# Bypassing immunization in an attempt to develop $\beta_1$ specific monoclonal human antibodies from semi-synthetic repertoires

By

Chantal Binda

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

Department of Medical Microbiology

University of Manitoba

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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# Bypassing immunization in an attempt to develop $\beta_1$ specific monoclonal human antibodies from semi-synthetic repertoires

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of Master of Science

*Remuer ciel et terre,*

*to leave no stone unturned.*

*Euripide*

# ABSTRACT

Immune selection can be mimiced *in vitro* by displaying antibody repertoires on the surface of a filamentous bacteriophage and selecting phage by binding to antigen. The development of recombinant DNA methods and the knowledge that antibody fragments could be functionally expressed and assembled in *E. coli* have led to the development of phage-antibody display technology.

Integrins are conserved proteins involved in tissue morphogenesis, tissue integrity, development, inflamation, control of cell growth, and cell mobility. Highly conserved regions of the integrins are thought to play an important role in their function.

Failure to raise antibodies against those regions using conventional hybridoma technology has led to the use of semi-synthetic repertoires in an attempt to develop $\beta_1$ integrin specific monoclonal antibodies.

Large diversity is an essential feature of semi-synthetic repertoires of antibodies. Seven semi-synthetic phage-antibody human libraries were used, where HCDR3 was randomised over 7 or 13 amino acid residues. These randomized heavy chains where cloned with a unique Humkv 325 light chain as Fab fragments into pComb 3H vector for phage display.

The libraries were panned by binding to a biotinylated $\beta_1$ integrin peptide know to be the epitope of an inhibitory antibody called JB1A, or to purified human $\beta_1$ integrin. Seven rounds of panning, with increasing stringency, were done in an attempt to increase the proportion of highly specific clones.

Modest enrichment was observed for the selection on peptide and no enrichment was observed on purified human $\beta_1$ integrin. Clones selected using these semi-synthetic antibody libraries do not seem to have any specificity for the desired epitope. Improvement of the method of selection and system outlined in this thesis should be done in any future attempt of selection. Nevertheless phage-antibody display technology has a promising future as a complement to the well established hybridoma technology.

## ACKNOWLEDGMENTS

I would sincerely like to thank my supervisor, Dr. John A. Wilkins, for his judicious guidance, his patience, and his scientific expertise throughout this project. I would also like to thank Dr. Ma Luo for her help with the phage display as well as her judgment and her friendship. I am also thankful to my coworkers and friends Dr. Wang Di, Heyu Ni, Yang Long Mou, and Heather. I would specially thank my parents, Yolande and Jean-Pierre and my brother Olivier, who supported me without compromises all my life.

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## INTRODUCTION



## RESULTS

# ABBREVIATIONS

Carb: carbenicillin

cDNA: complementarity deoxyribonucleic acid

CDR: complementary determining region

$C_H$: constant domain of the heavy chain

$C_L$: constant domain of the light chain

CY: cytoplasmic domain

dsDNA: double strand DNA

ECM: extracellular matrix

Hc: heavy chain

HCDR3: third complementarity determining region of the heavy chain

Ig: immunoglobulin

Kana: kanamycin

Lc: light chain

LCDR3: third complementarity determining region of the light chain

ORI: origin of replication

Pfu: plaque forming units

RA: rheumatoid arthritis

ssDNA: single strand DNA

Tet: tetracyclin

TM: transmembrane domain

$V_H$: variable domain of the heavy chain

$V_L$: variable domain of the light chain

# INTRODUCTION

## I. Discovery of antibodies.

As an assistant to Robert Koch in 1890, Dr Emil Behring was a codiscoverer of antibodies which at that time were called antitoxins and the first to use antibody preparations, on animals, as a therapeutic agent of infectious diseases, such as diphteria or tetanus. A few years later, in 1893, treatment of humans with antitoxin was begun (Gronski, P., Seiler, F.R., and Schwick, H.G., 1991). Behring was awarded for his pioneering work in 1901 with the first Nobel Prize for physiology and medecine. It was at the beginning of the century that a branch of biology that is today called immunochemistry appeared. From this time, enormous developments have been made on antibodies and there use in therapy. Today, monoclonal antibodies which have specificities against various antigens are being more and more rapidly developed. The future of antibodies tend toward the search for highly specific and *in vitro* produced monoclonal antibodies. Behring said a hundred years ago, as translated in Gronski et al., 1991, "Considering that antitoxin is an inanimate chemical substance, the possibility cannot be discounted that it may, at a later date, be able to be produced without the aid of an animal body" (Behring, 1894). This is now realistic due to the development of synthetic antibody repertoires.

## II. Antibody structure and antigen recognition

Antibodies are Y-shaped molecules and are expressed as surface molecules or soluble forms by B lymphocytes (Cellular and molecular immunology, Abbas, A.K., Lichtman, A.H., Pober, J.S., 1997). Each antibody molecule is composed of 4 polypeptide chains arranged in two identical pairs. 1 partner is known as the heavy chain and the other partner of a pair is called the light chain.

The heavy chain is 55 Kd and light chain is 25 Kd. One Lc is covalently linked to the N-terminal region of one Hc by one disulfide bond, and the two Hc are linked together by two disulfide bonds (Figure 1a). The IgG Hc is composed of 3 constant domains called $C_H1$, $C_H2$, and $C_H3$ and one variable domain at the N-terminal region. The Lc is composed of one constant domain and one variable domain. Within the variable domains of Hc and Lc, there are three hypervariable regions called CDR (complementarity determining regions). The antigen binding site is formed by CDRs pairing of Hc and Lc. CDRs are responsible for the antibody specificity. The HCDR3 is the most variable (Figure 1b) and consequently the one most responsible for antigen recognition (Wu, T.T. et al., 1993). The amino acid residues of the hypervariable regions form loops that are exposed on the antibody surface and are thus available to interact with the antigen. The constant regions are important for the antibody mediated effector mechanisns, they are responsible, for example for some effector functions such as complement binding and Fc receptor mediated phagacytosis.

Antibody molecules can be separated in different fragments. Fv fragment is composed of the $V_H$ and the $V_L$. Fc fragment is composed of both $C_H2$ and $C_H3$ domains. The Fab fragment consists of the Lc, the $V_H$ and $C_H1$,

A)

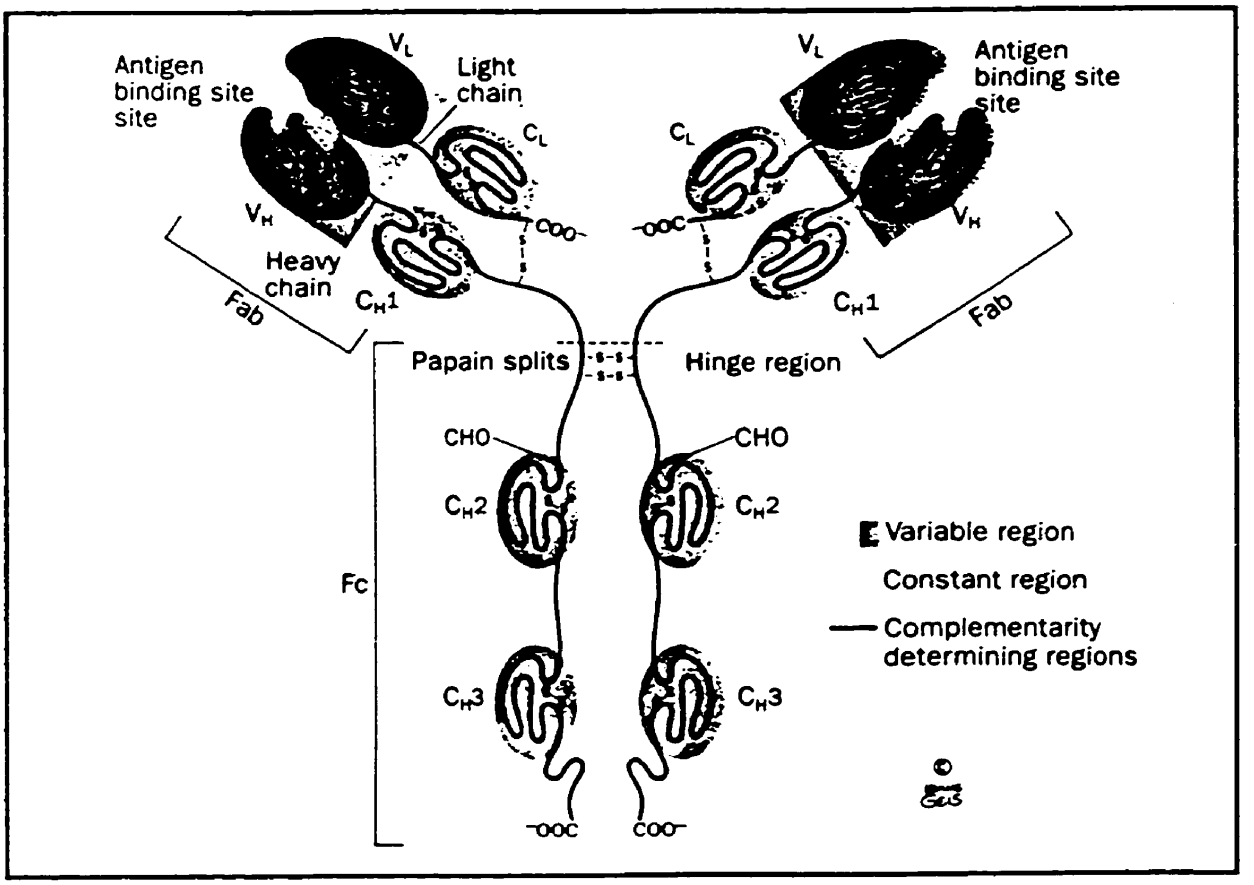
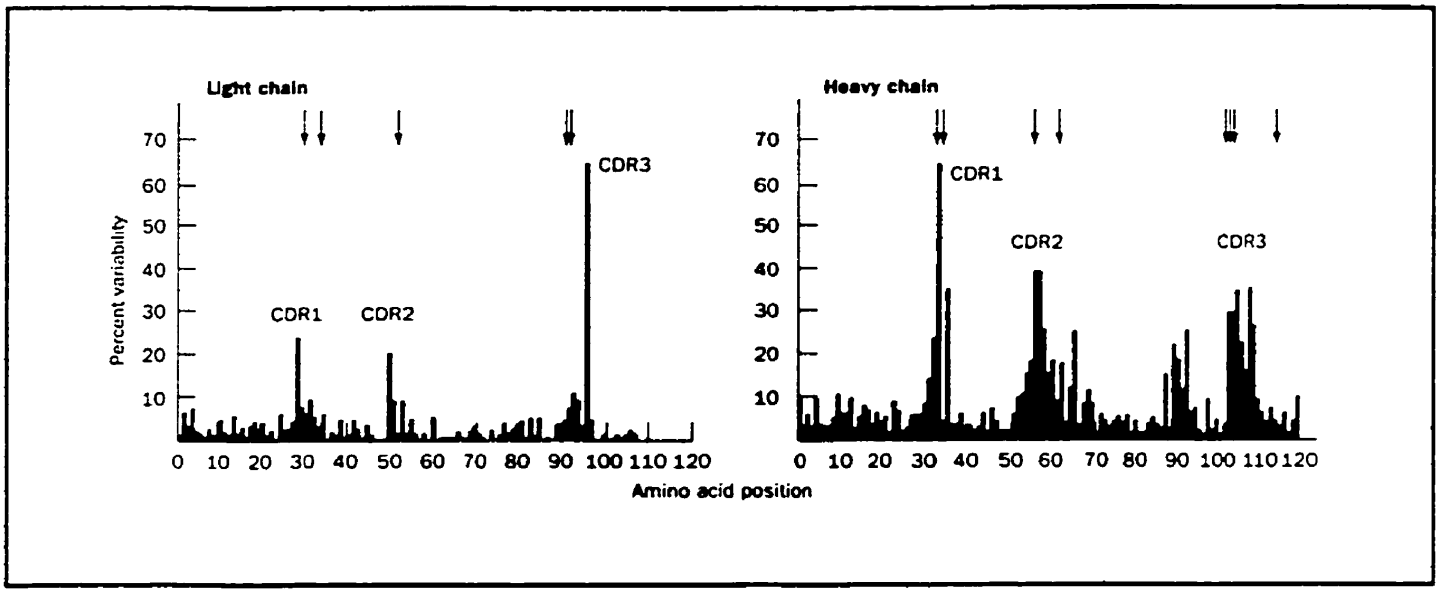



