	(30 mer)

Human Heavy Chain constant domain *control* 5' Primers

IgG1	ConGa	5′ - TCC ACC AAG GGC CCA TCG - 3′	(18 mer)

Human Light Chain Primers

Kappa chain variable domain 5' primers

VK1a	5′ - GAC ATC GAG CTC ACC CAG TCT CCA -3′	(24 mer)
VK1s	5′ - GAC ATC GAG CTC ACC CAG TCT CC -3′	(23 mer)
VK2a	5′ - GAT ATT GAG CTC ACT CAG TCT CCA -3′	(24 mer)
VK3a	5′ - GAA ATT GAG CTC ACG CAG TCT CCA -3′	(24 mer)
VK3b	5′ - GAA ATT GAG CTC AC(G/A) CAG TCT CCA -3′	(24 mer)

Kappa chain constant domain 3' primers

CK1d	5′ - GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G -3′	(57 mer)

Kappa chain constant domain *control* 5' primers

CONKa	5′ - ACT GTG GCT GCA CCA TCT G -3′	(19 mer)

Lambda chain variable domain 5' primers

VL1	5′ - AAT TTT GAG CTC ACT CAG CCC CAC -3′	(24 mer)
VL2	5′ - TCT GCC GAG CTC CAG CCT GCC TCC GTG -3′	(27 mer)
VL3	5′ - TCT GTG GAG CTC CAG CCG CCC TCA GTG -3′	(27 mer)
VL4	5′ - TCT GAA GAG CTC CAG GAC CCT GTT GTG TCT GTG - 3′	(30 mer)
VL5	5′ - CAG TCT GAG CTC ACG CAG CCG CCC -3′	(24 mer)
VL6	5′ - CAG ACT GAG CTC ACT CAG GAG CCC -3′	(24 mer)
VL7	5′ - CAG GTT GAG CTC ACT CAA CCG CCC -3′	(24 mer)
VL8	5′ - CAG GCT GAG CTC ACT CAG CCG TCT TCC -3′	(27 mer)

Lambda chain constant domain 3' primers

CL2	5′ - CG CCG TCT AGA ATT ATG AAC ATT CTG TAG G -3′	(30 mer)

Lambda chain constant domain *control* 5' primers

CONL1	5′ - AAG GCT GCC CCC ACG GTC ACT CTG -3′	(24 mer)

Underlined nucleotides represent primer-encoded restriction sites

2. 6 PREPARATION OF *E. coli* STRAIN XL1-Blue FOR CLONING

The preparation of bacterial cells for high efficiency transformation involves growing the bacteria to early-to-midlog phase. This offers a period of greatest competence where the cells are still growing rapidly at densities (and volumes) high enough to recover very large number of cells. The cells are harvested by chilling and centrifuging, and for electroporation, washed several times with water and resuspended in 10% Glycerol in deionised water to reduce the ionic strength of the final cell suspension (Gene Pulser Controller, Operating Instructions and Applications Guide, BIO RAD, USA). Increasing cell concentration increases yield of transformants, therefore cells are resuspended to very high concentration, aliqouted and stored at -80 ^{o}C. The *E. coli* strain XL1-Blue (Stratagene, U.S.A) has the following genotype:
recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac^{-}
F' (proAB^{+}*, lacI*^{q}*, ZdelM15, Tn10).*

XL1-Blue allows high efficiency transformation, up to 10^9 colonies per μg of pUC DNA when using electroporation (Cold Spring Harbor Laboratory Course Manual, 1993). XL1-Blue is recombination-deficient (*rec A1*) which is necessary to maintain stability of plasmids propagated in this strain. In addition, transposon 10 (*Tn 10*) which harbors the gene coding for resistance towards the antibiotic tetracycline (tet^{r}) has been intergrated into the F' factor. This ensures the expression of the F' factor in media containing tetracycline and maintains the ability of the bacteria to act as a host for male-specific phages such as M13.

300 μl glycerol stock of *E. coli* strain XL1-blue (Stratagene Cloning Systems, La Jolla, Ca., USA) was resuspended in 5 ml SB medium supplemented with 20 μl of 10 mg/ml tetracycline (Merck, Germany) and incubated overnight with shaking (300 rpm) at 37 ^{o}C. 3 ml of the overnight culture was transferred into a 37 ^{o}C pre-warmed 300 ml SB medium containing 600 μl tetracycline (10 mg/ml) and incubated as above until mid-log phase (for ca. 3 h), when the OD_{600} = 0.8 - 1.0. The culture was incubated on ice for 20 minutes and divided into 6 pre-chilled 50 ml tubes, and centrifuged at 4,800 rpm (3,838 xg) for 10 minutes at 4 ^{o}C. The supernatant was discarded and the pellet resuspended in 10 ml ice-cold water, topped to 50 ml and re-centrifuged as above. The pellet was resuspended in 25 ml ice-cold water and centrifuged again, and was resuspended in 1 ml 10% glycerol (Carl Roth, Germany). The cells were pooled into two tubes and centrifuged as above, pooled and resuspended in 3 ml of 10% glycerol and aliqouted at 200 μl into 1.5 ml microfuge tubes directly onto dry ice and stored at -80 ^{o}C. To test whether the cells were viable and devoid of contamination, 50 μl were plated on carbenicillin (Merck, Germany) [100 μg/ml LB medium], kanamycin (Sigma, USA) [70

μg/ml LB medium], and tetracycline (10 μg/ml LB medium) plates, and incubated overnight at 37 ^{o}C. Inspection of the plates next morning showed that there were no growth on the carbenicillin and kanamycin plates indicating that the XL1-blue strain had not acquired resistance to these two markers whilst there were clones on the tetracycline plates showing that the cells were viable. 1 μg wildtype phagemid, pComb3HSS (Dr. Barbas, Scripps Research Institute, La Jolla, USA) was electroporated into 200 μl of the cells to determine transformation efficiency of the cells. This was determined to be 3.2 x 10^{7} colonies/μg DNA.

2. 7 PREPARTION OF pComb3H VECTOR

2. 7. 1 Transformation of E. coli strain XL1-blue by High Voltage Electroporation (Electrotransformation).

Transformation of bacteria by high voltage electroporation is a convenient and reliable way to achieve success in cloning. Frozen glycerol stock of bacteria are thawed on ice and used for transformation. Electroporation is carried out in a special high voltage mini-electrode and chilled cuvettes. An optimal balance between the strength of the electrical field, the length of the electrical pulse and the concentration of DNA results in higher transformation efficiencies (Sambrook *et. al.*, 1989).

1 μg of pComb3HSS DNA resuspended in 10 μl sterile deionised water was mixed with 200 μl thawed glycerol stock of *E. coli* strain XL1-blue cells and transferred into a chilled 0.2 cm gap cuvette (Bio-Rad, USA), and pulsed at 2.5 kV, 25 μFD and 200 ohms for 4.5 seconds at a field strength of 12.5 kV/cm in a Gene Pulser (Bio-Rad, USA). The electrotransformed cells were rapidly flushed with 3 ml SOC medium into a 10 ml tube and incubated at 37 ^{o}C for 1h, shaking (200 rpm), in a G 24 Incubator shaker (New Brunswick Scientific Co. Inc., Edison, NJ, USA). The culture was transferred into a 50 ml tube containing pre-warmed (37 ^{o}C) 10 ml SB medium supplemented with 2.6 μl carbenicillin,100 μl of 1M $MgCl_2$ (Merck, Germany), and incubated as above. Prior to the incubation, about 80 μl was saved for determining the efficiency of the transformation by plating 50 μl, 1.0 μl, and 0.1 μl on LB plates containing 100 μg/ml carbenicillin. The culture was transferred into a 1l conical flask containing 100 ml SB medium supplemented with 56.0 μl of 100 μg/ml carbenicillin, 1 ml of 1M $MgCl_2$ and incubated overnight as above. The plasmid DNA was extracted with the QIAGEN MIDI Plasmid kit the next day.

2. 7. 2 pComb3HSS: QIAGEN MIDI plasmid purification

pComb3HSS DNA was purified using the QIAGEN MIDI plasmid purification kit according to the manufacturers instructions. The QIAGEN plasmid kit is an alkaline lysis based purification protocoll. It allows the purification of ultrapure plasmid DNA (yield ca. 100 μg DNA) without the use of phenol, chloroform, or cesium chloride (QIAGEN Plasmid Purification Handbook). Essentially, the protocol involves lysis of bacterial cells under alkaline conditions and after centrifugation, the lysate is applied under defined salt conditions to a pre-equilibrated QIAGEN resin tip. The resin ensures that only plasmid DNA is bound while degraded RNA, cellular proteins, and metabolites are washed down the column under gravity. A high salt buffer efficiently elutes plasmid DNA from the resin and the DNA is desalted and precipitated by isopropanol. Finally, it is washed in 70% ethanol, dried briefly and resuspended in an appropriate buffer.

The overnight pComb3HSS electrotransformed culture was divided 50 ml each into two 50 ml centrifuge tubes and centrifuged at 3,000 rpm (1,500 xg), for 20 minutes at 4 ^{o}C, Beckmann GS-15R centrifuge. One pellet was used for purification of the wildtype plasmid using the Qiagen Plasmid purification kit. 1 μg of the plasmid DNA was analysed on 1% agarose gel with 1 kb DNA ladder (Gibco BRL, USA) as a marker.

2. 8 PREPARATION OF LIGHT CHAIN VECTOR FOR CLONING

The pComb3H-SS[1] vector contains a light chain stuffer sequence of about 1,200 bp and a heavy chain stuffer of 300 bp

10 μg pComb3H-SS DNA was restricted with Sac I and Xba I (6.25 U/μg and 12.5 U/μg respectively) at 37 ^{o}C, ethanol precipitated, run on 0.7% low melting agarose (Sigma, USA) at 90 V, for 1 h and both vector DNA and light chain stuffer fragments excised out, and electroeluted (seperately) in an S & S Biotrap. The DNA was ethanol precipitated, resuspended in TE buffer, the amount measured and stored at -20 ^{o}C.

2. 9 TEST LIGATION-LIGHT CHAIN INSERT

Important parameters to consider during ligation of an insert (foreign DNA) to a vector include an optimal ratio of insert to vector and the concentration of each DNA in the reaction mixture (Sambrook *et. al.*, 1989). To monitor the efficiency of ligation, it is invaluable to carry out test ligations which should include the optimal ratio of cut vector DNA to insert, the cut vector with ligase but without insert, and the cut vector DNA

[1] pComb3H-SS: wild type plasmid
pComb3H-S: light chain vector (with the light chain stuffer fragment excised out)

only without neither insert nor ligase. These serve as a useful guide for the subsequent ligation.

The light chain insert test ligation was as follows:

a. 43.2 ng pComb3H-S vector + 12.1 ng light chain insert + ligase
b. 86.4 ng pComb3H-S vector + 12.1 ng light chain insert + ligase
c. 86.4 ng pComb3H-S vector + ligase (no insert)
d. 86.4 ng pComb3H-S vector (no insert, no ligase)

Each reaction was carried out in a total volume of 5 µl, incubated at 16 ^{o}C overnight, electroporated into 200 µl of *E. coli* strain XL1-blue, and titrated overnight as above. Phenol/chloroform prepartion of (total) DNA and restriction enzyme analysis using the light chain enzymes Sac I and Xba I for each test ligation was carried out to determine which of the test ligations was (more) successful.

2. 9. 1 Light Chain Library construction: ligation of Light Chain insert to Light Chain vector

2.2 µg pComb3H-S DNA was ligated to 0.605 µg light chain DNA overnight at 16 ^{o}C i.e. 50x the concentrations of both light chain vector and insert from *test ligation (a)* above, was used in the construction of the light chain library. 50 µl, 1.0 µl, and 0.1 µl of the library were plated on carbenicillin plates (100 µg/ml LB medium). The light chain library total DNA was extracted by using the QIAGEN Midi Plasmid preparation kit.

2. 9. 2 Light Chain Library: Single clones minipreps culture

Single clones from the carbenicillin plates were cultured overnight in SB medium supplemented with 100 µg/ml carbenicillin. Minipreps were prepared by phenol/chloroform extraction and the DNA analysed on agarose gel by light chain restriction enzymes to determine the percentage of clones that had the light chain fragment (insert).

2. 10 <u>THE COMPLETE FAB LIBRARY CONSTRUCTION</u>

A series of background test ligations, and restriction enzyme analyses of the ligations, revealed that a 3:1 vector to insert molar ratio gave a higher percentage of desired clones with about 70% having both the light and heavy chain inserts and this was used during the actual ligation of the heavy chain insert to the heavy chain vector.

4.95 µg heavy chain vector DNA and 1.65 µg heavy chain insert were ligated overnight, ethanol precipitated the next day, resuspended in 10 µl water and electroporated into XL1-blue cells. The cells were grown at 37 ^{o}C for 1h with shaking and, transferred into a prewarmed (37 ^{o}C) 10 ml SB medium containing 2.6 µl carbenicillin (low concentration), and 100 µl 1M $MgCl_2$. 50 µl, 1.0 µl, and 0.1 µl were titrated on

carbenicillin LB plates, and the rest incubated for 1h as above. The culture was transferred into a 37 ^{o}C prewarmed 100 ml SB medium supplemented with 56 µl carbenicillin (high concentration) and 1 ml 1M $MgCl_2$ and incubated for 1 h as above. 200 µl (7.0 x 10^{11} t.u/ml) of VCSM13 helper phage (Stratagene, Ca., USA) was added to the culture and incubated for 2 h as above. 160 µl kanamycin was added to the culture and incubated under the same conditions as above, overnight.