**Figure 1.** Structure of the human IgG molecule (a). Sequence comparaisons showing the variability of CDRs regions within the Lc and the Hc (b) (Voet, D., Voet, J.G., 1990).

B)


Light chain
Heavy chain
Percent variability
70
60
50
40
30
20
10
CDR1
CDR2
CDR3
0 10 20 30 40 50 60 70 80 90 100 110 120
Amino acid position

whereas Fd fragment are made up the $H_V$ and $C_H1$. Fab fragments behave as whole antibodies in terms of antigen recognition, affinity, and specificity (Huse et al., 1989).

The recognition of an antigen by an antibody molecule implies interaction that is non-covalent and reversible. The force or strength of the binding occuring between an antigen and an antibody combining site is called affinity. An antigen can be any substance that is specifically bound by an antibody molecule or a T lymphocyte receptor. Antigens include polysaccharides, lipids, hormones, phospholipids, complex carbohydrates, nucleic acids, and proteins. The specific portion of a molecule that is recognized by an antibody is called an epitope. Epitopes can be either linear or conformational. Linear epitopes are formed of a portion of the linear sequence that is accessible to the antibody when the protein is folded or denatured. Conformational epitopes are formed by sequentially noncontiguous residues that are spatially adjacents only when the protein has acquired a specific conformation.

## III. Generation of diversity and affinity maturation.

### 1. Generation of diversity of the antibody repertoire.

The naive antibody repertoire of an individual is larger than $10^9$. Ig genes undergo an effective mechanism of somatic DNA recombination during B lymphocytes ontogeny for generating a large and highly diverse repertoire from a limited number of genes. Different genetic mechanisms contribute to the diversity of antibody repertoire (Cellular and molecular immunology,

Abbas, A.K., Lichtman, A.H., Pober, J.S., 1997). The first one is the combinatorial diversity involving somatic recombinations of gene segments. The second mechanism arises from junctional diversity caused by the inaccurate DNA rearrangement that occurs at the junctions between the V genes and the J or D genes. Junctional diversity is also caused by the random addition of nucleotides at the junctions of rearranged VJ or VDJ genes, these new sequences being called N regions. The third mechanism contributing to the diversity of a repertoire is the combination of the Hc and Lc, because both chains are involved in the antigen binding.

Hc, lambda, and kappa Lc genes are encoded on three different chromosomes (Figure 2). The Hc and Lc loci are composed of several genes that by rearrangements will give rise to the V and C regions of the Ig. Each Hc and Lc locus is composed of 100-200 V genes each about 300bp. 5' of each V region exon there is a small exon that codes for the translation initiation signal and the leader sequences. 3' of the V genes are the C genes. The Hc C region gene contains 3 to 4 exons to make the C region and other exons coding for the transmembrane domain and the cytoplasmic domain. The kappa Lc locus contains one C gene, and the lambda locus is composed of 3-6 genes. The J and D (only for the Hc) genes segments code for the C-terminal ends (the most variable) of the V regions are composed of small exons of 30-50bp which are located between the V and C genes locus.

Rearrangements of Ig genes in the developing B cell involves first the Hc locus (Figure 3). First one D and one J segments are joined, then one V genes is joined to the DJ complex to form a VDJ gene. Introns between the VDJ gene and the C genes are spliced out giving rise to a functional Hc mRNA which then lead to the production of a Hc. The second rearrangement involves the Lc locus. One V segment is joined to one J segment followed by the splicing of the introns located between the VJ complex and the C region.

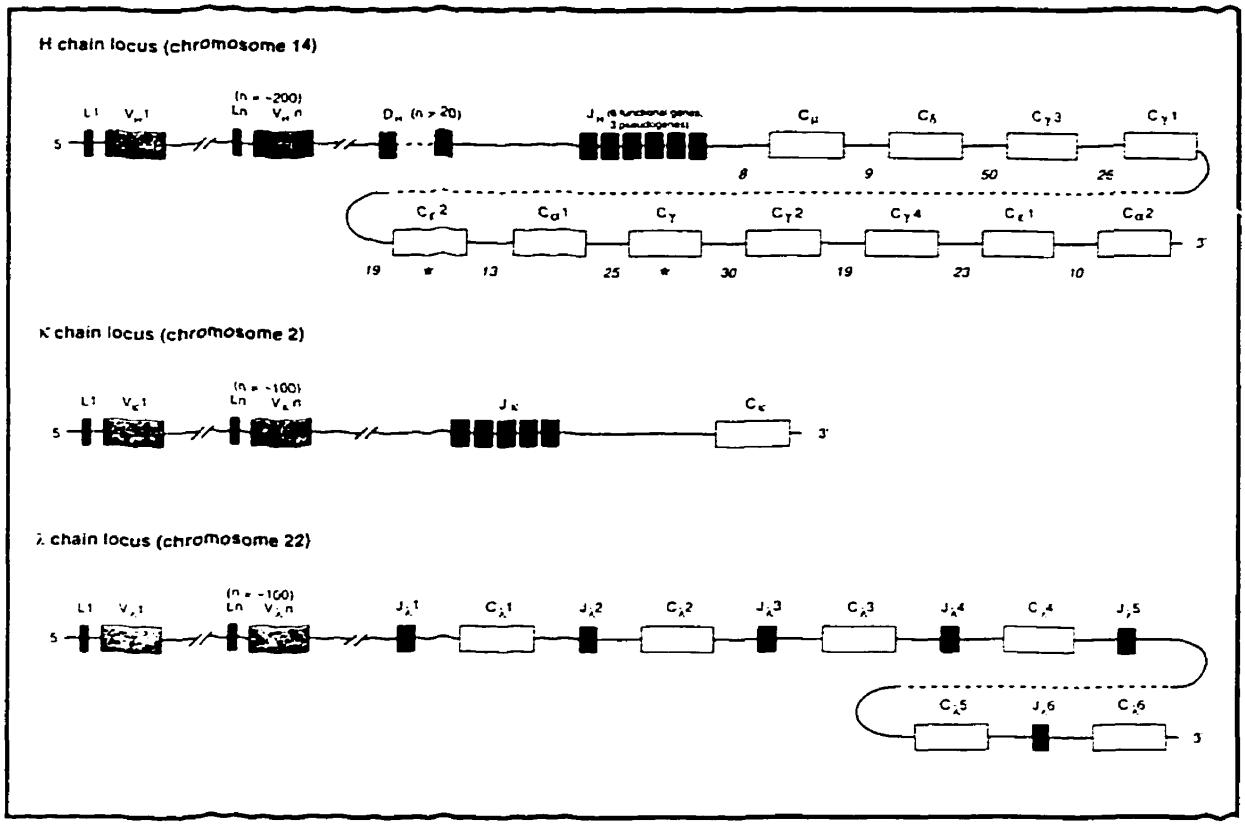



**Figure 2.** Organization of the human immunoglobulin genes in the germline. Each $C_H$ gene shown here as a single exon is composed of multiple exons. * nonfunctional pseudogenes. Italic numbers refer to approximate lengths of DNA in Kb. (Abbas, A.K., et al., 1997).