2. 11 PREPARATION OF THE NAIVE SURFACE DISPLAYED FAB PHAGES

The overnight culture was divided into two 50 ml tubes and centrifuged at 3,000 rpm (ca. 1,500 xg) for 20 minutes at 20 ^{o}C in a Beckmann GS-15R centrifuge. The supernatant was transferred into a GSA rotor flask and 4 g (4% w/v) PEG-8000 (Sigma, USA) and 3 g (3% w/v) NaCl (Merck, Germany) was added and incubated on ice for 20 minutes. The bacterial pellet containing the unselected (total) library DNA was saved for DNA isolation using the QIAGEN DNA Preparation kit. The PEG precipitated phage suspension was centrifuged at 8,600 rpm for 20 minutes at 4 ^{o}C (in a Sorvall RC 5B Plus centrifuge using a GSA rotor). The supernatant was discarded and the pellet was resuspended in 2 ml of 1% Casein (Sigma)/TBS (supplemented with 0.02% NaN_3 [Merck, Germany]), and divided 1 ml each into two 1.5 ml microfuge tubes and centrifuged for 5 minutes at 12,500. The two Fab phage suspensions (supernatants) were stored at 4 ^{o}C (for biopanning), and -20 ^{o}C (for long term storage) whilst the pellets were discarded.

2. 12 BIOPANNING

2. 12. 1 Coating of antigens

Maxisorp immunotubes (Nunc,Wiesbaden, Germany) were coated with 9 µg (300 µl) IVIG (30 mg/ml) preparation (Sandoglobin, Sandoz, Basel, Switzerland) and stored at 4 ^{o}C.

2. 12. 2 Day 1

2. 12. 2. 1 Bacterial culture

200 µl glycerol stock of *E. coli* strain XL1-blue was thawed on ice and resuspended in 10 ml SB medium supplemented with 20 µl tetracyclin (10 µg/ml, and incubated at 37 ^{o}C for about 1 h (until mid-log phase, Absorbance at OD_{600}nm = 0.8 - 1.2) with shaking.

2. 12. 2. 2 Panning methodology

Antigen-coated immunotube number 1 was washed 3x with wash buffer (0.2% PBS/Casein-0.05% Tween 20 (Fluka, Switzerland) and once with PBS, blocked

completely with blocking buffer (1% Casein/PBS) and incubated at 37 ^{o}C for 1 h. The blocked tube was washed 2x with wash buffer and once with PBS, and 300 µl of the Fab phage suspension was added and incubated at 37 ^{o}C for 2 h inside a humidified chamber.

Using a Pasteur pipette, the Fab phage suspension were pipetted from the tube and unbound phages washed off as follows: Panning 1; 1 ml wash buffer was pipetted into the tube, and allowed to stand for 10 minutes at room temperature and aspirated. This was repeated again and then rinsed with PBS. During subsequent panning rounds however, the wash buffer was allowed to stand for 2 minutes (repeated 10x) and finally rinsed once with PBS. 300 µl elution buffer [0.1M HCl (Merck)/glycine (Sigma), pH 2.2] was added to the tube, vigorously pipetted up and down, and incubated 10 minutes at room temperature. The eluted phages were collected from the tube, and neutralized with 60 µl neutralization buffer containing 2M Tris (Carl Roth, Germany)/HCl pH 9.0, and used to infect 3 ml of *E. coli* strain XL1-blue culture (from above) and incubated at 37 ^{o}C for 20 minutes in a shaking incubator.

50 µl of the bacterial culture was plated on carbenicillin and tetracyclin plates and incubated at 37 ^{o}C overnight as a control to ascertain that no phagemid and helper phage contaminations had taken place in the bacterial hosts. The transformed 3 ml *E. coli* was added to a 37 ^{o}C pre-warmed 10 ml SB medium supplemented with 2.6 µl carbenicillin and 100 µl of 1M $MgCl_2$, and briefly vortexed. 60 µl was saved for output titration of the Fab phage (by plating 50 µl, 1.0 µl, and 0.1 µl on carbenicillin plates) with the rest of the culture incubated further at 37 ^{o}C for 1 h, with shaking. Similarly for input Fab phage titration, 10 µl of 10^{-7}, 10^{-8}, and 10^{-9} c.f.u/ml serial dilutions of the infecting (PEG-precipitated) phage suspension were incubated with 50 µl of the bacterial culture at 37 ^{o}C for 20 minutes and were plated on carbenicillin plates for Fab phage input titration. The transformed 13 ml culture was added to a 37 ^{o}C-prewarmed 100 ml SB medium supplemented with 56 µl carbenicillin and 1 ml of 1M $MgCl_2$, and incubated at 37^{o}C for 1 h, with shaking, and 200 µl (7.0×10^{11} t.u/ml) VCSM13 helper phage was added, and incubated at for 2h as above. 160 µl kanamycin (70 µg/ml) was added to the culture and grown overnight as above.

2. 12. 3 DAY 2 (and other subsequent rounds of panning)

Preparation of surface-displayed Fabs for the next round of panning was done exactly as under section 2.10, above. For subsequent panning rounds, 300 µl of the PEG precipitated Fab Phage suspension (from the previous panning) was incubated with the coated antigen in the immunotubes, washed, eluted, neutralized and used to re-infect

freshly grown bacteria (as in Panning 1), and the panning steps continued as above. Total library DNA was isolated from the bacterial pellet from the last round of panning for further analysis, unlike the earlier panning rounds when the bacterial pellet was discarded (during PEG/NaCl precipitation of the phages).

At the end of the last round of panning, single clones growing on carbenicillin plates were cultured in 48-well plates containing 300 µl SB supplemented with carbenicillin and 10 mM $MgCl_2$ for 5 - 6 h at 37 ^oC with shaking. VCSM13 helper phages were added and grown further for 1 h. Thereafter, kanamycin was added and grown overnight. The overnight culture was centrifuged at 2000 rpm in a Beckman GS-15R centrifuge using a plate rotor (Beckman Instruments, Palo Alto, Ca., USA). The supernatant was transferred into a blocked 96-well microtitre plate and used in Fab phage ELISA. DNA were prepared from the pellets from clones that were positive in the ELISA and analyzed by restriction enzyme on agarose to identify clones that had both heavy and light chain inserts.

2. 13 ELISA PROCEDURES

Microtitre plates (Coastar, Corning, Wiesbaden, Germany) were coated with equal concentrations of antigens (600 ng/well) and kept in a humidified chamber at 4^oC overnight. Wells were blocked with 1% Casein-PBS for 1 h at 37 ^oC and incubated with Fab phage supernatants for 1 h. Bound Fab phages were detected by HRP-conjugated mouse anti-M13 phage antibodies (Pharmacia, Freiburg, Germany), and read in a DYNATECH MR 5000 ELISA reader.

2. 14 DETERMINATION AND ANALYSIS OF NUCLEOTIDE AND AMINO ACID SEQUENCES

Fab phage clones that were positive in the ELISA and had light and heavy chain DNA inserts as determined by restriction enzyme analysis were cultured overnight in SB medium containing carbenicillin. DNA preparations were done either by the Promega Wizard Miniprep kit (Promega, Madison, WI) or Qiagen miniprep kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. Cycle sequencing reaction was performed using Amersham cycle sequencing kit (Amersham, Braumscheig, Germany) with infrared flourophore (IRD41)-labelled primers (MWG Biotech, Eberserg, Germany) (Fischer *et. al*., 1999). The sequencing primers were PelB and SeqGb for the heavy chains, and OmpA, SeqLb or SeqKb for the light chains , (Fischer *et. al*., 1999; Jendreyko *et. al*., 1998; Graus *et. al*., 1997).

Table 1.2 Sequencing primers.

A. HEAVY CHAIN

(+) strand:	*SEQGb*	5′- GTC GTT GAC CAG GCA GCC CAG -3 ′	(21-mer)
(-) strand:	*Pelb*	5′- ACC TAT TGC CTA CGG CAG CCG - 3′	(21-mer)

B. LIGHT CHAIN

(+) strand:	*SEQkb*	5′- ATA GAA GTT GTT CAG CAG GCA - 3′	(21-mer)
(+) strand:	*SEQlb*	5′- GAA GTC ACT TAT GAG ACA ACA C - 3′	(22-mer)
(-) strand:	*OmpA*	5′- AAG ACA GCT ATC GCG ATT GCA G - 3′	(22-mer)

Nucleotide sequences were analyzed with the MacDNAsis program, and manually controlled by scf-files. Sequencing was repeated for ambiguous or unclear sequences. Clones expressing functionally rearranged heavy and light chain sequences were further evaluated by VBASE via the Internet (http://www.mrc-cpecam.ac.uk/imt-doc/publicINTRO.html) using the program DNA-Plot developed by W. Muller and H.-H. Althaus, University of Cologne, Germany for determination of germline segments and mutations. To enable easy comparison of our findings with those of others in this field, we applied the widely used Kabat definition of complementarity-detemining regions (CDRs) and framework regions (FRs) as well as the V-BASE nomenclature. Thus we used the full length of the germline V-genes in these calculations.

3. 0 RESULTS

3. 1 LIBRARY CONSTRUCTION

3. 1. 1 Isolation of RNA

10^7 cells/ml B lymphocytes - antibody producing cells - were isolated from 20 ml peripheral blood from an 8-1/2 year-old girl (SH) suffering from acute myocarditis with clinical signs of cardiac failure for library construction. She was diagnosed as a patient suffering from systemic lupus erythematosus and rheumatic fever. The blood was drawn 24 days after the patient had had IVIG therapy. RNA from peripheral blood lymphocytes after administration of IVIG would provide DNA dominated by currently secreted specificities, as cells actively secreting antibodies will contribute substantially more immunoglobulin RNA than from resting B cells that sit stationary in lymphoid tissues (Persson, 1993). Total RNA was extracted from the B lymphocytes for cDNA synthesis (Fig. 3.1).

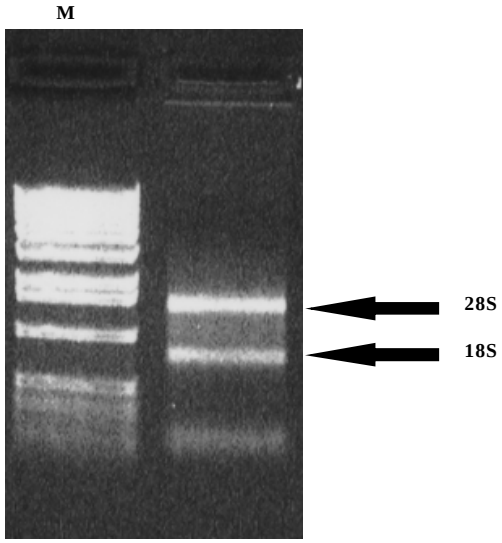

Figure 3. 1. Gel electrophoregram of total RNA isolated from the peripheral blood lymphocytes of a patient with SLE and rheumatic fever. The quality of undegraded total RNA is shown by the double-sized 28S rRNA band to that of the 18S rRNA band. M, ΦX 174 molecular marker.

Since RNA molecules are unstable and denature readily, and since DNA molecules are stable to handle in cloning, the RNA was reverse-transcribed into cDNA, for subsequent use in PCR and routine cloning purposes.

3.1.2 Amplification and cloning of Fd, λ, and κ chain genes

First strand cDNA syntheses in separate reactions (γ, κ and λ chains) were performed using 3'specific primers that were subsequently used in the PCR. To generate sufficient amount of DANN for cloning, the cDNAa was first amplified in PCR. The PCR primers are designed such that they incorporate restriction enzyme sites allowing for directed cloning with the proper reading frame. Analysis of the heavy chain PCR products on 2 % agarose gel revealed bands of expected sizes between 660-680 bp. The varying intensities may represent the level of expression of different antibody gene families presented by the patient at the time the material was collected (Fig. 3.2).

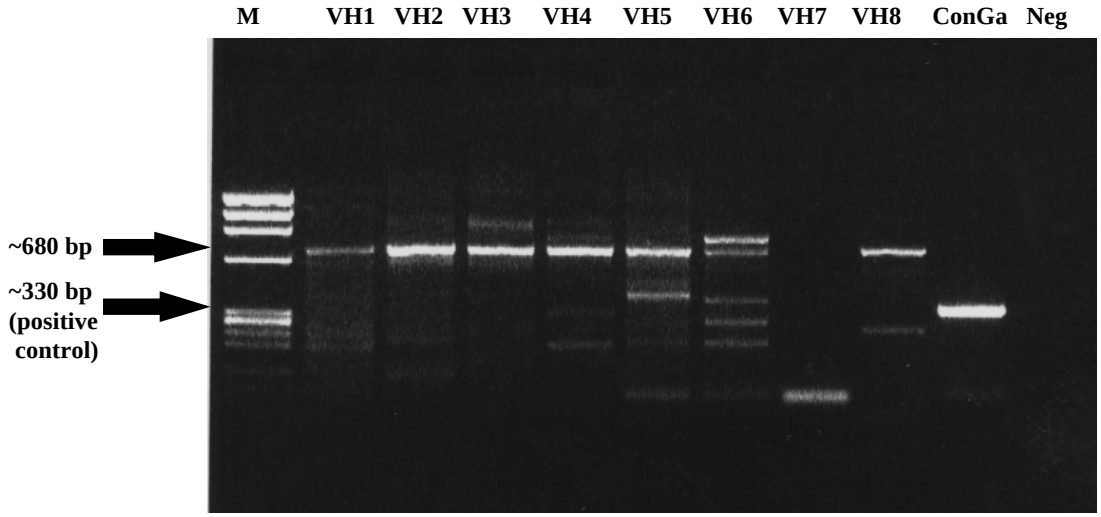

Figure 3. 2. Gel analysis of PCR amplified human genomic immunoglobulin heavy chain variable region segment. Amplification conditions are described in the materials and methods. M, ΦX 174 molecular marker; VH1, …..VH8, family specific Ig genes; ConGa, positive control primer for constant regions; Neg, negative control without template

The heavy chain PCR products were pooled, electrophoresed on 2% agarose, and the expected size of the heavy chain fragments excised and purified from the gel, Fig. 3.3 (A) and (B). The fragments were restricted by the heavy chain restriction enzymes, subjected to agarose gel electrophoresis, and finally purified from the agarose gel, Fig. 3.3 (C) and (D). Similarly, all light chain PCR fragments (kappa and lambda) were also pooled, restricted, and purified from agarose.