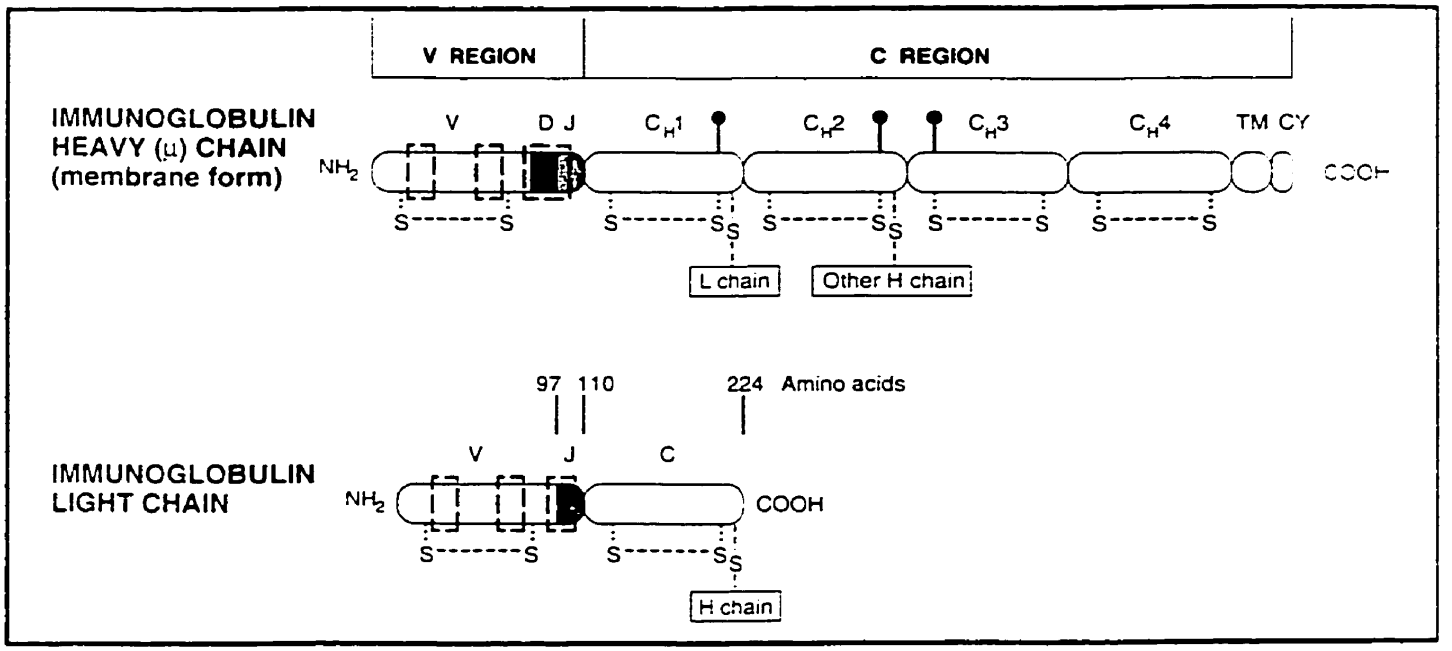



**Figure 3.** Relation of Ig gene segments to domains of Ig polypeptide chains. Dashed boxes indicate CDR regions. (Abbas, A.K., et al., 1997).

The Hc and Lc proteins are assembled in the endoplasmic reticulum to form a functional cell surface expressed Ig molecule.

The successful production of an Hc stimulates the Lc genes rearrangements. Production of Hc, or either kappa, or lambda Lc on one chromosome inhibits rearrangement on the other chromosome, accounting for allelic exclusion of Ig Hc and Lc in individual B cells.

## 2. Affinity maturation of antibodies.

Affinity maturation is the increased average affinity of a secreted or membrane associated (Cellular and molecular immunology, Abbas, A.K., Lichtman, A.H., Pober, J.S., 1997) Ig after repeated antigenic stimulations. Affinity maturation occurs during responses to antigens and is caused by somatic point mutations in the sequence coding for Ig V region exons of both Lc and Hc, concentrated particularly in the hypervariable regions. A selection process follows which ensures the positive selection of the best high affinity cells. The lymphoid follicles germinal centers in peripheral lymphoid tissues are the main sites of this fine tuning of the antibody response.

# IV. Approaches and limitations of conventional and monoclonal antibody production.

Different methods are now available for the production of human antibodies. These methods are discussed here briefly.

## 1. Hyperimmune human serum.

As was discussed previously, Dr Emil Behring was the first to use antibody preparations as a therapeutic agent of infectious diseases. At that time, immunized animals such as sheep, were used as the source of serum, which caused side effects such as serum sickness. Later, hyperimmune human serum was used for treatment. This technique is extremely inefficient because only a small proportion of the serum preparation will consist of specific antibodies to a particular antigen. However, this technique is still useful in developing countries, where more expensive and advanced techniques are not available to decrease the incidence of infectious diseases. Today, immunotherapeutic approaches require larger quantities of specific human antibodies with high affinities (Gronski,P., Seiler,F.R., and Schwick, H.G., 1991).

## 2. Hybridoma technology.

For the past 20 years, monoclonal antibodies have been available since their first production by Köhler and Milstein (1975). They described in 1975 the fusion of mouse myeloma and mouse spleen cells from an immunized donor. This cell line secreted anti-sheep red blood cell antibodies. In this way, immortalized cell lines could be generated for the production of antibodies of known specificities. The methods are well established and they offer unlimited supplies of specific rodent monoclonal antibodies against a variety of antigens. Unfortunately, hybridoma technology is less successful for the production of human monoclonal antibodies or for the production of antibodies against molecules with high degree of sequence homology between

species which render the immune response of the host difficult and often unsuccessful (James, K., and Bell, G.T., 1987).

## 3. Humanization of mouse monoclonal antibodies.

Because of the failure to produce human monoclonal antibodies by the hybridoma technology, mouse monoclonal antibodies are being humanized using recombinant DNA techniques to circumvent the limitation of the use of rodent antibodies in clinical applications especially when repeated administration is required for therapy due to the high immunogenicity of rodent antibodies in human. Humanization can be achieved by transferring the antibody hypervariable regions (CDRs) from the mouse monoclonal antibody into the constant backbone of a human antibody (Jones, P.T., et al., 1989; Riechmann, L., et al.,1988) or by transferring the variable domain (Boulianne, G.L., et al., 1984; Morrison, S.L., et al., 1984; Neuberger, M.S., et al., 1985). CDR grafting is the most frequently used approach for the humanization of rodent antibodies. CDR grafting often yields antibodies with a lower affinity because framework residues are involved in antigen recognition by direct contact with the antigen or by keeping the conformation of the CDR loops (Foote, J., Winter, G., 1992) These humanized monoclonal antibodies still contain parts of the mouse sequence which might cause some immunogenicity problems. The transfer of CDRs may also need some adjustment to the human framework. Another stategy for humanization is the method of resurfacing where only the surface residues of a rodent antibody are humanized (Pedersen, J.T., et al., 1994). The surface residues are the most exposed and are highly variable, therefore they are important for antigen recognition. Humanization based on selection from phage display libraries has also been reported. Selection from libraries of randomized small

sets of framework residues (Rosok, M.J., et al., 1996; Baca, M., et al., 1997), and also by sequential chain shuffling of either rodent Lc and Hc is replaced by a human polypeptide library (Jespers, L.S., et al., 1994) have been reported. Humanization of rodent monoclonal antibodies by a combination of CDR grafting and V gene shuffling using selection by phage display has been demonstrated (Rader, C., et al, 1998).

## 4. Transgenic mice.

Recently, transgenic mice that carry large human immunoglobulin minilocus (1300 kb human Ig kappa Lc locus or human VH26 heavy chain gene) on a yeast artificial chromosome (YAC) were made possible (Xiangang Zou, et al., 1996; Harmer, I.J., et al., 1996). These miniloci introduced into the mouse germline undergo functional rearrangements resulting in the production of chimaeric antibodies in which a human Hc is paired with a mouse Lc *in vivo*. The use of transgenic mice as a humanization strategy has also been reported by Mendez, M.J., et al. (1997). These experiments give the hope for introduction of both human Lc and human Hc locus into transgenic mice with an Ig knockout. This would allow the production of nearly authentic human antibody repertoires in transgenic mice in an *in vivo* context. This system could probably mimic more accurately the immune system in the generation of repertoire diversity and affinity maturation of antibodies. These antibodies would be less immunogenic as therapeutic agents.

## 5. Selected lymphocyte antibody method (SLAM).

A novel strategy for the production of monoclonal antibodies has been reported recently (Babcook, J.S., 1996) which takes advantage of the *in vivo* mechanisms that generate high-affinity antibodies. It is based on the identification, within a very large population of rabbit or mouse antibody forming cells (AFC), of a single cell producing specific antibodies, by means of the hemolytic plaque assay (Jerne, N.K., and Nordin, A.A., 1963). Sheep red blood cells coated with streptavidin and a biotinylated antigen were used. Then, cDNAs of variable regions of the Hc and Lc of the clone are amplified by RT-PCR and cloned into a human expression vector that contain human Ig constant regions. Myeloma cells are then transfected with these vectors. This allows for the expression of chimaeric Ig. This technique, if reproducible, should be useful for the production of human monoclonal antibodies. But it may not be very efficient at producing human monoclonal antibodies to self antigens or unknown proteins. This technique can only be used for antigens that can be purified and biotinylated. This means that the selection has to be performed against a known target.

## 6. Phage-antibody libraries.

Phage-antibody display is a promising technology which has been developed over the last few years for the generation of human and mouse monoclonal antibodies. This technique is based on the cloning of antibody fragments (Fab or scFv) in phage libraries. This new technique is the subject

of this thesis, and will be discussed in a more detailed and critical way in the following sections.

## V. Phage display concept and systems.

### 1. Random peptide libraries.

The powerful technology of phage display was first described in 1985 by George P. Smith (Smith, G.P., 1985). George Smith found that peptides could be displayed on the surface of filamentous bacteriophage by fusing them to the N-terminus of the coat protein pIII. He fused a fragment of the gene for EcoRI restriction enzyme to the N-terminus of pIII and did the affinity selection on antibody against EcoRI enzyme. This original approach provided a link between recognition and replication, because the foreign polypeptides are associated with the phage itself by its fusion with pIII coat protein which is in contrast with the conventional lambda phage expression vector in which fusion proteins are not incorporated into the infection particle. Selected clones can be identified quickly. Billions of clones can be easily surveyed whereas tens of plaque-lifts would be required to screen a phage lambda library of a million clones.

Smith's group used this technique to make the first random peptide library. Degenerate synthetic oligonucleotides coding for all possible short peptides (hexamer) were cloned into phage display vector (Scott, J.K., and Smith, G.P., 1990). Only small modifications were required to display a variety of bigger molecules such as proteins, antibodies, drugs, and nucleic

acids at the surface of filamentous phage. Phage display is impressive in its broad range of applications. The ability to display peptides and proteins on the surface of filamentous bacteriophage has impacted on many fields such as immunology, cell biology, protein engineering, and pharmacology. It is a technology that is applicable to a wide range of problems.

## 2. Combinatorial antibody libraries.

Immunoglobulin gene combinatorial libraries are generated by the combination of separate Hc and Lc cDNA libraries, from natural repertoire of Hc and Lc genes, into a single expression library in which all permutations of Hc and Lc combinations are theoretically possible and may be functionally expressed. Combinatorial libraries enable the simultanous screening of millions of related compounds and the rapid identification of novel compounds without any prior structural or sequence knowledge.

The cornerstone of the expression of combinatorial antibody libraries is the demonstration in 1988 (Better, M., et al., 1988; Skerra, A., Plückthun, A., 1988) that antibody fragments scFv and Fab could be expressed and functionally assembled in *E. coli*. There are many advantages to using *E. coli* as an expression system. These include the high level of expression, an excellent knowledge of the genetics of *E. coli*, and it is cheap to grow. However *E. coli* is unable to do post translational modification such as glycosylation (Makrides, S.C., 1996).

Protein transport to the periplasm of *E. coli* is functionally equivalent to the transport of a protein to the lumen of the endoplasmic reticulum of an

eukaryotic cell. Expressed antibody fragments must be secreted into the periplasm of *E. coli* for the necessary disulfide exchanges to occur. Targetting of the antibody fragments to the periplasm is achieved by fusing them to the carboxyl terminus of a leader peptide. The most commonly used leader peptides are *Staphylococcus aureus* protein A, *E. coli* outer membrane leader peptide (omp A), and *Erwinia carotova* leader peptide (pel B) (Barbas, C.F., Burton, D.R., 1994). The mechanism of expression of antibody fragments libraries will be discussed in more detail in following sections.

The first combinatorial antibody libraries were made possible by the availability of PCR which allowed for the rapid cloning of the antibody genes (Larrick, J.W., et al., 1989; Orlandi, R., et al., 1989). This in conjunction with a detailed knowledge of IgG structure and V region sequences allowed for the production of primers to amplify most of the IgG genes required to make libraries that covered all of the IgG repertoire.

The first combinatorial antibody library to be created was published by Huse et al. in 1989. This library was expressed in phage lambda and contained approximately $2.5 \times 10^7$ clones. In that experiment they combined repertoires of Hc and Lc to clone and express functional Fab fragments. They made this library from mRNA isolated from an immunized mouse with KLH-coupled p-nitrophenyl phosphonamidate antigen I (NPN) and the transition state analog hapten. The screening of the library was done using a radioactive probe and nitrocellulose filter lifts. They screened 1 million phage plaques and identified approximately 100 clones that bound NPN. The screening capacity was limited and laborious unlike the filamentous phage fusion system first proposed by Smith in 1985, where the genotype and phenotype are linked, this approach did not allow for the selection and enrichment of the positive clones.

The first repertoires of antibody fragments were cloned into phage lambda based vectors (Potolano, S., et al., 1991; Huse, W.D., et al., 1989; Persson, M.A.A., et al., 1991; Caton, A.J., and Koprowski, H., 1990; Mullinax, R.L., et al., 1990). The most commonly used type of vector is now phagemid based system (McCafferty, J., et al., 1990; Clackson, T., et al., 1991; Barbas, C.F., et al., 1991; Kang, A.S., et al., 1991). Phagemids are plasmids which contain the origin of replication of filamentous phage. Phagemid system is more advantageous compared to lambda phage vectors because they are double stranded, so there is less chance of mutation and also because the efficiency of transformation is 100 times higher which allows for the creation of larger libraries. The screening method also limits the proportion of the library that can be surveyed relative to the size of the normal B cells repertoire. The antigen must also be available in significant quantity and in a purified form. This is discuss in more details in the discussion.

## 3. Synthetic repertoires.

When dealing with the production of human antibodies against self proteins ,or when producing antibodies against antigens that are conserved between species, or novel antigens that are not identified or available in a purified form, the synthetic approach is the most suitable method, it has increased our ability to select human monoclonal antibodies. Semi-synthetic libraries are created by using cloned V genes used as a framework, to which randomized CDR3 regions of the Hc or Hc and Lc are fused *in vitro*. It allows for the production of monoclonal antibodies with specificities to a broad range of antigens without immunization. Ultimately, completely synthetic repertoires over the 6 CDRs with variable framework regions have the

potential to replace immunization for the antibodies against virtually any conceivable given epitope, completely independant of the immune system.

In the past few years, efforts have been put into the production of semisynthetic libraries that vary over the HCDR3 or over both HCDR3 and LCDR3. the rationale behind this approach is that it is mainly the variability of these CDR sequences that confers specificity to antibodies. As the HCDR3 is the most variable CDR with lengths that vary from 2 to 26 aa residues (Wu, T.T., et al., 1993). It was also demonstrated that some $V_H$ domains bind to antigen without a Lc (Ward, E.S., et al., 1989). Whereas the synthetic approach results generally in antibodies with lower affinities than the antibodies obtained by immunization it is possible to increase their affinity by chain shuffling and CDR walking (Crameri, et al., 1996; Yang, W.-P., et al., 1995; Burton, D.R., and Barbas, C.F., 1994). Both semisynthetic Fab and scFv fragments have been produced, these have been screened against a variety of antigens with shapes and sizes as different as metal ions, haptens, peptides, proteins, carbohydrates, DNA, inorganic surfaces, cell surface antigens (directly selected on cells) foreign antigens, and human antigens.

The first synthetic antibody was published by Barbas et al. in 1992. These authors were able to change an anti-tetanus human antibody into an anti-fluorescein antibody by randomizing only the HCDR3, over 16 aa residues. The size of the library, constructed in pComb3 vector, was $5 \times 10^7$ and they selected Fab with affinities up to 0.1μM. This anti-tetanus toxoid antibody used as a backbone possessed a universal Lc encoded by Humkv 325 germline sequence which is over-represented in the repertoire of Lc (Burton, D.R., Barbas, C.F., 1994). Because of its over-representation (bias

toward it in the immune system) this Humvk 325 Lc was used in the construction of semisynthetic libraries.

A year later, the same group published (Barbas, C.F., et al., 1993) the results of selection on metal ions and haptens from HCDR3 libraries of length 5, 10, and 16 aa residues. All the clones that were obtained derived from libraries of HCDR3 length 16, which diversity of shapes was probably higher than the libraries with shorter HCDR3. They also tested libraries with semisynthetic LCDR3 and libraries with both HCDR3 and LCDR3 randomized. For the synthetic LCDR3 they did not obtain any clones, and the number of positive clones selected from libraries of HCDR3/LCDR3 was higher than from HCDR3 libraries. In this report human anti-tetanus toxoid Fab 7E (Barbas, C.F., et al, 1992) was also used as a backbone. Each of the libraries, expressed into pComb3, were at least $10^8$ in size.

The same group also reported in 1994 the construction of semisynthetic Fabs as adhesive ligands for integrins. In a previous paper (Barbas, C.F., et al., 1993) they inserted the integrin recognition motif RGD into the HCDR3 of a human antibody against the human HIV coat protein gp120, and randomized the 6 residues flanking the RGD motif, resulting in a phage library. A series of antibodies was obtained which bound to $\alpha_v\beta_3$, $\alpha_{IIB}\beta_3$, $\alpha_5\beta_1$. Fab-9 clone was obtained but did not appear to distinguish between $\alpha_v\beta_3$ and $\alpha_{IIB}\beta_3$. They reported that further rounds of selection and engineering on the RGD sequence could create specificity for either $\alpha_v\beta_3$ or $\alpha_{IIB}\beta_3$ from the Fab-9 clone which could previously not distinguish between the two.