The heavy and light chain PCR fragments were ligated into the pComb3H phagemid for the construction of the SH library which contained 3.3 x 10^7 members. The pComb3H phagemid used in this combinatorial library construction allow for display of the

repertoire of cloned antibody fragments on the surface of helper phage (VSCM13), that in its genome carries the corresponding antibody DNA.

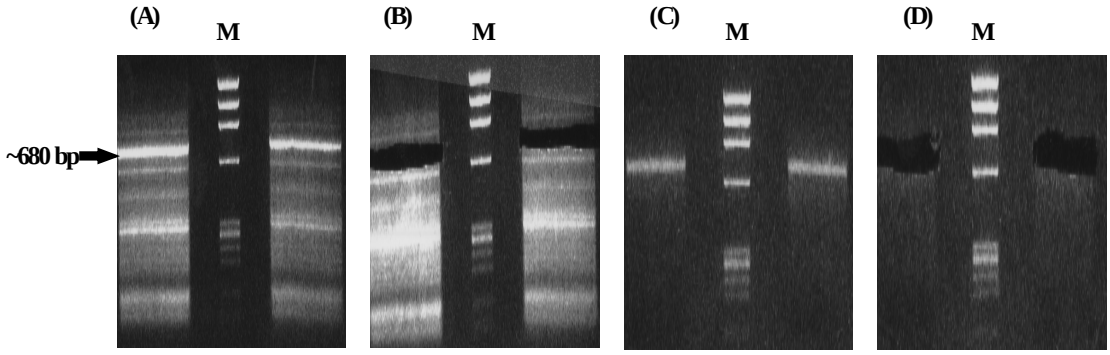

Figure 3. 3. 2 % agarose gel of, (A) pooled heavy chain PCR products, (B) gel showing excised heavy chain PCR products, (C) Xho I/Spe I restricted heavy chain PCR products, and (D) gel showing excised restricted heavy chain products. M is the molecular marker ΦX 174.

In a random screening, 7/10 (70 %) clones contained both heavy and light chain DNA inserts as analysed by restriction enzyme analysis. Gene sequencing analysis of randomly picked clones (unpanned or non-antigen selected) from the unselected library revealed that the heavy chain antibody genes were in the right reading frame, but many of the light chain sequences had one or two stop codons in the reading frame. Therefore, light chain shuffling was done by retaining the heavy chain of the original repertoire, and new light chain genes were amplified and ligated into the plasmid. The new library contained 6.9 x 10^7 members and 8/10 clones had both heavy and light chain fragments as revealed by restriction enzyme analysis. To determine whether the unpanned SH library contained many different clones, the diversity was monitored by digesting 12 individual clones with BstN1 (New England, Biolabs, Berverly, MA., USA) restriction enzyme and the fragments analyzed on 2 % agarose (Fig. 3.4).

Each of the clones analyzed by BstN1 restriction had a distinct restriction pattern from the other showing that the library was made up of different clones with diverse heavy and light chain gene usage. Cloning of target genes results in the presence of 'clones of interest' and inevitably wildtype plasmids which outcompete the desired clones. In order to successfully isolate clones that express antibody genes it was necessary to optimize the unselected library before its usage.

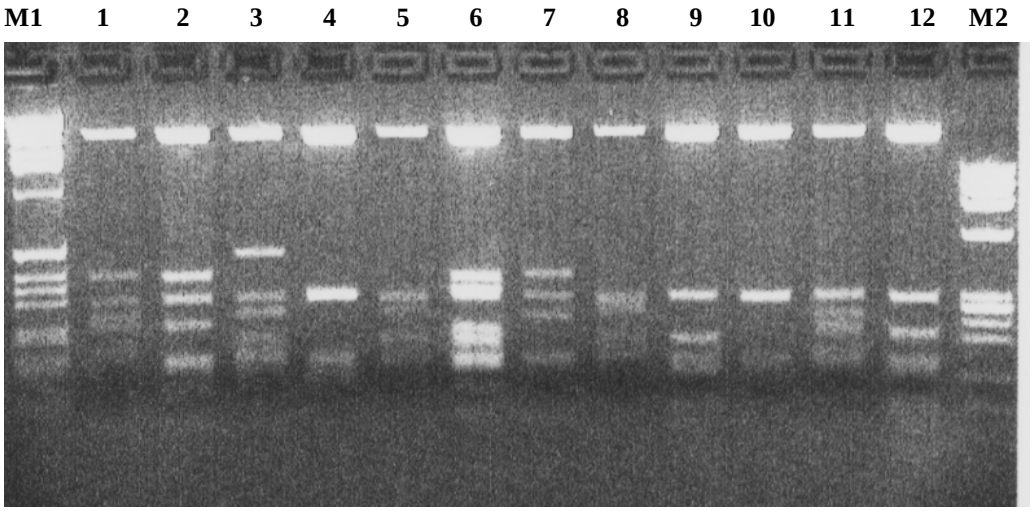

Figure 3. 4. BstN1 digestion of 12 randomly picked unselected clones from the library of patient SH. BstN1 frequently cleaves in the V regions but only 2 sites in the pComb3H phagemid vector. M1, 1 kb molecular marker; M2, ΦX 174 molecular marker.

3. 2 LIBRARY OPTIMIZATION

Agarose gel electrophoresis of the total DNA from the unselected (original) library revealed 2 bands at or near the expected size of the library DNA (Figure 3. 5, lane 1). More often, the presence of 'bald' clones (clones containing no inserts but which have selective growth advantage) in a library results in over-amplification of the these clones to the disadvantage of clones containing inserts during further culture of the library (Fischer *et. al*., 1999). To find out which of these 2 bands contain clones with heavy and light chain inserts, the upper and lower bands were separately excised from the gel, purified, restricted and analyzed on agarose.

Fig. 3.5 shows that the upper band contains both heavy and light chain inserts whilst the lower band was 'bald'. Hence, the upper band was used to transform bacteria, and the resultant library contained 5.6×10^6 members. This became the final constructed library from the patient with systemic lupus erythematosus and rheumatic fever (SH library), from which panning on an antigen (IVIG) resulted in the isolation of antigen specific Fabs, as well as the isolation of unselected clones.

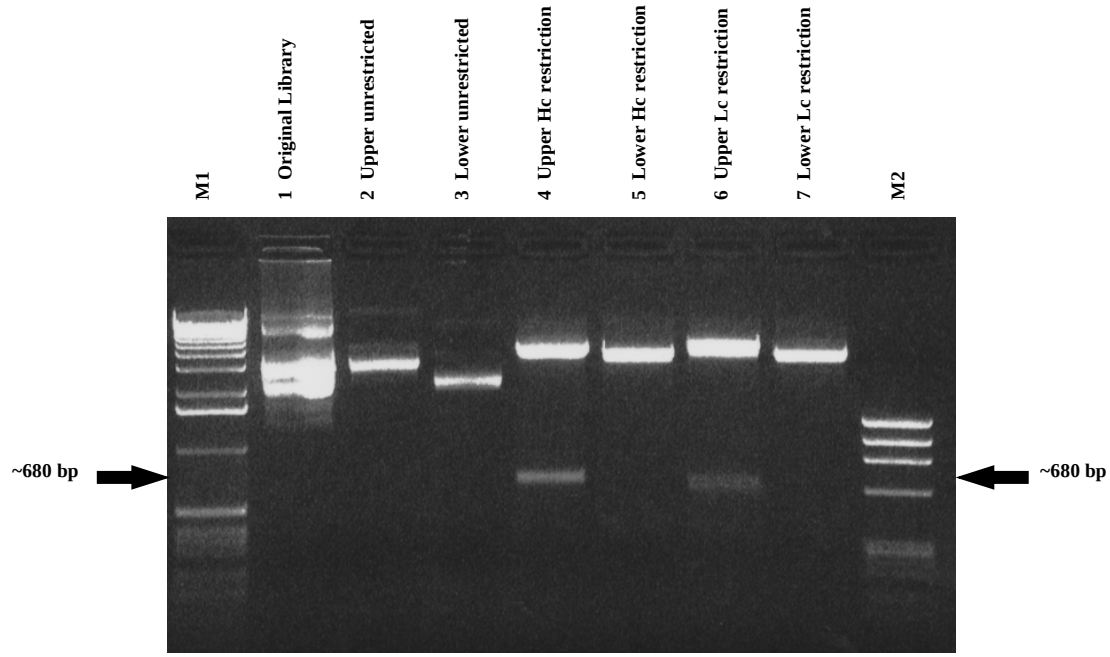

Figure 3. 5. Restriction enzyme analysis of the original/unselected library to eliminated bald clones from the original library (lane 1). Lanes 2 and 3 respectively, are the unrestricted upper and lower bands purified from the original library. Lanes 4 and 6 show restricted heavy and light chain DNA fragments from the unrestricted upper fragment band (lane 2). Lanes 5 and 7 show that the unrestricted lower band (lane 3) contain no immunoglobulin chain DNA fragments. Xho I/Spe I were the heavy chain restriction enzymes whilst the light chain restriction enzymes were Sac I/Xba I. The molecular markers M1 and M2 have already been described in Figure 3. 4

3. 3 BIOPANNING ON IVIG

Panning of the SH library for monoclonal Fab phages bound by IVIG was performed using 300µl of (30mg/ml) IVIG preparation (Sandoglobin, Sandoz, Basel, Switzerland) which were coated in 5ml Nunc immunotubes. A selection strategy was used in which the Fab phages were captured by the immobilized IVIG, and unbound phages washed off. Bound phages were then eluted with HCl/glycine buffer pH 2.2, neutralized with Tris HCL pH 9.0, and used to re-infect bacteria for propagation for the next rounds of panning, until a highly enriched population of phages carrying IVIG specific Fabs were obtained. The SH library was subjected to four rounds of panning on IVIG. To monitor the progress of the panning procedure, the percent of phage recovered at every round, as well as the enrichment of phages at a particular round over the previous were calculated from the number of clones that were plated on phage input and output carbenicillin plates, Table 3.1 (Siegel *et. al*., 1997).

Table 3.1: Panning of phage displayed Fabs from SH combinatorial antibody library on IVIG (After Siegel *et. al*., 1997).

Panning round	Phage Input (c.f.u)[a]	Phage Output (c.f.u)[b]	% Bound[c]	Enrichment[d]
1	2.8×10^{12}	5.7×10^{5}	2.0×10^{-5}	-
2	1.5×10^{12}	3.9×10^{5}	2.6×10^{-5}	x1.3
3	1.2×10^{12}	2.6×10^{6}	2.2×10^{-4}	x8.5
4	1.3×10^{12}	9.8×10^{6}	7.5×10^{-4}	x3.4

[a]Number of colony forming units (c.f.u) of phage incubated with antigen

[b]Total number of c.f.u of phage eluted after incubation with antigen

[c](phage output/phage input) x 100 i.e. % phage bound (or recovered)

[d]Fold increase (enrichment) in % bound phage compared to previous round of panning

Titration of phage input and output revealed that there was an enrichment of phages bound by IVIG at every round of panning (from panning 1 to panning 4). There was a 38-fold increase in percent phage bound in panning 4 over that obtained during the first round of panning. These data seemingly revealed that bacterial colonies that were infected with phages from a previous round of panning resulted in the enrichment of specific phage population expressing Fabs bound by IVIG. This gave the indication that the possibility to isolate monoclonal Fabs bound by IVIG exists.

To isolate single clones producing monoclonal antigen-specific antibodies (Fabs bound by IVIG), total DNA from the fourth round of panning need to be transformed into bacteria. However, before the transformation, it was important to restrict the total DNA obtained from the fourth round on panning on IVIG to ascertain that the population of antibodies selected during the panning process contain both heavy and light chain inserts. In addition, it is also worthwhile to perform a polyclonal Fab phage ELISA on the antigen (IVIG) used in the selection process (panning) to gain a first hand knowledge about the specificity of the selected phages in the biopanning process.

3. 4 POLYCLONAL FAB PHAGES BOUND BY IVIG

Though 'bald' clones in the unselected library were 'eliminated' or reduced as much as possible during the optimization of the library (section 3.2, Fig. 3.5), the re-emergence of such clones as well as clones with single inserts (light or heavy chain DNA, only) which have growth advantage over clones with double inserts (heavy and light chain DNA inserts) during various rounds of panning could not be underrated. Therefore, to have an idea whether clones selected at the end of the panning process were clones

containing both heavy and light chain DNA inserts before transforming bacteria for the isolation of Fabs, the total DNA at the end of the last round of panning (panning four) was subjected to both heavy and light chain restriction enzyme analysis, and compared with the total DNA from the unselected library, Fig. 3.6

As visualized on the agarose gels, both Figures 3.6 (A) and (B) show that the IVIG-selected clones contain both antibody heavy and light chains DNA inserts of expected sizes of approximately 680 bp. After the digestion of the total DNA from panning 4 but before transforming bacteria with it for isolation of monoclonal Fab phages, it was also necessary to verify whether the selected clones during the panning process contained phages that bind IVIG. This was important to establish that the selected library is not full of 'plastic-binding' phages which inevitably are present in a library (Adey *et. al.*, 1995). This was done by polyclonal Fab phage ELISA in IVIG-coated wells for the unselected library, as well as the selected libraries for all four rounds of panning on IVIG (Fig. 3.7).

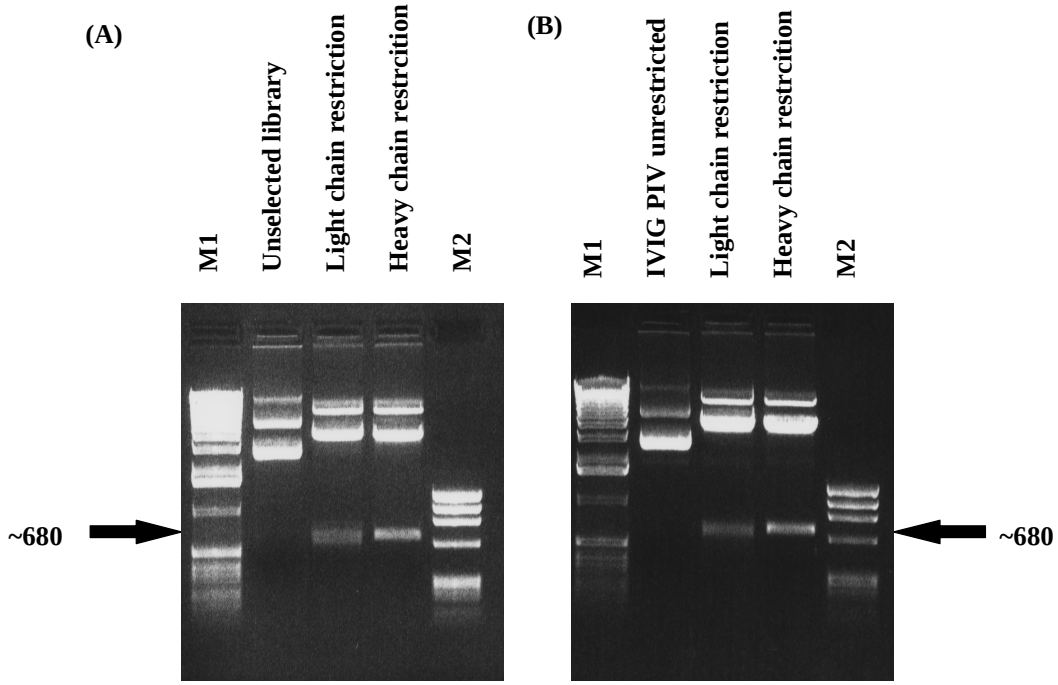

Figure 3. 6. Enzymatic double restriction. (A) Total unselected library DNA, (B) total DNA IVIG panning IV. Restriction enzymes: light chain - Sac I/Xba I; heavy chain - Xho I/Spe I. M1, M2 molecular markers described in Figure 3.4

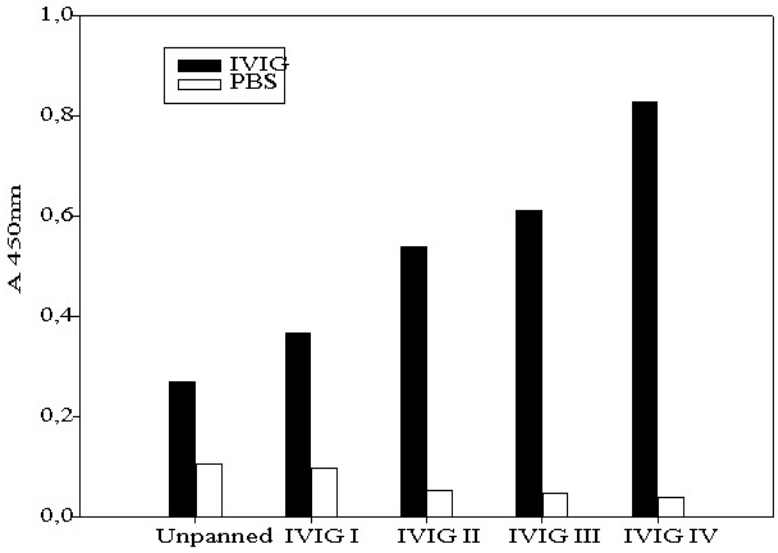

Figure 3. 7. ELISA of polyclonal phages of the unselected (unpanned) repertoire of the systemic lupus erythematosus and rheumatic fever library, and selected against IVIG in panning round 1 (IVIG I), panning round 2 (IVIG II), panning round 3 (IVIG III), and panning round 4 (IVIG IV). Coated proteins consisted of IVIG as well as PBS/1% casein blocking buffer to detect nonspecific binding. Bound Fab-phages were detected with HRP-labeled anti-M13 antibodies. Results are expressed as absorbance at 405 nm with a multiscan automatic plate reader (MR5000, Dynatech Labs, Chantily, VA., USA)

Typical IVIG binding phage enrichment were observed from panning 1 to 4. As can be seen in figure3.7, the initial 'high' background on PBS (plastic binding clones) gradually decreased from panning 1 to panning 4 indicating that the stringency of washing led to the retention of Fab phages specifically bound by IVIG. Only a few of sticky, non-specific binders remained after round four of panning. These phages were present at a much higher proportion in the unselected repertoire. This also demonstrated one of the objectives of the Fab phage panning method, that is to say, to increase the 'signal-to-noise ratio' of specific versus non-specific binding ratio during the selection process (Siegel *et. al*., 1997). This result indicated that there was a probable chance of successful isolation of monoclonal Fabs bound by IVIG which would lead to their further characterization.

3. 5 ISOLATION OF MONOCLONAL FAB PHAGES BOUND BY IVIG

Total DNA from panning 4 was used to transform bacteria and individual colonies were picked and grown for 5 - 6 h, and super-infected with VCSM13 helper phage for Fab-phage rescue. This allowed for the display of cloned antibody fragments on the surface of phage particles that in its genome carries the corresponding antibody DNA. Culture supernatants from individual colonies from the fourth round of panning were analyzed for their reactivity with IVIG in ELISA. Plasmid DNA from positive clones were digested by restriction enzymes and analyzed by agarose electrophoresis. Clones that contain both heavy and light chain inserts (data not shown) were sequenced and only clones that contain functional heavy and light chain antibody variable region sequences were used further in the study. Representative clones showing specific reactivity to IVIG are shown in figure 3.8. This results also show that the panning procedure finally resulted in a highly enriched population of phage carrying specific antibody fragments with 'little' or 'minimal' unspecific binding.

One of the aims of this study was to compare the IVIG-binding Fabs isolated from the patient (SH) with rheumatic fever and systemic lupus erythematosus to those isolated from 3 patients with idiopathic thrombocytopenic purpura (Fischer *et. al*., 1999; Jendryeko *et. al*., 1998) cloned in our laboratory. One of the patients developed SLE. The IVIG-binding Fabs from patient SH was tested for their reactivity to human Fc fragments. Rheumatoid factors are antibodies that bind to the Fc portion of IgG immunoglobulins. They are found in patients with arthritis as well as patients with inflammatory and infectious diseases. Rheumatoid factors are also found in healthy people following immunization suggesting that they may play a role in normal immune functions such as in the clearance of circulating immune complexes (Potter and Capra, 1995). Rheumatoid factors in idiopathic thrombocytopenia are thought to stabilize (enhance) low affinity anti-platelet antibodies in binding to platelets. Yang *et. al*. (1999) postulated that some IgG components in IVIG interact with these IgG rheumatoid factors and thus interfere with their enhancing effects on anti-platelet antibodies.

None of the IVIG-binding Fabs from the patient with systemic lupus erythematosus and rheumatic fever library bound Fc fragments (figure 3.8) in contrast to IVIG-binding Fabs isolated from patients with idiopathic thrombocytopenic purpura (Fischer *et. al*., 1999; Jendryeko *et. al*., 1998).

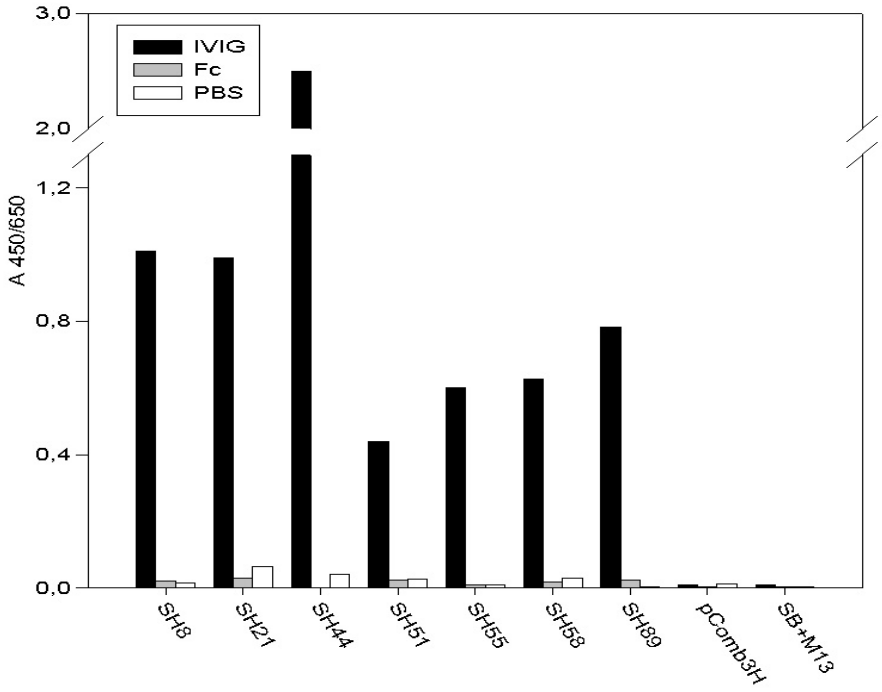

Figure 3.8. ELISA of representative individual (monoclonal) Fab-phage antibodies obtained after the fourth round of panning on IVIG. Wells were coated with IVIG, Fc fragments and PBS/1% Casein blocking buffer. Negative controls were pComb3H transfected *E. coli* phage supernatant (pComb3H), and SB medium containing VCSM13 helper phage (SB+M13). Bound Fab-phages were detected with HRP-labeled anti-M13 antibodies. Results were expressed as under Figure 3. 7. Clones selected repetitively included SH8 and SH44 (2X each); SH21 (9X), and SH89 (7X).

As a further step in characterizing and comparing the IVIG-binding Fabs isolated from patient SH to those from the patients with idiopathic thrombocytopenic purpura, the Fabs from SH was tested for their binding to platelets. Since platelets require a different surface (for coating) from IVIG for ELISA, the testing of the Fabs for their reactivity to platelets was done in a separate ELISA.

3. 6 REACTIVITY OF IVIG-BINDING FABS ON PLATELETS

Idiopathic thrombocytopenic purpura is mediated by autoantibodies that react to antigens expressed on platelets leading to their clearance by phagoctosis. Thrombocytopenia and platelet dysfunction are frequently found in patients with SLE, in particular, in association with anti-platelet antibodies. These antibodies react with the major platelet glycoproteins, gpIIb-IIIa, interfering with platelet aggregation and

adhesion (Xu *et. al*., 1995). To compare the extent of the repertoire used by the Fabs bound by IVIG from the library generated from the patient with systemic lupus erythematosus/rheumatic fever (SH) library, to that from the patients with autoimmune thrombocytopenia, the panel of selected Fabs from the SH library were tested also for binding to platelets in ELISA. IVIG-binding Fabs with reactivity to platelets had earlier been isolated from patients with autoimmune thrombocytopenia (one of whose illness progressed to SLE) in our group.

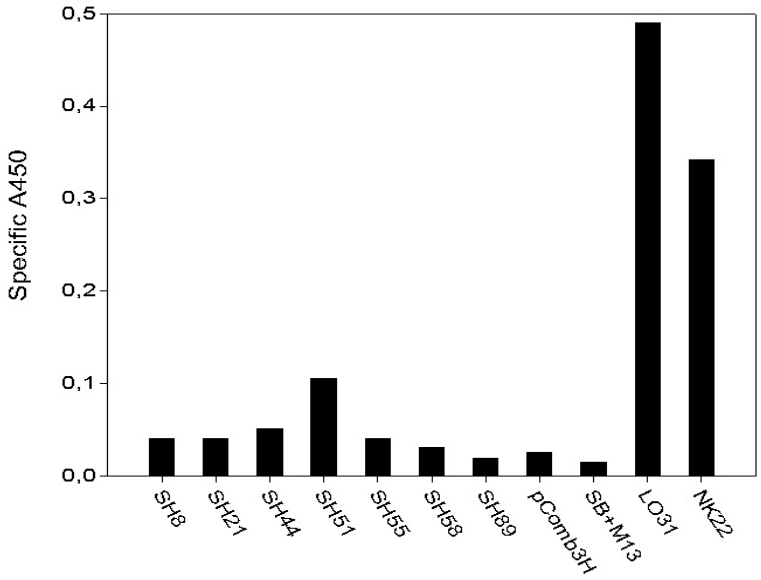

Figure 3.9 Summary of two repetitive ELISAs with representative Fab phage supernatants of sequenced clones after the 4th round of panning with IVIG on human platelets. 50 µl/well of 3 x 10^7 cells/ml of purified platelets were coated by drying to flexible plates (Microtest III, Falco, Oxnard, California). Incubation with Fab phages and further development were as described for the IVIG ELISA. The blood group was A, Rh positive. Shown is the mean A450/650 after subtraction of the background staining on PBS/Casein coated wells. Positive controls were LO31 (V3-30 locus) and NK22 (V4-b locus), platelet-binding IVIG-derived Fabs from patients with autoimmune thrombocytopenia (Fischer *et. al.*, 1999). Negative controls were pComb3H transfected phage supernatant (pComb3H) and SB medium containing VCSM13 helper phage (SB + M13). Bound Fab phages were detected with HRP-labeled anti-M13 antibodies.

None of the SH library IVIG-binding Fabs bound platelets. IVIG has a weak interaction with clone LO31, which was isolated from a patient with autoimune thrombocytopenia

whose illness progressed to SLE (Jendreyko *et. al*., 1998; Fischer *et. al*., 1999). To analyze the IVIG-binding Fabs further, the DNA were sequenced. The sequencing showed that indeed full length antibody DNA has been cloned. This permitted their further characterization.