Around the same time Barbas et al. published their first semisynthetic Fab library, Hoogenboom and Winter (1992) published the first semisynthetic scFv library. Two libraries were built from 49 human

germline $V_H$ gene segments and a single unmutated Vλ3 Lc. The $V_H$ segments were rearranged *in vitro* to create synthetic HCDR3 of 5 or 8 aa residues. Each of the libraries had a size of approximately $10^7$ clones. They selected semisynthetic scFv with specificity against two haptens, phOx (2-phenyl-5-oxazolone) and NIP (3-iodo-4-hydroxyl-5-nitrophenyl-acetate) reaching affinities up to the μM range. They also tried selection against whole proteins such as TNF (tumor necrosis factor), BSA (bovine serum albumine), turkay egg-white lyzozyme and human thyroglobulin. They isolated 1 TNF binding scFv but failed to get specific scFv for the others proteins. They suggested that these two libraries, which were randomized over short HCDR3 lengths of 5 or 8 a.a. residues and contained only one Lc, did not contain a large enough repertoire. Short HCDR3 might create pocket-like binding sites specific for haptens whereas large HCDR3 create flat surfaces specific for proteins (Hoogenboom, H. R., Winter, G., 1992).

Another group worked extensively on semisynthetic repertoire of scFv fragments (de Kruif, J., and al., 1995; de Kruif, J., et al., 1995). They created a human library of $3.6 \times 10^8$ different clones. This library was made of 49 human germline $V_H$ genes with synthetic HCDR3 varying over 6 and 15 residues and 7 different Lcs. The HCDR3 were randomized over stretches of a.a. residues flanked by regions of limited a.a. variability that were encountered frequently in natural antibodies. The rationale behind this approach is that within HCDR3, some residues are more variable than others. Fully randomized HCDR3 may not be suitable because only a small proportion of the synthetized antibodies will be represented in a library due to the efficiency of transformation that limits the size of the libraries. The same library was also used by Boel, E., et al. (1998). Competitive selection was performed in the presence of a complement sensitive strain of *Moraxella catarrhalis* as a competitor to enrich for phage directed against differentially expressed structures on two different strains of the same bacterial species.

They obtained 10 different phage-antibodies with specificity for complement-resistant but not for complement sensitive strain, recognizing the high-molecular weight outer membrane protein (HMW-OMP). The same semi-synthetic library was also used by Van Ewijk, W., et al. (1997). A subtractive approach was used to select scFv with binding properties against thymic stroma which was used as a target tissue and lymphocytes and splenocytes as absorber cells.

The group of Griffiths et al. (1994) published the isolation of human Fab fragments from a large semisynthetic library ($6.5 \times 10^{10}$). They surprisingly isolated Fab fragments with specificities to a large range of antigens, including 5 haptens, 14 various foreign antigens, and 17 human antigens. To create a repertoire of that size, they used a method called combinatorial infection based on the Cre catalyzed recombination at the loxP sites. Bacteria containing a donor Hc repertoire cloned on a plasmid were infected with an acceptor Lc repertoire encoded on a phage vector. The two chains were then combined in the bacteria by the lox-Cre site-specific recombination system of P1 bacteriophage to bring together the Hc and the Lc into the same replicon (Sternberg, N., and Hamilton, D., 1981; Hoess et al., 1982). They obtained semisynthetic scFvs with specificities against a broad range of antigens (13 different antigens) including haptens such as DNP and proteins such as ICAM-1 and the Von Willibrand factor (VWF). Affinities obtained with this system ranged from 2μM to 100nM. They did selection in the presence of competitor antigens in order to help direct the specificity toward a particular region of an antigen.

The same group published an interesting report where they selected semisynthetic scFv fragments specific for cell subpopulation (De Kruif, J., et al., 1995). They isolated fragments specific for subpopulation of an heterogeneous mixture of blood leukocytes by flow cytometry, by incubating

peripheral blood leukocytes with the semisynthetic phage antibody library (De Kruif, J., et al., 1995) and fluorochrome-labeled CD3 and CD20 antibodies. This approach might be useful in obtaining human antibodies against cell surface antigens in their native conformations. They also suggest that the nonselected cells in the mixed population appeared to absorb nonspecific phage providing a substractive procedure to target specific antibodies and discarded phage that bind to common epitopes.

Another semi-synthetic antibody library was constructed (Braunagel, M., and Little, M., 1997) using trinucleotide oligos. The HCDR3 was encoded by a random 24bp oligonucleotide sequence synthetized from a mixture of presynthetized codons, where a precise ratio of amino acids can be achieved. The repertoire complexity was of $8x10^8$ independant clones. Binders where obtained against 3 haptens conjugated to BSA, DNP (dinitrophenol), FITC (fluorescein isothiocyanate), and NIP (3-nitro-4-hydroxy-5-iodophenylacetic acid).

## VI. Potential advantages and limitations of phage display.

Any new technology has the ability of improving or complementing an already existing methods but rarely to completely replace it. The main or most important contributions of phage-antibody display technology will be discussed in the following section. The limitations of that approach will also be presented.

## 1. Advantages.

Phage-antibody display technology offers the ability of sorting large libraries by powerful enrichment and selection methods that allow for the ready screening of up to $10^{12}$ phage-antibody in contrast to the conventional monoclonal antibody method which is limited to the screening of a small proportion of the repertoire (approximately $10^3$ clones) that can be examined.

If the library selected is from a non-immunized source or is a synthetic repertoire, phage-antibody display bypasses immunization and the use of animals.

One of the most interesting contributions of phage-antibody display is the potential to isolate antibodies to nonimmunogenic self-molecules or to highly conserved proteins. This may provide new therapeutic opportunities for cancer treatment, immunosuppression, anti-inflamation therapy, regulation of disease states, but also for the development of reagents for magnetic resonance imaging, delivery of radioisotopes and purification of recombinant proteins (Barbas, C.F., Lerner, R. A., 1990), diagnostic reagents and drug-discovery tools, and to block viral infection where vaccination is not possible (Hodits, R. A., et al., 1995; Burioni, R., et al., 1994).

Another advantage of phage-antibody display is the stability of the cDNA which can be used as a continuous source of antibody production by *E. coli* whereas hybridomas are often unstable. Because the genes coding for the antibody fragment have already been cloned in the phagemid vector, it is rapidly available for further manipulations by genetic engineering or conversion of the antibody fragment to full length immunoglobulins.

Also, because there is no need for a purified form of the antigen to which antibodies are selected against, it is possible to isolate antibodies against novel antigens which have yet to be identified or characterized. Even cell surface antigens in their native configurations can be directly used in the selection with intact cells (Cai, X., and Garen, A., 1995; de Kruif, J., and al., 1995; Zhang, H., et al., 1995).

Furthermore antibody fragments produced by phage display are less immunogenic than murine antibodies or monoclonal antibodies that are mouse-human chimereas (humanized antibodies) and might be better as therapeutic agents. Also as reported by Bruggeman et al. (1996), phage-antibody display might be useful in isolating antibodies against antigens that are unstable under physiological conditions of the serum. Consequently hardly achievable by conventional immunization because of the impossibility to immunize with such antigens.

## 2. Limitations.

Phage-antibody display technology has several limitations, some of them inherent to the system itself and some limitations that could be solved with a better knowledge and understanding of that technology.

The phage titer in a culture of *E. coli* will reach up to $10^{12}$ pfu/ml, so the bigger a library is in terms of diversity (the number of different members in that library), the fewer the number of each member is represented in the library. Consequently a particular binding clone has a good chance of being lost and never be recovered in the first round of panning. Low stringency has

to be used in the first round. The diversity of a library is also limited by the efficiency of bacterial transformation. This can be partly solved by a system employing combinatorial infection by the lox-Cre site-specific recombination (Griffiths, A. D., et al., 1994). The diversity can also be affected by the choice of primers used for the construction of the library, therefore some families of sequences might be absent.

The $V_H$ and $V_L$ pairing being random, the original pairing selected for high-affinity by immunization is lost, but is likely to be present in large random libraries. Unfortunately, it is impossible to differentiate original from artificial pairings.

One major problem of phage-antibody display technology is not inherent to this particular system but mostly to all bacterial expression systems. Problems can occur due to incorrect expression, folding and transport in *E. coli*. The antibody fragment has to be successfully expressed and folded properly on the phage surface to allow binding to the antigen. These problems are not readily solved due to the uniqueness of each antibody fragment in a library. Conditions have to be set so the proportion of expressed and properly folded antibody fragments is maximal. Comparison of expression of different forms of antibody fragments of the same antibody have shown that the Fab expression yield is always lower than that of Fv or scFv fragments in *E. coli* (Pluckthun, A., 1991). It has been suggested that the periplasmic folding process is not as efficient for larger fragments such as Fab compared to Fv or scFv (where the two chains are linked by a linker) fragments.

Even if the expression and folding of the antibody fragment is properly achieved, many of the phage are "bald" (no antibody fragment is expressed at

the phage surface) due to their proteolysis by the proteases of *E. coli*. Filamentous phage pIII is resistant to *E. coli* proteases but it is not the case for antibody-pIII fusions. Hc alone or Hc-Hc pairing is also found at the surface of phage in the case of Fab expression systems.