3. 7 HEAVY CHAIN AMINO ACID HOMOLOGY

Sequencing of clones that were positive in the IVIG monoclonal Fab phage ELISA revealed 23 clones that had both functional heavy and light chain genes. Amino acid homology tree was constructed to show that the sequences were different from each other. Four individual clones were repetitively selected, these varied from two to nine, figures 3.10 and 3.11.

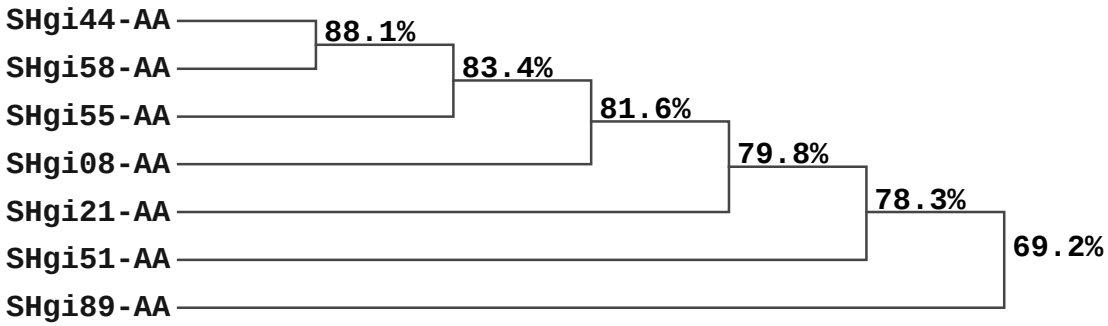

Figure 3. 10. Amino acid homology tree of representative V_H Fab phages bound by IVIG from the SH library constructed by the McDNASIS software. Clones SH8 and SH44 (2X each), SH21 (9X), and SH89 (7X) were repetitively selected.

Comparison of the complete overlapping heavy chain amino acid sequences between all seven individual V_H 3 family Fab phage loci bound by IVIG showed that Fabs 44 and 58 were the most closely related (88.1%) whilst the lowest homology was with Fab 89 (69.2%).

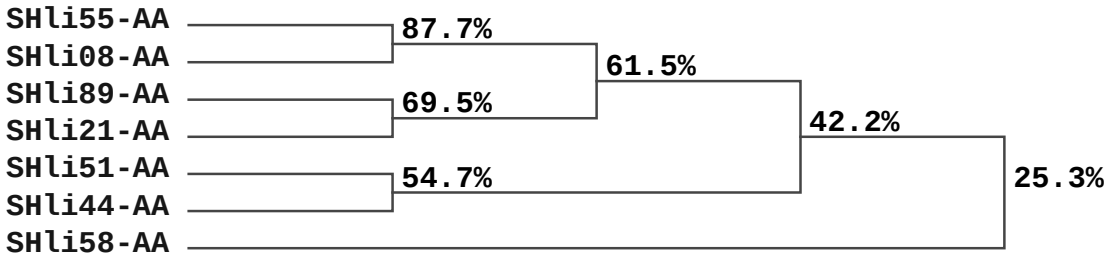

Figure 3. 11. Amino acid homology tree of representative V_L Fab phages bound by IVIG from the SH library constructed by the McDNASIS software. Clones SH8 and SH44 (2X each), SH21 (9X), and SH89 (7X) were repetitively selected.

The light chain amino acid homology revealed that the most closely related Fabs were clones 55 and 8 which shared a homology of 87.7% whilst the lowest homologous clone was clone 58 (25.3%).

3. 8 V_H AND V_L GENE SEGMENT USAGE OF IVIG SPECIFIC FABS

The variable region gene sequences from the Fab phages were aligned to human germline variable region sequences compiled in the VBASE directory to assign them to their closest germline counterparts with the aid of the DNAPLOT alignment software. A homology table was built to categorize the variable region genes into families, loci, and their deviation from their closest germline gene, table 3. 2. The D and J_H genes of each Fab are also provided. With regard to V_H gene usage, only two V_H3 germline genes were selected, namely V3-23 and V3-30, with 3-23 being the most frequently used 16/23 (70%). These two V_H loci genes were the most frequently observed in IVIG-bindig Fabs from patients with autoimmune thrombocytopenia (Fischer *et. al*., 1999; Jendreyko *et. al*., 1998). Comparison of the 3-23 heavy chain sequences with known V_H germline sequences revealed less divergence from the closest V_H germline genes with homologies ranging between 96.7-99.6 %. The remaining 7/23 (30%) derived their V_H loci from the gene segment V3-30, sharing 99.6% homology with their closest germline gene. Comparison of the SH IVIG Fab phages light chain variable region sequences with their closest germline gene segments revealed that the $V_\lambda1$, $V_\lambda2$, $V_\lambda3$, and $V_\kappa2$ families were represented. Among these, the V_L 3 family predominated with about 19/23 (83%) clones being represented. Unlike the V_H segments where only two V_H loci were used, the light chain gene segments were derived from 4 different V_L loci.

3. 9 SH NON-ANTIGEN SELECTED (UNPANNED) CLONES

5 non-antigen or unpanned clones were sequenced to determine the diversity of the systemic lupus erythematosus and rheumatic fever library (Table 3.2). 4 clones belonged to the V_H 3 family but only 1 (clone SH-u8a) was derived from the 3-23 locus compared with the IVIG binders where 16/23 were from this locus. The unselected clone SH-u8a, used a similar V-D-J rearrangement to that of the IVIG-selected clone SH-55. Nevertheless, they were two different clones as each paired with light chains from different V_L families. The V_H region segment from one non-antigen selected clone (SH-u2b), was not assigned to any particular locus after the database search. This clone however, paired with an identical light chain that was observed with nine (V 3-23) IVIG Fab clones. Another unpanned clone (SH-u3a) that belongs to V_H1 family paired with a λ light chain that utilized a λ gene segment identical to that of the IVIG binders that used the V_H 3-30 loci. The V_λ gene usage from the unpanned clones were $V_\lambda1$, $V_\lambda2$, $V_\lambda3$, and $V_\lambda6$.

Table 3. 2 Putative germline sequences of SH IVIG-selected and non-antigen selected antibody heavy and light chain variable regions from the patient with systemic lupus erythematosus and rheumatic fever[a]

Clone	V_H family	V_H	V_H locus	Homology %†	D	J_H	V_L family	V_L	V_L locus	Homology %†	J_L
IVIG-selected Fab phages											
SH8‡	3	DP-47/V3-23…+ (Z12347)*	3-23	97.4	D6-19 (X97051)	JH4b (X86355)	VL3	DPL16/VL3.1…+ (Z22202)	3l	99.2	JL2/JL3a (M15641)
SH21‡	3	DP-47/V3-23…+ (Z12347)	3-23	99.6	D4 (J00232)	JH3b (X86355)	VL3	IGLV3S2+ (X71966)	3h	99.2	JL3b (D87017)
SH44‡	3	DP-47/V3-23…+ (Z12347)	3-23	97.4	D2-8/DLR1 (X97051)	JH4b (X86355)	VL2	2b2.400B5+ (Z73665)	2b2	91.7	JL2/JL3a (M15641)
SH51	3	DP-47/V3-23…+ (Z12347)	3-23	96.7	D4-11/DA1 (X97051)	JH4b (X86355)	VL1	DPL7/VL1.2 (IGLV1S2)+ (Z22193)	1e	99.6	JL3b (D87017)
SH55	3	DP-47/V3-23…+ (Z12347)	3-23	97.0	D6-25 (X97051)	JH4b (X86355)	VL3	DPL16/VL3.1…+ (Z22202)	3l	98.5	JL3b (D87017)
SH58	3	DP-47/V3-23…+ (Z12347)	3-23	99.3	D3-3/DXP4 (X97051)	JH1 (J00256)	VK2	DPK18/A17+ (X93635)	A17	97.8	JK2 (J00242)
SH89‡	3	DP-49/1.9III…+ (Z12349)	3-30/3-30.5	99.3	D3-22/D21-9 (X97051)	JH6b (X86355)	VL3	3r.9C5/DPL23…+ (Z73647)	3r	99.6	JL2/JL3a (M15641)
Randomly picked clones from the unpanned SH library											
SHu2a	3	DP-48/13-2+ (Z12348)	3-13	94.8	D6-19 (X97051)	JH5B (X86355)	VL2	2c.118D9/V1-2+ (X97462)	2c	95.9	JL2/JL3a (M15641)
SHu8a	3	DP-47/V3-23…+ (Z12347)	3-23	97.4	D6-25 (X97051)	JH4b (X86355)	VL6	6a.366F5/V1-22…+ (Z73673)	6a	98.1	JL2/JL3a (M15641)
SHu2b	3	p6 (M77305)	-	94.4	D4 (J00232)	JH4b (X86355)	VL3	IGLV3S2+ (X71966)	3h	96.4	JL3b (D87017)
SHu3b	1	DP10/hv1051…+ (Z12312)	1-69	97.0	D5-12/DK1 (X97051)	JH6b (X86355)	VL3	3r.9C5/DPL23…+ (Z73647)	3r	89.7	JL2/JL3a (M15641)
SHu7b	3	DP-38/9-1…+ (Z12338)	3-15	99.0	D3-10/DXP·1 (X97051)	JH4b (X86355)	VL1	1b.366F5/DPL5…+ (Z73661)	1b	96.4	JL2/JL3a (M15641)

***Numbers in parenthesis indicate European Molecular Biology Laboratory/GenBank accession numbers. Sequences are named according to the VBASE databank. Dots indicate abbreviated names and/or existence of synonyms; + indicates a mapped chromosomal location.**

†Homology of the variable region genes

‡Clones selected repetitively included SH8 and SH44 (2X each); SH21 (9X), and SH89 (7X)

3. 12 MUTATION RATES

A table of mutation rates (Table 3.3) was built to show where in the variable region (CDRs and FRs), the deviation (i.e. < 100% homology) from their germline counterparts observed in the previous section, occured for each clone. Somatic hypermutations leading to a single base substitution may either lead to replacement (R) of an amino acid or remain silent (S) if the resulting codon encodes for an identical amino acid. R/S ratios above 2.9 are considered high and indicative of antigen selection. However, the role of CDR3 which lies at the center of the antigen-binding site is difficult to access and were not included in this type of analysis. In addition, the first eight codons of the FR1 region were excluded from the analysis of somatic mutations because this region is complementary to the primers used for amplification (see figures 3.12 and 3.13) . Sequences from representative clones for the 23 Fabs were aligned on themselves to reveal individual variations among the clones (figures 3.12 and 3.13)

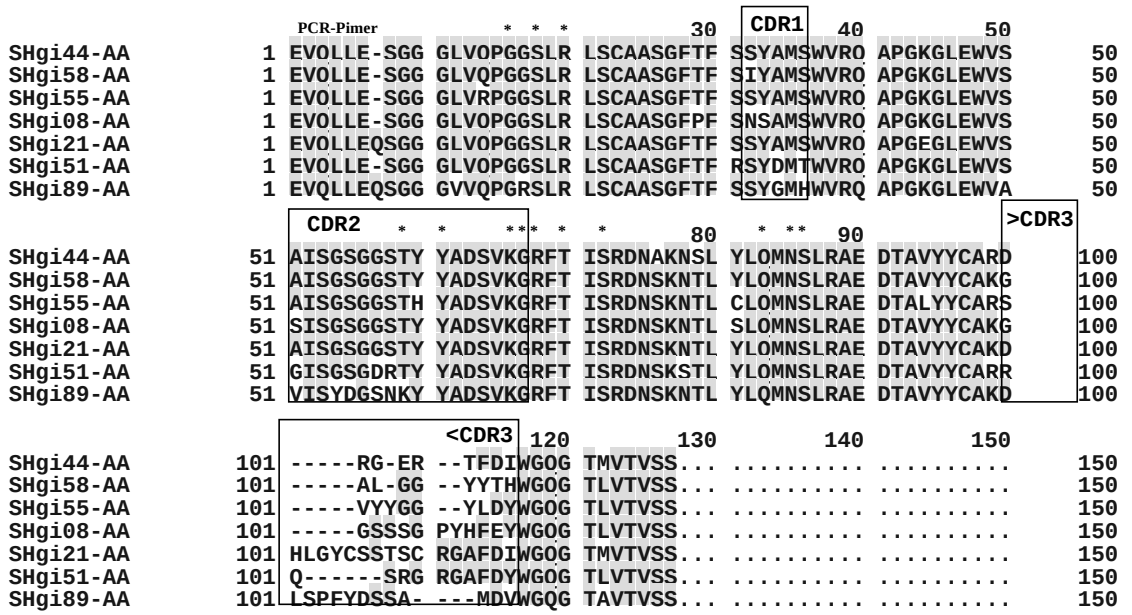

Figure 3. 12. Alignment of V_H amino acids of representative clones SH8, 21, 44, 51, 58, and 89 from IVIG-selected Fabs according to their highest homology. Complementarity determining regions (CDRs) according to the Kabat definition are indicated (Kabat *et. al*., 1991). The beginning of the sequences (ca. 8 amino acids) was determined by the PCR primers. * = critical amino acid positions known for binding to staphylococcal protein A (SpA) Graille et. al., 2000). The sequences are designated according to the library from which they were obtained, i.e., SH is the initials of the patient; g means IgG heavy chain; i means (IVIG), the antigen used in isolating the antibody. These sequence data are available from the European Molecular Biology Laboratory nucleotide sequence database under accession numbers AJ298606 - AJ298612.

The V_H CDR3 regions displayed an overall high degree of variation in length and sequence, ranging between nine (e.g. SHgi44) and 17 (SHgi21) amino acids.