Another serious difficulty with phage-antibody display is the instability of the libraries. The host cells tend to diminish the stress caused by the expression of the protein fusion by eliminating the plasmid, or the genes or part of the antibody fragment by plasmid rearrangement or mutation. This can result in the surface expression of only part of the antibody fragment and such deletants are often "sticky" (Method in enzymology vol. 267, 1996). It is then difficult to select for a rare clone when its frequency is lower than the nonspecific background binding to the antigen (Rapoport, B., et al., 1995). One of the consequence is that there will be outgrowth of phage-antibody that have lost all or parts of the gene because the amplification is in solution. Deletions have been found to happen more often with Fab than scFv systems (Method in enzymology vol 267, 1996) probably because in Fab constructs the Lc is not fused to the pIII and can be deleted.

The isolation of phage-antibody, from semi-synthetic repertoires, specific for proteins has proven more difficult to achieve (Hoogenboom, H. R., and Winter, G., 1992) probably because of a lack of structural diversity in the libraries which are not broad enough to get any possible specificity and because of the larger size of a protein compared to small antigens such as haptens and peptides (where you can get a higher concentration of antigen for the same surface of selection).

Synthetic repertoires can bypass the problem that occurs with libraries constructed from PBLs or splenocytes where there is a bias against self-antigens and intracellular or tumor antigens (Molecular Immunology, second edition).

If the biological function of an antibody is needed, phage-antibody display technology might not be the method of choice. Antibody fragments have no effector function due to the lack of the Fc portion and the lack of glycosylation pathway in *E. coli* unless it is engineered into it after selection process to get a full length immunoglobulin.

## VII. Pathway for Fab assembly in *E. coli.*

### 1. Filamentous M13 bacteriophage.

M13 bacteriophage are filamentous bacterial viruses that infect F+ strains of *E. coli*. M13 phage are long and cylindral viral particle with a size of approximately 1-2 μm in length and 6-7 nm in diameter, but the length is dependant on the size of the ssDNA that is encapsidated in a particle. Phage genome encodes for 10 different proteins, 5 of which are structural capsid proteins, 3 that are involved in protein synthesis, and two that are needed for assembly purposes. One end of the phage particle attaches to the tip of F pilus of the host via the pIII protein. The coat proteins can be reutilized by the newly formed phage as they are deposited on the cytoplasmic membrane of the host cell when the phage particle is stripped. The ssDNA is converted to dsDNA when entered into the cell by the host synthesis apparatus. This

dsDNA called replicative form (RF) is used as a template for the transcription and expression of the phage proteins. The pII gene product specifically nicks one strand of the RF, and elongation proceeds from the 3' terminus created by displacing the strand. This displaced strand is then cut and circularized. The new ssDNA is either used as a RF or is covered by a ssDNA binding protein (pV) which is available for virion assembly. Assembly of the phage particles takes place at the host membrane. The pV proteins are stripped off and replaced by the structural coat proteins (localized at the membrane of the host cell) as the phage particle is extruded through the cell envelope.

Two structural proteins are involved in phage display, being the products of genes III and VIII. They have exposed N-terminal domains that tolerate foreign inserts. The phage genome also encodes for a region called the intergenic region which is inserted into phage display vectors. This region contains the DNA origin of replication, the packaging signal, and the termination of RNA synthesis. The fusion protein of interest for the pComb 3H system is the pIII gene product which is a 42 kD protein expressed as a number of 3-5 copies at the tip of the virion particle. Monovalent display system tolerates bigger foreign inserts than multivalent display as they interfere with pIII functions such as infection, assembly, and structural stability (Smith, G.P., 1993). This protein is essential for infectivity. The C-terminal portion of pIII is anchored into the membrane of the virion capsid and also to the membrane of the host cell (before assembly of the virion). The N-terminal portion of pIII is important for infectivity of host cells by binding to the F-pili (Model, P., Russel, M., 1988). The pIII protein is a flexible protein, because of this and also its exposure at the end of the phage particle it is an excellent fusion partner for phage display (Makowski, L., 1993).

## 2. Assembly of Fab fragment into the periplasm of *E. coli.*

Figure 4a shows how the Fab fragments are directed to the periplasm of the host cells and assembled. The phagemid vector pComb 3H encodes for both Lc and Hc/pIII fragments which are respectively expressed as fusions with the ompA and pelB leader peptides. These leader peptides direct both chains of the Fab fragment separately to the periplasmic space of the host cells. The Lc is secreted in the periplasm whereas the Hc/pIII fusion is anchored to the inner membrane of the host cell by the C-terminal portion of the pIII protein. The leader peptides are cleaved upon reaching the periplasm. Phagemid vectors do not encode for phage proteins needed for replication, assembly, and native pIII proteins needed for infection of host cell. A helper phage is necessary to produce phage particles (figure 4b). Helper phage VCS M13 is modified to have a defective origin of replication which result in a packaging that is less efficient for helper phage than for phagemid DNA. Using a phagemid system over cloning directly into the phage genome has the advantage that the valency of the expression of Fab fragments can be controlled to have monovalent display. Monovalent display increase the ability to distinguish between low and high affinity, therefore better selection can be achieved (Levitan, B., 1998).

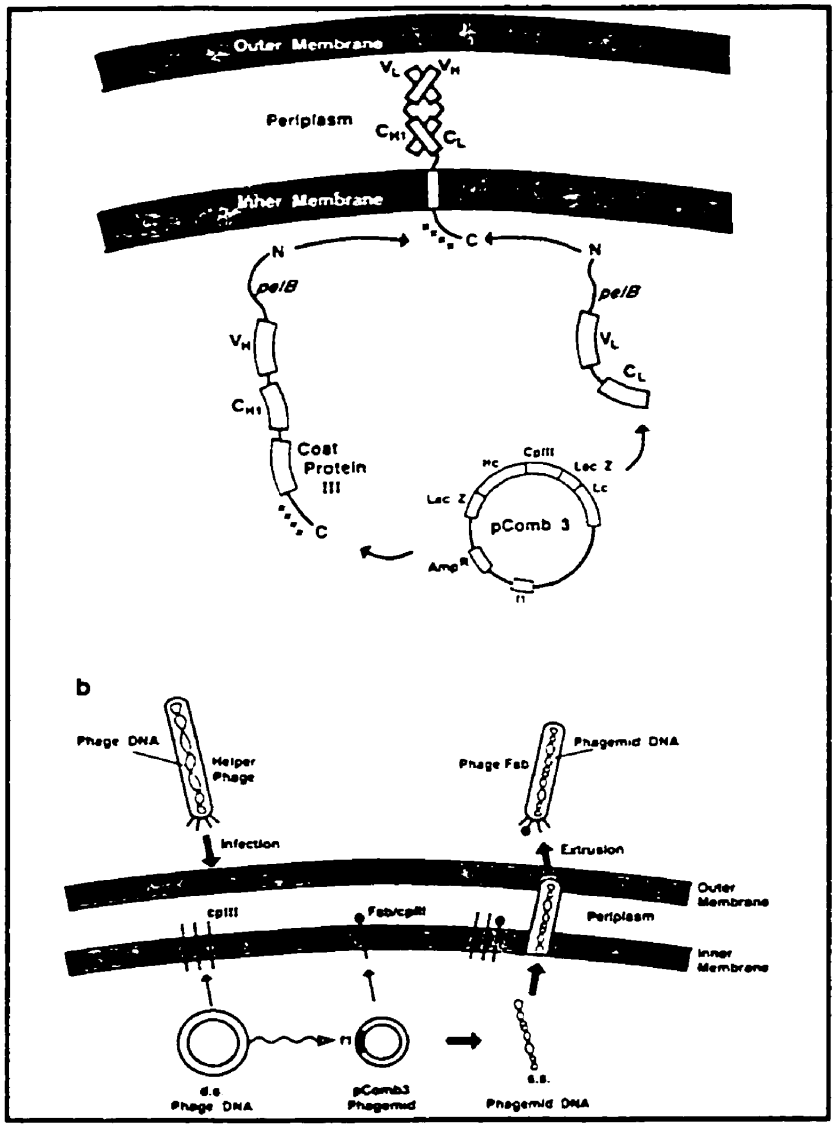



**Figure 4. Fab assembly in *E. coli.*** (Barbas, C.F., Burton, D.R., 1994).

**A) Pathway for Fab assembly:**

- Lc and Hc/pIII fusion are directed to the periplasmic space by pel B or omp A signal sequences.
- The Hc is anchored to the membrane by the pIII fusion and the Lc is secreted into the periplasm. The 2 chains then assemble.

**B) Helper phage rescue of phagemid to give a phage display library:**

- pComb3H needs a helper phage to provide the phage proteins necessary for replication and assembly and native pIII necessary for infection.
- Helper phage DNA is poorly packaged in competition with the phagemid due to a defective ORI.

## VIII. By-passing immunization to develop $\beta_1$ integrin specific antibodies.

It is known that monoclonal antibodies against self-antigens or proteins highly conserved between species are difficult to raise using conventional immunization. Moreover hybridoma technology tends to be less effective in the production of human monoclonal antibodies. There is a bias toward epitopes with highly diverse sequences using methods based on immunization and a bias against less divergent sequences. Consequently, these conserved regions which may have an important role or significance are not as well understood or studied because of the frequent failure to raise antibodies that might be useful for the understanding of the function of such molecules. Semisynthetic repertoire is then a method of choice to produce human monoclonal antibodies specific for conserved proteins.

### 1. $\beta_1$ integrin.

Control and regulation of $\beta_1$ integin are being investigated in Dr J. A. Wilkins' laboratory. Integrins are adhesion receptors that mediate cell-ECM and cell-cell adhesion. They are conserved proteins involved in tissue morphogenesis, tissue integrity, development, inflamatory response, control of cell growth, and cell motility (Gumbiner, B. M., 1996; Lauffenburger, D. A., 1996). Integrins are heterodimeric transmembrane proteins in which one of the $\alpha$ chains is associated non-covalently with one of the $\beta$ subunits (Gumbiner, B. M., 1996; Lauffenburger, D. A., 1996) (Figure 3 and 4). Integrins have a large extracellular domain, a membrane domain, and a short cytoplasmic domain, the last being involved in integrin-specific

signalling probably by interacting with intracellular components (Hynes, R. O., 1992). It is also thought that highly conserved regions of the integrins play an important role in their function (Leung-Hagesteijn, C. Y., 1994).

In Dr J. A. Wilkins laboratory, a panel of stimulatory and inhibitory antibodies have been developed over the past years against $\beta_1$ integrin on the T leukemic cell line Jurkat (Stupack, D. G., et al., 1991; Gao, J. X., et al., 1995; Wilkins, J. A., 1996). One inhibitory antibody called JB1A inhibits adhesion of $\beta_1$ integrin to fibronectin, which is an ECM protein. JB1A antibody recognize a continous epitope of the $\beta_1$ chain spanding from amino acid residue 82 to 87. The amino acid sequence of this epitope is TAEKLK. This epitope was determined by screening a $\beta_1$ epitope library (Novatope system, Novagen Inc.) and by selecting from a 15 mer random phage peptide library (Chiron Corporation). This epitope, being well studied in Dr J. A. Wilkins laboratory, was thought to be an ideal target for establishing the semisynthetic phage-antibody library system in the laboratory. By establishing this system in the laboratory and proving its feasibility, it might be possible in the near future to develop novel monoclonal antibodies against conserved regions of the $\beta_1$ integrin or other molecules involved in the inflammatory response (at the site of inflammation) for the early detection of RA directly on biopsie taken from patient, as an example. Selection on purified $\beta_1$ integrin from human placenta has also been attempted.

## 2. Description of the semi-synthetic antibody human libraries.

To achieve this goal, we were generously provided with 7 semisynthetic phage-antibody human libraries from Dr Carlos F. Barbas III (department of molecular biology. The Scripps Research Institute, La Jolla, California). These libraries are namely V13/V1G, V13/V3G, V13/8H, V13/Si#1, V7/V3G, V7/8H, and V7/Si#1. The sequence of the $V_H$ of the libraries labelled Si#1 was published by Barbas, S. M., (1995). The others libraries are from libraries of $V_H$ genes with HCDR3 randomized over 7 or 13 a.a. residues. The libraries possessed a single universal unmutated Lc, the Humkv 325 germline sequence which is over-represented in the repertoire of Lc (Burton, D. R., Barbas, C. F., 1994). These Lc were cloned in the human expression vector pComb 3H. The libraries have a diversity greater to 1 x $10^8$. These Fab libraries are unpublished at this time explaining why only partial information is available. The HCDR3s of these libraries contain all amino acids with the exception of cysteine. The CDR3 were completely randomized using a synthetic oligonucleotide with an NNS doping strategy (Scott, J. K., Smith G. P., 1990). N is any of the 4 nucleotides (G,A,T,C) and S is G or C. NNS oncodes all 20 amino acids. The lack of Cys prevents the formation of non-desirable intra-CDR3 disulfide bounds.

## 3. Human expression vector pComb 3H.

The vector used for the construction of the libraries is pComb 3H (Figure 5). PComb 3H is a phagemid vector derived from pBluescript (Stratagene) specially designed for the expression of human Fab fragments. PComb 3H contains two origins of replication, one from the plasmid Col E1

and one from the filamentous bacteriophage f1. PComb 3H contains the gene for the enzyme beta-lactamase which confers resistance to ampicillin and carbenicillin. It provides as well a human consensus sequence to the amino-terminus of the Hc which sequence is EVQLLE. Leader sequences for transportation of the Fab fragment to the periplasm is also provided. The Lc is fused at the carboxy-terminus of the OmpA gene (*E. coli* outer membrane protein A leader peptide) and the Hc fragment is fused to the carboxy-terminus of the PelB gene (*Erwinia cavotora* peptate lyase leader peptide). The Hc fragment is also fused to the amino-terminus of the carboxy-terminal domain of the gene III of filamentous phage coat protein.

## 4. Strategy of selection.

The strategy employed to select specific Fabs from the semi-synthetic antibody libraries is shown in figure 6. The libraries were first preadsorbed with soluble avidin in an attempt to divert the specificity toward the JB1A peptide. Low stringency was used in the first panning round to decrease the chance of losing rare positive binding phage-antibodies. The stringency of washes was increased in further rounds of selection. After the final washing step, the phage that remained bound to the immobilized antigen were eluted in a two-steps procedure. They were first eluted with low pH buffer, then secondly host cells XL1-Blue were added directly to the panning wells to elute the phage that could not be eluted with low pH. For the selection on purified $\beta_1$ integrin, no preadsorption step was performed. Several rounds of panning were done in a attempt to increase the proportion of highly specific clones.

**pComb 3H**

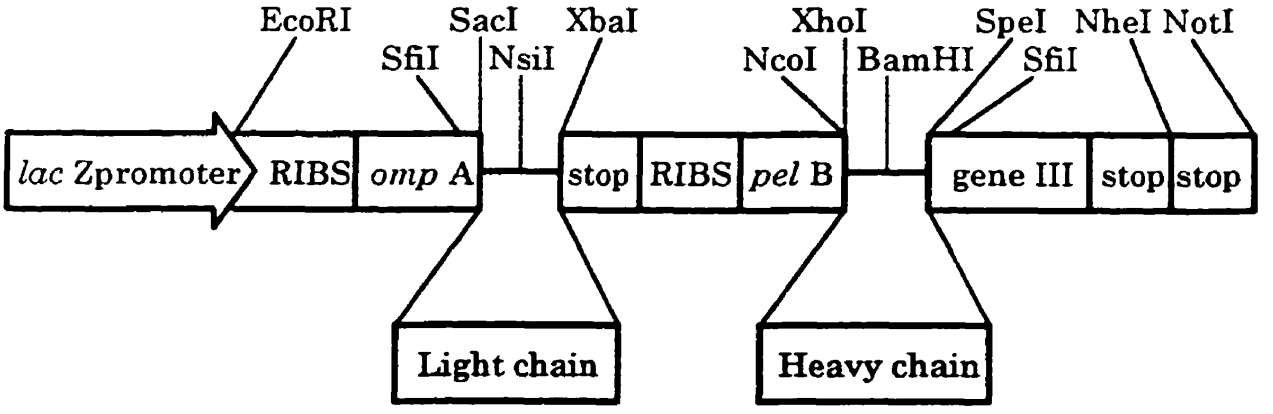



**Figure 5. Human expression vector pComb 3H.**

- Provides a human consencus sequence to the amino terminus of the Hc.
- Contains ORI of replication of plasmid ColE1 and of the filamentous bacteriophage f1 ( IR ).
- Carbenicillin resistance ( beta-lactamase gene ).
- Pel B gene ( *Erwinia cavotora* pectate lyase ).
- Omp A gene ( *E. coli* outer membrane protein A ).
- Carboxy-terminal domain of the gene III.

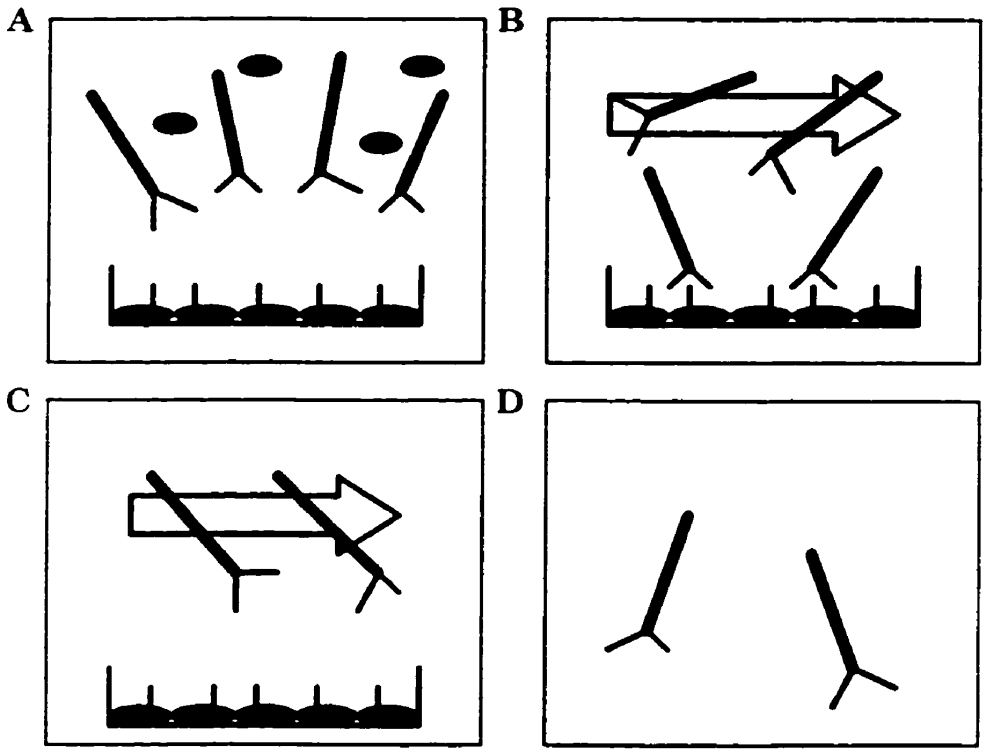



**Figure 6. Strategy of selection selection of specific Fabs from semisynthetic antibody libraries.**

A) Immobilized Ag is exposed to the phage display library.
B) Specific phage-Fabs bind and the rest of the phage are removed by washing.
First panning -> 1 wash
Second -> 5 washes
Third and subsequent -> 10 washes
C) Specific phage-Fabs are eluted with 0.1M HCl pH 2.2 and XL1-Blue cells.
D) Isolation of specific phage-Fabs.
E) Production of soluble Fabs.