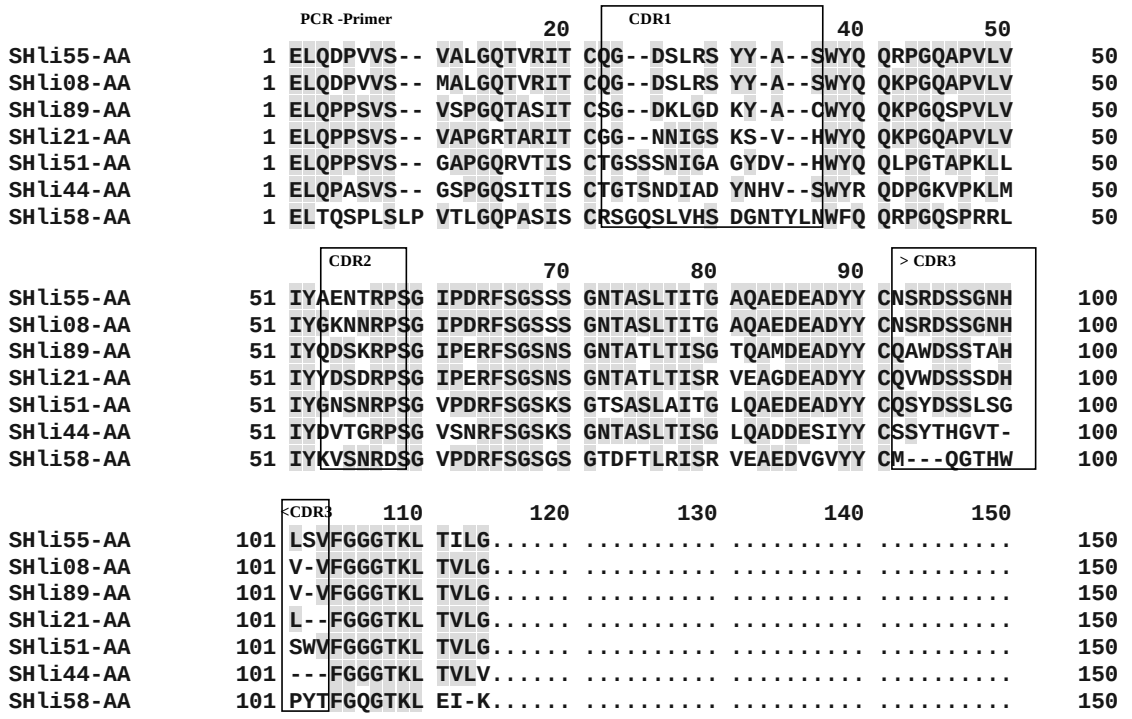

Figure 3. 13. Alignment of V_L amino acids of representative clones SH8, 21, 44, 51, 58, and 89 from IVIG-selected Fabs according to their highest homology. Complementarity determining regions (CDRs) according to the Kabat definition are indicated (Kabat *et. al.*, 1991). The beginning of the sequences (ca. 8 amino acids) was determined by the PCR primers. The sequences are designated according to the library from which they were obtained, i.e., SH is the initials of the patient; l means a light chain; i means (IVIG), the antigen used in isolating the antibody. These sequence data are available from the European Molecular Biology Laboratory nucleotide sequence database under accession numbers AJ298613 - AJ298619.

The pattern of somatic mutations in the CDR1 and CDR2 of the V_H genes was indicative of replacement events in four Fabs namely SH8, 51, 55, and 58 with a high R/S ratio of >2.9 (Table 3.3 Mutation Rates). The remaining three Fabs had 0 R/S ratios. Two light chains, SH25 and SH55 had a higher R/S ratio >2.9 in the entire V_L CDR1 and CDR2. On the whole, only two Fabs displayed a R/S ratio >2.9 over the entire V_H region (SH51 and SH55) whilst one Fab (SH44) had a R/S ratio above 2.9 in the entire V_L region.

Analysis of the V_H and V_L genes in this study and earlier reports (Jendreyko *et. al.*, 1998; Fischer *et. al.*, 1999; Hoffman *et. al.*, 2000) revealed that the interaction of IVIG

with the Fabs might be dependent on the heavy chain since the light chains were from diverse families. In some cases, the Fabs bound by IVIG had no functional light chains. The interaction of IVIG with Fabs solely from the V_H3 family in this study was characteristic of the interaction of B cell superantigens with immunoglobulins from the V_H3 family (Silvermann, 1997). Thus, we next tested the IVIG-binding Fabs for their reactivity with B cell superantigens.

Table 3.3. Mutation rates in Variable region genes*

Clone	V_H CDRs 1 and 2		V_H FRs 1 - 3		Entire V_H region	V_L CDRs 1, 2, (3)		V_L FRs 1 - 3		Entire V_L region
	No./total no. (%)	R/S (ratio)	No./total no. (%)	R/S (ratio)	No./total no. (%)	No./total no. (%)	R/S (ratio)	No./total no. (%)	R/S (ratio)	No./total no. (%)
IVIG-selected Fab phages										
SH8†	4/66 (6.1)	3/1 (3)	3/204 (1.5)	1/2 (0.5)	7/270 (2.6)	0/78 (0)	0 (0)	2/177 (1.1)	1/1 (1)	2/225 (0.8)
SH21†	0/66 (0)	0 (0)	1/207 (0.5)	1/0 (∞)	1/273 (0.4)	1/78 (1.3)	0/1 (0)	1/177 (0.6)	1/0 (∞)	2/225 (0.8)
SH44†	0/66 (0)	0 (0)	7/207 (3.4)	4/3 (1.3)	1/273 (2.6)	12/75 (16.0)	9/3 (3)	9/177 (5.1)	6/3 (2)	21/252 (8.3)
SH51	5/66 (7.6)	5/0 (∞)	4/204 (2.0)	3/1 (3.0)	9/270 (3.3)	1/93 (1.1)	0/1 (0)	0/180 (0)	0 (0)	1/273 (0.4)
SH55	1/66 (1.5)	1/0 (∞)	7/204 (3.4)	4/3 (1.3)	8/270 (3.0)	3/84 (3.6)	3/0 (∞)	1/177 (0.6)	1/0 (∞)	4/261 (1.5)
SH58	1/66 (1.5)	1/0 (∞)	1/204 (0.5)	1/0 (∞)	2/270 (0.7)	5/90 (5.6)	2/3 (0.7)	1/186 (0.5)	1/0 (∞)	6/276 (2.2)
SH89†	1/66 (1.5)	0/1 (0)	1/207 (0.5)	0/1 (0)	2/273 (0.7)	1/78 (1.3)	0/1 (0)	0/177 (0)	0 (0)	1/255 (0.4)
Randomly picked clones from the unpanned SH library										
Shu2a	7/63 (11.1)	7/0 (∞)	7/204 (3.4)	4/3 (1.3)	14/264 (5.3)	8/90 (8.9)	7/1 (7.0)	3/180 (1.7)	1/2 (0.5)	11/270 (4.1)
Shu8a	2/66 (3.0)	2/0 (∞)	5/204 (2.5)	2/3 (0.7)	7/270 (2.6)	2/84 (2.4)	2/0 (∞)	1/186 (0.5)	1/0 (∞)	3/270 (1.1)
Shu2b	6/66 (9.1)	4/2 (2.0)	9/204 (4.4)	6/3 (2.0)	15/270 (5.6)	5/72 (6.9)	3/2 (1.5)	4/177 (2.3)	3/1 (3.0)	9/249 (3.6)
Shu3b	4/66 (6.1)	4/0 (∞)	4/201 (2.0)	3/1 (3.0)	8/267 (3.0)	10/66 (15.2)	8/2 (4.0)	15/177 (8.5)	8/7 (1.1)	25/243 (10.3)
Shu7b	4/72 (5.6)	4/0 (∞)	2/204 (1.0)	0/2 (0)	6/276 (2.2)	4/75 (5.3)	3/1 (3.0)	5/174 (2.9)	4/1 (4.0)	9/249 (3.6)

*CDRs = complementarity determining regions; FRs = framework regions; no./total no. = number of mutations/total number of bases in the region; (%) = percent; R/S = number of replacement mutations/number of silent mutations; ratio = R/S ratio; ∞ = infinity (mathematically incorrect).

†Clones selected repetitively included SH8 and SH44 (2X each), SH21 (9X), and SH89 (7X). Refer Table 3.2 for other definitions.

3. 13. BINDING OF IVIG-SELECTED FABS TO KNOWN B CELL SUPERANTIGENS

A control study involving an investigation of IVIG Fabs from IgG and IgM phage display libraries from a healthy individual, and the results observed in this study as well as in previous studies involving interaction of IVIG with Fabs from 3 patients with autoimmune thrombocytopenia indicated that the preferential binding of IVIG to Fabs from the V3-23 and V3-30 loci might not be related to the healthy state of the donor or treatment with IVIG. Rather it was indicative of the unique binding of B cell superantigens to immunoglobulins from the VH3 family. This prompted us to examine the binding of the Fabs to Staphylococcal protein A (SpA) and HIV-1 gp120, two of the most characterized B cell superantingens (Silverman, 1997).

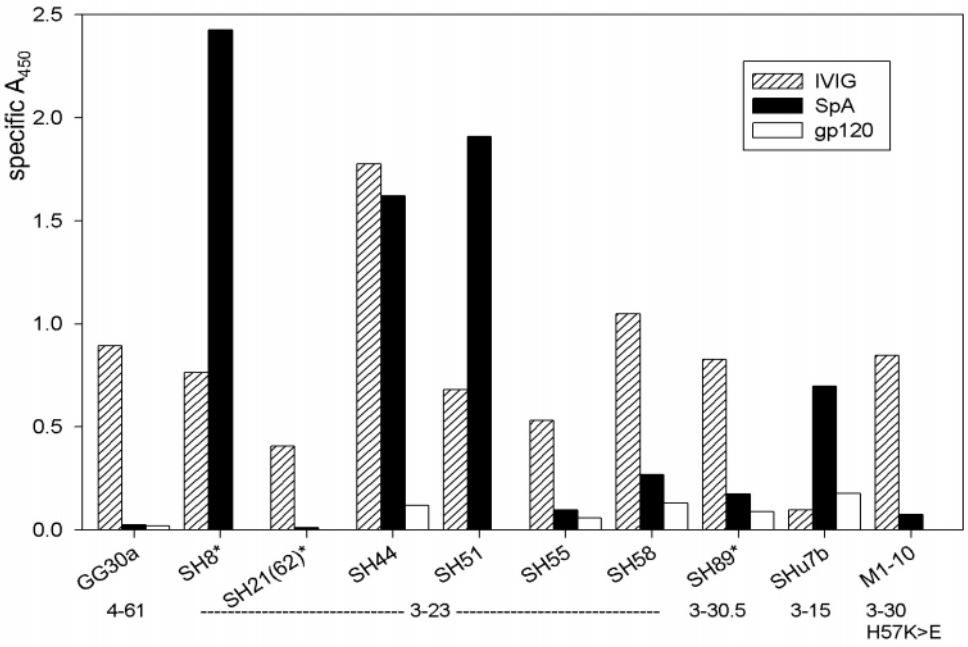

Fig. 3. 14 Summary of ELISAs with Fab phage supernatants comparing the binding to IVIG and the B cell superantigens staphylococcal protein A (SpA) and gp120. The V_H germline origins of the Fabs are indicated. Each specific A_{450} was calculated by subtracting the value obtained for pComb3H phage supernatants on the respective antigen from the measured A_{450}. Values on IVIG and SpA for clones marked with * were obtained in a second ELISA with appropriate controls, and all values on gp120 were obtained in an independent assay. However, all relative values were confirmed repeatedly in independently assays. SHu7b is a V3-15 unselected control Fab. GG30a is a V4-61 derived platelet-reactive IVIG binder isolated from a patient with idiopathic thrombocytopenic purpura. (Jendryeko *et. al.*, 1998). M1-10 is a V3-30 unselected control Fab from a patient with Kawasaki disease (Leucht *et. al.*, 2001). This clone has an amino substitution of glutamine (E) for lysine (K) at position V_H57.

As demonstrated in Fig 3.14, some of the IVIG-selected Fab phages (SH8, SH44, and SH51) displayed a very strong binding to SpA, whilst interactions with gp120 were

weak. Except SH44, binding to SpA did not correlate to binding with IVIG, for example, clones SH8 and SH51 showed stronger reaction to SpA than to IVIG. On the other hand, clones SH21(62), SH55, SH58, and SH89, showed higher binding to IVIG than SpA. Fab GG30a which is a V_H4 family clone was negative on SpA, this is not an unexpected result. SH7ub an unselected clone, was nearly negative on IVIG but clearly bound SpA.

4. 0 DISCUSSION

4.1 GENERAL REMARKS

Molecular analyses of phage-displayed antibody Fab fragments bound by IVIG cloned from patients with autoimmune thrombocytopenia in our laboratory often originated from the V3-23 and V3-30/3-30.5 V_H germline genes (Fischer *et. al*., 1999; Jendreyko *et. al*., 1998). One of the autoimmune thrombocytopenic patients' illness progressed to systemic lupus erythematosus (SLE). Furthermore, the same germline genes were also observed to be the most frequently used V_H genes for Fabs bound by IVIG from a library constructed from a patient with Kawasaki disease (Leucht *et. al*., 2001), and also from a healthy individual in a control study (Hoffmann *et. al*., 2000). The aim of this study was to learn more about the interaction of IVIG with Fabs by analyzing at the molecular level, Fabs bound by IVIG from a phage display library constructed from a patient with SLE and rheumatic fever, and at the same time compare these Fabs to those from the autoimmune thrombocytopenic patients.

4.2 GENETIC ORIGIN OF IVIG-SELECTED HEAVY AND LIGHT CHAINS

The library for this study was constructed from peripheral blood lymphocytes isolated from an 8-1/2 year-old girl. The blood was drawn 24 days after the patient had undergone IVIG therapy. The combinatorial antibody library system, instead of engineering single antibodies, creates millions of different clones by random processes and use the powerful selection panning process to isolate antibodies with the desired characteristics (Persson, 1993). However, the technique is not without problems. Plastic-binding clones which often are enriched over desired clones during the panning process makes it difficult to isolate antigen-specific clones. One of the factors that contributed to the success of this study was the 'elimination' of (as much as possible) bald clones that had no light and heavy chain DNA fragments. This optimization step during the library construction contributed heavily to the isolation of unselected clones with both functional heavy and light chain genes and paved the way for completion of the study. A total of 23 Fab phages bound by IVIG were isolated and characterized. The V_H sequences from all 23 antibodies bound by IVIG were derived from two putative germline genes namely V3-23 and the V3-30/3-30.5 polymorphic variant. In contrast, the V_H sequences from five unselected clones were derived from at least five different V_H region loci. There were no specific enrichment of a particular light chain loci as observed for the heavy chain. This purported to show that the heavy chain may be the major target of IVIG during the selection process, since IVIG-selected Fabs devoid of light chains, or those without functional light chains have been isolated before in our laboratory (Leucht *et. al*., 2001; Hoffmann *et. al*., 2000).

None of the IVIG-binding Fabs from the library of the patient with systemic lupus erythematosus and rheumatic fever bound platelets, in contrast to IVIG-binding Fabs isolated from patients with idiopathic thrombocytopenic purpura (Fischer *et. al*., 1999; Jendryeko *et. al*., 1998). 8 of 16 Fab fragments from the three libraries constructed from the patients with thrombocytopenia bound platelets, though the majority of them were derived from the V_H4 family (Fischer *et. al*., 1999). This perhaps suggest that the platelet-specific antibodies from the patients with thrombocytopenia were those that were relevant for the disease at the time the material was obtained. Mutation rates varied between the extremes of zero to high replacement rates, and CDR3 composition was between short to long residues. The mechanisms of replacement mutations which are thought to be initiated by unknown antigen selection may influence binding through the positioning of side chains that contact antigen, or by providing new contact residues in the antigen combining sites, or by replacing repulsive or low affinity residues with more favorable contact residues (Hoogenboom, 1997).

4.3 BIASED V_H GENE REPRESENTATION IN THE Ig REPERTOIRE

The V3-23 and V3-30/3-30.5 genes have been observed to be the most frequently rearranged and expressed V_H gene in the immune repertoire (Matsuda *et. al*., 1998; Cook and Tomlinson, 1995). It has been observed that there is not an equal representation of the various V_H families in the fetal and adult repertoire. The immune system is biased with some V_H families being over-represented and others under-represented. The known maximum number of functional segments of the human immunoglobulin heavy chain repertoire is 51 (Cook and Tomlinson, 1995), though another study by Matsuda *et. al*. (1998) reported 44 functional genes. The difference between these two studies is attributed to insertion/deletion polymorphism which when present results in the gain of some functional genes. 22 V_H3 family segments account for 43.1 % of the 51 functional V_H segments (Tomlinson, 1997). Some genes, such as V3-23 and V3-30 have been demonstrated to be over-represented at all stages of B cell development. The V3-23 gene which is well represented in the fetal repertoire and comprising about 25 % of all V_H3 clones in the normal adult peripheral B cells, is present in all individuals tested (Kraj *et. al*., 1997). De Wildt *et. al*., (1999) investigating the pairings of V_H and V_L chains from 365 human IgG+ B cells observed that the most frequently utilized V_H germline genes were from the V3-23 and V3-30/3-30.5 locus. In a large study involving more than 4,500 independent V_H3 family gene segments from 12 unrelated individuals, Huang *et. al*. (1996) observed that V3-23 and V3-30 were most frequently expressed in the normal repertoire with mean representations of 24.3 % and 16.1 % respectively. The significance of the study affirmed that the representation of the adult repertoire is not by random V_H rearrangement by which the frequency of utilization of variable region segments of a

family may be proportional to family size/complexity. In a more recent study, the V3-23 and V3-30 gene segments were found to be the most frequently rearranged V_H3 family genes at the pro-B cell stage (Rao *et. al.*, 1999). In B cell differentiation and development, the pro B cell is a precursor B cell that has productively rearranged V_HDJ_H genes but has not as yet expressed the μ (mu) protein. For this reason, pro-B cells are unaffected by selection mechanisms arising out of contact with antigen. In the B cell lineage, pro-B cells that have successfully rearranged a productive V_HDJ_H gene may differentiate into pre-B cells which expresses the pre-B cell receptor consisting of μ-surrogate light chain complex (Melchers *et. al*., 1993). Further differentiation takes place through immature and mature B cells (with the disappearance of the pre-B cell receptor), and the expression of surface immunoglobulin (sIgM) and IgD respectively. Rao and colleagues reasoned that earlier observations indicating the preferential rearrangement and over-representation of certain V_H segments and families in the repertoire were made from B cells that have already rearranged productive chains and have already been committed to selection (De Wildt *et. al*., 1999; Kraj *et. al*., 1997; Huang *et. al*., 1996). In their study, they demonstrated that even in pro-B cells that do not express antigen receptors, the V3-23 and V3-30 gene segments were the most frequently rearranged V_HDJ_H segments representing about 20 % and 24 % respectively among 760 V_H3 family genes. The study showed that the repertoire is already biased long before the expression of antigen receptors on the surface of the B cell (Rao *et. al*., 1999).

The detection of solely V_H3 Fabs (seven independent clones comprising six V3-23 and one V3-30) in the library from the patient with systemic lupus erythematosus and rheumatic fever does not seem to reflect an artifact. To make the library as diverse as possible, the V genes from the patient were isolated using degenerate PCR primers that can amplify all human V_H germline genes (Kang *et. al*., 1991b). To access the clonal diversity of the library and to access that the library was not dominated by clones arising from an artifact of preferential amplification of a few clones, 12 unselected clones were analyzed by BstN1 restriction. BstN1 frequently cuts in the V_H region but only at 2 sites in the pComb3H vector. The restriction pattern which was confirmed by sequencing of 5 unselected clones revealed that the library was heterogenous. Whereas the IVIG library utilized only two V_H3 family loci namely V3-23 and V3-30, the immunoglobulins isolated from the unselected library used different V_H3 family genes namely 3-13, 3-23, 3-15, and one that has not as yet been assigned to a V_H locus, as well as a V_H1 family segment 1-69, emphasizing that the unselected library from the patient did not comprise V_H genes solely from the V3-23 and V3-30 loci. Similarly, sequencing of unselected clones from libraries studies in our laboratory showed no bias

for a certain germline gene loci; rather a variety of different germline genes not observed among the IVIG-bound Fabs have been observed (Leucht *et. al*., 2001; Hoffmann *et. al*., 2000).

The normal/healthy immune system frequently produces autoantibodies, however autoimmunity is a rare event. This implies autoimmunity results from a perturbation of the control mechanisms (Potter and Capra, 1995). It has been observed that the germline gene loci dominating the IVIG-selected Fabs (V3-23 and V3-30) were also frequently utilized for coding other autoantibodies though it has not been established that only limited V_H segments could be used for autoantibodies (Matsuda *et. al*., 1993). For example, the V3-30 sequence is homologous to RF-TS2, RF-SJ1 and RF-SJ2 cDNA for rheumatoid factors (Pascual *et. al*., 1990). This same germline V_H segment was 99.7 % identical to cDNA for Kim4.6 autoantibody (Cairns *et. al*., 1989) which has DNA binding activity. The V3-23 segment is identical to the anti-DNA autoantibody (18/2) (Dersimonian *et. al*., 1987) and 30P1 cDNA found in fetal liver (Schroeder and Wang, 1990, Schroeder *et. al*., 1987). In addition, the V3-15 segment, which is the germline gene of 20P1 cDNA expressed in fetal liver is 99.7 % identical to cDNA for 4B4, an anti-Sm antibody encountered in systemic lupus erythematosus (Sanz *et. al*., 1989). Matsuda *et. al*. (1993) hypothesized that the correlation between autoantibody V_H genes and the early repertoire V_H, indicated that the preferred usage of autoantibody V_H segments in early stages of ontogeny may be due to positive selection by self-antigens. Though autoantibodies displaying given specificities are most often associated with different human autoimmune diseases, several studies in mice and humans have demonstrated that healthy individuals regularly produce several autoantibodies encoded by germline immunoglobulin variable regions with no somatic mutations. These autoantibodies do not provoke autoimmune pathology, in contrast to patient-derived autoantibodies that are highly specific for their respective autoantigens (Potter and Capra, 1995; Hoffmann *et. al*., 2000). The presence of autoantibodies in the normal immune system suggests that they may play some important physiological roles by augmenting the opsonization of infectious agents and the clearance of immune complexes and senescent cells. The importance of an autoreactive variable region gene in the development of the humoral immune system was demonstrated in a study where it was shown that a V_H gene frequently expressed in fetal liver was homozygously deleted in 20 % of patients with systemic lupus erythematosus and rheumatoid arthritis, but in only 2 % of healthy controls. This germline gene, designated Humhv3005, belongs to the V3-30 germline polymorphic gene variants, and encodes an amino acid sequence identical to the 56P1 V_H germline gene which is frequently expressed in fetal liver. The hv3005 gene is also 99 % homologous to the 1.9III germline V_H gene which in turn is

identical to the heavy chain of the kim4.6 anti-DNA antibody. Olee *et. al.* (1991) contended that homeostasis of the early B cell repertoire is maintained through interactions between autoreactive V genes and autoantigens, and subsequently through an interconnecting network of idiotypes-antiidiotypes. Thus a complete deletion of a cluster of developmentally important autoreactive V genes such as hv3005-like genes may alter the humoral immune response to bacterial and/or viral infections resulting in the overproduction of aberrant antibodies that may contribute to the initiation and/or perpetuation of autoimmune diseases.

4.4 BINDING OF IVIG COMPARED TO KNOWN B CELL SUPERANTIGENS

The V_H3 sequences expressed by the Fab phages isolated in this study utilized different D and J_H CDR3 region genes. The CDR3s were encoded by different D (D6-19, D4; D2-8; D4-11; D6-25; D3-3; and D3-22) and J_H (J_H4b; J_H3b; J_H1; and J_H6b) gene segments, and the lengths of the CDR3 regions were also variable. These features of the D and J_H genes indicated that the CDR3 region was not crucial in binding by IVIG. The interaction of IVIG with V3-23 and V3-30 from the V_H3 gene family was also demonstrated in a control study where the subjects were healthy individuals (Hoffmann *et. al*., 2000). In this study, 21/27 (78 %) of Fabs bound by IVIG were derived from V_H3 gene family. Of note, 14/21 (66.6 %) of these V_H3 Fabs were from the V3-23 and V3-30 loci which accounted for about 52 % (14/27) of the entire Fabs isolated. Hence the selection of these Fabs is linked to the preferential interaction of IVIG with V3-23 and V3-30. Thus, the results from Fischer, *et. al*. (1999); Jendreyko *et. al*. (1998); Hoffmann *et. al*. (2000), and this current study reveal that the preferential interaction of IVIG with Fabs from the V3-23 and V3-30/3-30.5 germline origins was not restricted to the healthy status of the donor, neither to treatment with IVIG. In conclusion, the observed interaction of IVIG with the Fabs was characteristic of binding of a B cell superantigen. In this regard, we compared the specific IVIG binding of our Fabs with the 2 best characterized B cell superantigens namely, staphylococcal protein A (SpA) and HIV-1 gp120.

B cell superantigens are proteins that interact with B cell and T cell receptors as well as immunoglobulins molecules outside the classical antigen binding sites (Silverman, 1997). They are potent activators of lymphocytes and have the capability of activating more than 5 % of the naive lymphocyte pool (an enormous population) in contrast to conventional peptide antigens that stimulate less than 0.01 %, representing a 500-fold enhanced lymphocyte activation potential (White *et. al*., 1989). Staphylococcal protein A (SpA) is a 42-kDa membrane protein of *Staphylococcus aureus* (Silverman, 1997). SpA, secreted by nearly all known clinical isolates of *S. aureus*, enhances virulence of the pathogen although the mechanisms have not been identified. SpA is a tandem of five

extra-membrane domain proteins E, D, A, B, C and an additional transmembrane domain named X. Each of the extramembrane domain has 58 - 61 amino acids and share a primary amino acid homology of about 80 % with each other. SpA has two distinct types of immunoglobulin binding specificities; one in the Fab portion of immunoglobulins of the IgM, IgA, IgG, and IgE isotypes and the other in the Fc portion of IgG1, IgG2, IgG3, and IgG4 immmunoglobulins (Potter *et. al*., 1997). Each extramembrane domain is thought to be capable of mediating Fcγ binding. The Fcγ binding property of Protein A is extensively utilized in purification and labeling of proteins (Graille *et. al*., 2000). All five extramembrane domais have also been demonstrated to show human Fab binding (Janssson *et. al*., 1998).

SpA specifically binds to antibodies from the human V_H3 gene family but not immunoglobulins from other V_H families (Silverman, 1997). Immunoglobulins bound specifically by SpA share conserved sequences in FR1, FR3 and the C-terminal portion of CDR2 outside the H2 loop unique only to the V_H3 gene family. Kirkham and Schroeder (1994), suggested that the C-terminal portion of CDR2 (from the V_H3 family that correlates with SpA binding activity) is an invariant V_H3 family-specific stretch sequence which could be more appropriately designated as part of the FR3 subdomain. Studies with three different phage display antibody libraries demonstrated that multiple rounds of panning on SpA resulted in selection of V_H3 Fabs that were encoded by the V3-23 gene (Silverman, 1998). Within the human genome, the V3-23 gene is closest to the consensus sequence of all inherited human V_H3 genes, and it encodes for V_H regions that contain one of the most highly conserved V_H FR1/FR3 surfaces (Schroeder *et. al*., 1990). Of all V_H3 antibodies with binding to SpA that have so far been evaluated, the V3-23 derived antibodies have the strongest binding interactions with the native SpA (Silverman *et. al*., 1997). The crystal structure of domain D of SpA complexed to a V3-30 derived Fab has confirmed earlier studies that the critical amino acids necessary for binding are located in the FR1, FR3 and CDR2 of the V_H3 immunoglobulin molecule (Graille *et. al*., 2000; Potter *et. al*., 1998). Silverman and colleagues investigating the unconventional nature of SpA binding demonstrated that 13 V_H3 amino acid residues in FR2/FR3 and CDR2 are essential for SpA binding (Graille *et. al*., 2000; Cary *et. al*., 2000). Among the 13 V_H residues implicated as SpA contacts, V_H3 genes include frequent germline sequence variations only at position 57. This position is not a core position and substitution of isoleucine (I) and threonine (T) for lysine (K) are permissive for SpA binding. The other 12 remaining V_H residues which are highly conserved in the V_H3 family have been found to be in direct contact with domain D of SpA. By comparison, other nonbinding germline V_H family genes have been found to contain two or more residue differences at the 13 V_H positions identified as SpA

contacts (Cary *et. al*., 2000). A detailed structural study by Graille *et. al*. (2000) identified 7 core residues among the 13 V_H contact residues: arginine/lysine (R/K)-19, glycine (G)-65, arginine (R)-66, threonine (T)-68, serine (S)-70, glutamine (Q)-81, and asparagine (N)-82a. These core of 7 V_H residues constitutes the structural motif of SpA binding, and conveys the restricted specificity for V_H3-encoded immunoglobulin and their homologoues. All Fabs isolated here expressed these 13 V_H amino acid residues and importantly, the 7 core residues as well. However, their binding to SpA varied in intensity. The observed binding differences could be due to subtle conformational variations which could be permissive for some immunoglobulins and not others.

Karray *et. al.* (1998), observed that gp120 immunoglobulin binders are highly sensitive to mutation. They demonstrated that V3-30 gene-encoded immunoglobulins with less than 96 % homology to germline genes lose the capacity to bind gp120, thus suggesting that hypermutation may have an adverse effect on gp120 binding. This inverse correlation between the degree of somatic mutation and gp120 binding is similar to the reported binding pattern described for SpA. V gene hypermutation associated with somatic selection of the B cell repertoire by exogenous antigens apparently often results in a loss of that B cell superantigen's binding capacity. The B cell receptor has a distinctive feature of undergoing somatic hypermutation, which influences the representation of superantigen binding B cell receptors in the somatic immune repertoire. Germline encoded V region structures demonstrate higher affinities (and avidities) in immature animals and in naive lymphocyte subsets than in IgG bearing B cells (Silverman *et. al*., 1997). While only about 80 - 85 % of V_H3 IgMs from adult sources bind SpA, SpA binding activity has been demonstrated for every non-mutated V_H3 immunoglobulin that has so far been tested (Hillson *et. al*., 1993 as reviewed in Silverman, 1997). Also, the frequency of superantigen binding in IgG populations which generally show greater levels of hypermutation is much lower than for IgM populations (Sasano *et. al*., 1993). Though many immunoglobulins (V_H3 family) that have undergone class switch and somatic mutation lose the ability to bind SpA, a substantial number of these antibodies still bind SpA. Silverman (1997) reported that at least 16 of the 22 (72 %) known functional V_H3 germline encoded genes have SpA binding activity while Sasano *et. al*. (1993) demonstrated that 60 % of V_H3 Fab fragments had SpA-binding activity. Since some somatically-mutated V_H3 encoded antibodies can bind SpA, both the position and nature of the mutations affect SpA binding (Potter *et. al*., 1997).

Thus, hypermutation somewhat adversely affects the frequency and avidity of V_H3 mediated SpA binding. However, from our results, the presence of mutations alone may

not account for the observed SpA binding pattern exhibited by the Fab phages. Fab phage SH51, maintaining all the residues known for SpA binding and with an R/S ratio greater than 2.9 showed strong interaction with SpA, whilst Fab phages SH21 and SH58 with 99.6 % and 99.3 % homologies to the V3-23 germline gene, respectively, did not show any appreciable binding to SpA. SH21 has an extremely long V_H CDR3 (17 amino acids), which could result in structural interference with binding to SpA. Could the association of different light chains influence these V_H3 non-SpA binding interactions? Potter and Capra (1995), reported the loss of a mouse monoclonal antibody LC1 reactivity against human monoclonal antibody with rheumatoid factor reactivity when some light chains were paired with an LC1 reactive heavy chain. LC1 detects a cross-reactive idiotope on the heavy chain of many non-pathogenic IgM antibodies and monoclonal IgM autoantibodies, many of which exhibit rheumatoid factor reactivity. The loss of LC1 reactivity indicates that the light chain influenced the availability of the LC1 determinant and provides another mechanism, other than mutation to explain the loss of an heavy chain isotype. The light chain could also provide conformational variations which could be non-permissive for some immunoglobulins and thus influence its binding abilities. In certain cases however, light chain pairing may influence the capacity of SpA to bind VH3 containing heavy chains (Domiati-Saad and Lipsky, 1997).

The critical residues for binding gp120 to V_H3 gene families, though not so well defined, (Neshat *et. al*., 2000) may be in overlapping regions to that of SpA. On the whole, our IVIG Fabs showed weak binding to gp120 whilst stronger reactivities on SpA were recorded for some clones. The observed binding differences between SpA and gp120 for the Fab phages could be that SpA and gp120 target different sites on V_H3 immunoglobulins, some of these contact sites partially overlap though (Karray *et. al*., 1998). In addition, none of the two superantigens tested in this study (SpA and gp120) was able to compete with IVIG in binding to the Fabs, indicating that at least some of the contact residues on IVIG must be different from those for SpA and gp120. Delineating the exact mechanism(s) of binding of IVIG to V3-23 and V3-30 Fabs is an enormous task, granted the huge variety of immunoglobulins present in the IVIG pool. As a solution, our IVIG-selected Fabs may now be used to clone antibodies representative of this IVIG subfraction from a healthy donor library. This will enable the study of the structural basis for recognition and possible regulatory influence on the B cell repertoire during normal development and disease (Osei *et. al.*, 2000).

4. 5 SUMMARY AND CONCLUSION

The beneficial therapeutic effects of IVIG are well documented, but the underlying molecular mechanisms are not fully understood. Recent investigations from our laboratory into the molecular analysis of Fabs bound by IVIG from patients suffering from autoimmune thrombocytopenia revealed that the most frequently selected Fabs originated from the V3-23 and V3-30 V_H germline genes. A subsequent study with IgG and IgM phage display libraries from a healthy donor also demonstrated a preferential reactivity of IVIG to Fabs of V3-23 and V3-30 origin. That study revealed that the unique reactivity of IVIG to Fabs of these two V_H gene loci was not restricted to the autoimmune nature of the donors, neither to previous treatment with IVIG. One of the thrombocytopenia patients developed lupus. This study was undertaken to study the molecular interaction of IVIG with antibodies selected from a patient suffering from systemic lupus erythematosus and rheumatic fever using phage display technology, and to compare the results with the previous studies.

Twenty-three Fabs representing seven independent clones were isolated. In contrast to ITP-derived clones, none of the Fabs selected in this study reacted with platelets. The Fab phages bound by IVIG were sequenced in order to determine their V_H gene usage and clonal relatedness. V3-23 and V3-30 V_H genes were found to be exclusively utilized by the Fab phages bound by IVIG. Moreover, different CDR3 regions including different D and J_H gene segments were observed to be used by these Fabs. The results further showed that the binding of IVIG to the Fabs was independent of the light chain since different light chains were observed to be associated with the V_H3 immunoglobulins. Detailed sequence analysis of the Fabs revealed the presence of amino acid residues at positions within FR1, FR3, and the 3' end of CDR2 that are known to be contacted by the B cell superantigen Staphylococcus protein A (SpA). Some of the Fabs were shown to bind strongly to SpA, but there was no correlation with the binding-intensity to IVIG. Some bound very weakly to HIV gp120, another B cell superantigen. This study, together with previous results, suggests that a subset of IVIG may function as a B cell superantigen that may significantly shape the B cell repertoire. The binding mechanism appears to be similar but not identical to the other tested B cell superantigens.

4.6 ZUSAMMENFASSUNG UND SCHLUSSFOLGERUNG

Therapeutische Erfolge von IVIG sind gut dokumentiert, aber die zu Grunde liegenden molekularen Mechanismen sind noch nicht vollständig erforscht. Molekulare Analysen unseres Labors über die Interaktion von IVIG mit Fabs von Patienten, die an einer autoimmunen Thrombozytopenie (ITP) leiden zeigten, dass die am häufigsten selektierten Fab von den V3-23 und V3-30 VH-Keimbahngenen abstammten. Eine weitere Studie mit IgG und IgM Phagen-Display Bibliotheken von einem gesunden Spender zeigten ebenfalls eine bevorzugte Reaktivierung von IVIG mit Fabs vom Ursprung der V3-23 und V3-30 Gene. Es konnte gefolgert werden, dass diese Interaktion von IVIG mit Fabs von diesen zwei VH-Genen weder alleine auf den Gesundheitsstatus des Spenders zurückzuführen war, noch auf eine zuvor erfolgte Behandlung mit IVIG. Diese Dissertation wurde unter Verwendung der Phagen-Display Technologie unternommen, um die molekulare Interaktion von IVIG mit Antikörpern zu erforschen, die von einem Patienten kloniert wurden, der an einem systemischen Lupus erythematodes und rheumatischem Fieber leidet. Die Resultate waren mit den früheren Studien zu vergleichen, insbesondere mit den Daten eines Patienten, der zu der ITP einen Lupus entwickelte.

23 Fabs, welche 7 unabhängige Klone repräsentierten, wurden isoliert. Im Gegensatz zu von Patienten mit ITP abstammenden Klonen reagierte keines von den in dieser Studie selektierten Fabs mit Thrombozyten. Die über IVIG gebundene Fab-Phagen stammten hierbei ausschließlich von den V3-23 und V3-30 VH-Genen ab. Darüber hinaus wurde beobachtet, dass von diesen Fabs verschiedene CDR3 Regionen einschließlich verschiedenen D- und JH-Gensegmenten benutzt wurden. Die Ergebnisse zeigten weiterhing, dass die Bindung von IVIG an die Fabs unabhängig von der Leichten Kette war. Ihrem Keimbahngen-Ursprung entsprechend hatten die Fabs Aminosäuren an Positionen in den FR1, FR3 und im 3'-Ende von CDR2, die dafür bekannt sind, dass sie für die Bindung des B-Zell-Superantigens Staphylococcus Protein A (SpA) essentiell sind. Es wurde gezeigt, dass sich zwar einige von den Fabs stark an SpA banden, aber keine Korrelation in der Intensität zur Bindung mit IVIG vorlag. Einige Fabs zeigten eine schwache Bindung an HIV gp120, einem anderen B-Zell-Superantigen. Zusammenfassend lässt sich aus der vorliegenden Studie und den vorherigen Ergebnissen schließen, dass ein Anteil von IVIG wie ein B-Zellen Superantigen funktionieren könnte, das für die Bildung und Regulation des normalen B-Zellen Repertoires wichtig ist. Der Bindungsmechanismus scheint ähnlich, aber nicht identisch mit dem der anderen getesteten B-Zellen-Superantigene zu sein.

5.0 APPENDIX

5.1 DECLARATION

I hereby declare that this is an original work carried out by me, and that, this work has not been submitted elsewhere for a degree.

Awuku Kwabena Osei

5.2 LIST OF PUBLICATIONS

1. Awuku Osei, Martina M. Uttenreuther-Fischer, Heike Lerch, Gerhard Gaedicke, and Peter Fischer (2000). Restricted VH3 gene usage in phage-displayed Fab that are selected by intravenous immunoglobulins. *Arthritis and Rheumatism*. **43**: 2722-2732

2. Sundsfjord, A.; **Osei, A**.; Rosenqvist, H.; Ghelue, M. V.; Silsand, Y.; Haga, HJ.; Rekvig, O. P.; and Moens, U (1999). BK and JC viruses in patients with systemic lupus erythematosus: Prevalent and persistent BK viruria, sequence stability of the viral regulatory regions, and non-detectable viremia. *J. Infect. Dis*. **180**: 1-9

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5.3 CURRICULUM VITAE

PUBLICATIONS

1. **Osei, A.,** Uttenreuther-Fischer, M. M., Lerch, H., Gaedicke, G., and Fischer, P (2000). Restricted VH3 gene usage in phage-displayed Fab that are selected by intravenous immunoglobulins. *Arthritis and Rheum.* **43**: 2722-2732

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POSTERS

1. Peter Fischer, Melanie Hoffmann, Nina Jendreyko, Stephan Leucht, **Awuku Osei**, Heike Lerch, Martina M. Uttenreuther-Fischer, Gerhard Gaedicke. Intravenous immunoglobulin (IVIG)-reactive IgG and IgM antibodies cloned by phage display reveal new insights in the network regulation of B cells. Immunobiol. 1999; 200: 703 (Vortrag, Poster)

2. Peter Fischer, **Awuku Osei**, Melanie Hoffmann, Nina Jendreyko, Stephan Leucht, Heike Lerch, Martina M. Uttenreuther-Fischer, Gerhard Gaedicke. Restricted gene use in phage-displayed human IgG and IgM Fabs selected with IVIG suggests a B cell superantigen-like interaction. Proc. Vakzine Meeting der DGfI, Münster 29.-30. June 2000. (Vortrag)

3. Peter Fischer, Stephan Leucht, **Awuku Osei**, Melanie Hoffmann, Nina Jendreyko, Heike Lerch, Martina M. Uttenreuther-Fischer, Gerhard Gaedicke. A novel B cell superantigen-like interaction of IVIG is suggested by the favoured selection of IgG and IgM VH derived from germ-line segments 3-23 and 3-30/3-30.5 Immunobiol. 2000, B6 (Poster)

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5.6 CITED LITTERATURE

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