- Process A-D is repeated for 7 rounds of panning.

Amplification of eluted phage-Fabs by infecting XL1-Blue host cell and rescue with VCSM13 helper phage.

avidin | JB1A biotinylated peptide (B-SGSGTAEKLK)

# MATERIAL AND METHODS

## I. Preparation of host cells XL-1 Blue.

### 1. Electrocompetent *E. coli* XL-1 Blue.

*E. coli* XL-1 Blue strain was purchased from Stratagene ( La Jolla, California, USA ).

Electrocompetent *E. coli* XL-1 Blue were produced using the method outlined in the manual prepared for the 1994 Cold Spring Harbor Laboratory course on: monoclonal antibodies from combinatorial libraries ( Barbas CF III, Burton DR, 1994 ).

A single colony from an LB plate ( Tet 40ug/ml ) was inoculated into 10ml of SB ( Tet 40ug/ml ) and incubated on a shaker overnight at 37°C. The next morning, 2.5ml of this 10ml culture was inoculated into 500ml of SB containing 10ml of 20% sterile glucose and 5ml of 1M sterile $MgCl_2$ and incubated for approximately 4 hours at 37°C on a shaker until an $OD_{600}$ of 0.700 to 0.800 was reached, the culture was chilled on ice for 15 minutes and then centrifuged at 2,691g ( Sorvall RC-5B, GSA rotor ) for 20 minutes at 4°C. The supernatant was poured off and the bacteria were resuspended in 1/2 volume of 10% glycerol in distilled water ( chilled at 4°C ). After resuspension, the cells were centrifuged at 2,691g for 20 minutes at 4°C and supernatant was poured off. The resuspension and centrifugation steps were repeated a second time. Once the supernatant was remouved the bacteria

were resuspended in 12.5ml of 10% glycerol in distilled water and transferred to a 50ml tube chilled on ice. The bacterial resuspension was centrifuged at 1628g for 15 minutes at 4°C. The supernatant was poured off as much as possible without losing any of the bacterial pellet and the pellet was resuspended in the residual glycerol solution left after pouring of the supernatant. 40ul aliquots were immediately placed in Ependorf tubes on ice. The tubes were frozen in liquid nitrogen then stored at -80°C ( variant from the original protocol where cells were frozen in EtOH/dry ice bath ). It is important to work quickly and always on ice.

## 2. Preparation of a -80°C bacterial glycerol stock of *E. coli* XL-1 Blue.

Protocol as proposed by Stratagene ( La Jolla, California, USA ).

1 or 2 colonies were inoculated from a LB plate ( Tet 12.5ug/ml ) into 10ml of LB ( Tet 12.5ug/ml ) and grown on a shaker until the culture reached an $OD_{600}$ of 1.0 to 2.0. 4.5ml of glycerol-media solution was added ( 5ml glycerol + 5ml LB ( Tet 12.5ug/ml )) to the bacterial culture. The cells were aliquoted into 500ul volumes into Ependorf tubes and frozen in liquid nitrogen. The tubes were then stored at -80°C.

## II. VCSM13 helper phage.

### 1. VCSM13 helper phage propagation.

VCSM13 helper phage was purchased from Stratagene ( La Jolla, California, USA ).

The following method is based on a modified protocol for M13K07 helper phage propagation from Pharmacia; molecular biology division.

VCSM13 helper phage was streaked on a B agar plate. Then 4ml of B top agar warmed up to 45°C containing 500ul of a culture of XL-1 Blue cells ( $OD_{600}$ > 0.800 ) was poured on the plate from the dilute side of the streak toward the more concentrated one. The plate was incubated for 6 to 12 hours at 37°C until plaques were visible. Areas of closely spaced single plaques were scraped into 30 to 200ml of 2XYT media ( Kana 70ug/ml ) in a conical plastic bottle. Then the culture was incubated overnight at 37°C on a shaker at 280 rpm. The next morning, the bacteria were pelleted by centrifugation at 13,290g ( Sorvall RC-5B, GSA rotor ) for 20 minutes, centrifugation was repeated until no more pellet was produced. Then the supernatant was heated at 55°C for 30 minutes ( variant from the original protocol ).VCSM13 helper phage was stored in 1ml aliquots at 4°C. This supernatant can be utilized as inoculum for more than 1 year.

## 2. Titration of VCSM13 helper phage.

Based on a protocol from Promega Protocols and applications guide ( 1989/90 ).

100ul of VCSM13 helper phage was added to 9.9ml of B broth. 5 serial 100x dilutions were made from this 10ml. 0.1ml of each of the 5 dilutions were added to 0.2ml of log phase XL-1 Blue cells and incubated at either RT or 37°C for 5 to 30 minutes. After infection, 4ml of B top agar, 45°C, was added to each tube and poured on B plates. The plates were incubated overnight at 37°C. The following day the number of plaque forming units/ml (pfu/ml ) was calculated.

dilution 1: $1/10^2$
dilution 2: $1/10^4$
dilution 3: $1/10^6$
dilution 4: $1/10^8$
dilution 5: $1/10^{10}$

Sample calculation: if 3 plaques were found on the dilution 5 plate, the titer would be $3 \times 10^{11}$ pfu/ml. $3 \times 10^{10}$ ( dilution 5 ) x 10 ( converts the 0.1ml plated to a per ml basis ).

## III. Selection for specific phage-antibodies by panning.

### 1. Phage-antibody libraries amplification.

The libraries and the protocol were provided by Carlos F. Barbas III ( The Scripps Research Institute, La Jolla, California, USA ).

Libraries V13/V1G, V13/V3G, V13/8H, V13/Si#1, V7/V3G, V7/8H, and V7/Si#1 were amplified by infecting 10ml of freshly cultured XL-1 Blue cells ( in SB Tet 10ug/ml until it reached $OD_{600}$ = 1.0 ) with 20ul of each libraries separately. Phage were allowed to infect bacteria for 15 minutes at RT or 37°C, then carbenicillin was added to a concentration of 20ug/ml. The infected cultures were incubated on a shaker for 1 hour at 37°C, then added to 200ml of prewarmed SB ( Carb 50ug/ml and Tet 10ug/ml ). The cultures were incubated for another hour on a shaker, and 1ml of $10^{11}$ to $10^{12}$ pfu/ml of VCSM13 helper phage was added afterward. The cultures were then shaken for 2 hours and kanamycin was added to a concentration of 70ug/ml. The cultures were shaken overnight at 37°C.

### 2. Phage-antibody libraries precipitation.

Precipitation was done using the method outlined in the manual prepared for the 1994 Cold Spring Harbor Laboratory course on: monoclonal antibodies from combinatorial libraries ( Barbas CF III, Burton DR, 1994 ).

Bacteria were pelleted by centrifugation at 2,691g ( Sorvall RC-5B, GSA rotor ) for 15 minutes at 4°C. Supernatant was transferred to a bottle and 4% ( w/v ) PEG-8000 and 3% ( w/v ) NaCl was added and dissolved properly. Phage were precipitated on ice for 30 to 60 minutes. Phage were pelleted by centrifugation at 10,760g ( Sorvall RC-5B, SS-34 rotor ) for 20 minutes at 4°C and supernatant was removed. Bottles were allowed to drain for 10 minutes on paper to remove as much PEG as possible because trace amounts of PEG have been thought to interfere with the binding of phage to various targets (Kay, B.K., Winter, G., and McCafferty, J., 1996). Phage pellets were resuspended in 2ml of 1% BSA in TBS and centrifuged for 5 minutes at 16,000g in a microcentrifuge. Supernatants were stored at 4°C with 0.02% $NaN_3$ or at -80°C with DMSO at a final concentration of 7%. For panning purposes, phage pellets were resuspended in 300ul of 1% BSA in TBS.

## 3. Phage-antibody libraries replication.

Replication was done following the method outlined in the manual prepared for the 1994 Cold Spring Harbor Laboratory course on: monoclonal antibodies from combinatorial libraries ( Barbas CF III, Burton DR, 1994 ).

2ml of freshly cultured XL-1 Blue ( $OD_{600}$ =1.0 ) were infected with the eluted phage from the previous round of panning and incubated at 37°C for 30 minutes to allow infection. For the first round of panning about 1 to 5 x $10^{10}$ pfu of each libraries were added separately to 10ml of fresh XL-1 Blue for infection. After infection, 10ml of prewarmed SB ( Carb 20ug/ml and Tet 10ug/ml ) was added and incubated for 1 hour at 37°C on a shaker. Then

carbenicillin concentration was adjusted to 50ug/ml and incubated for another hour at 37°C on a shaker. Aproximately 5 x $10^{11}$ pfu VCSM13 helper phage was added and the cultures were transferred to a prewarmed 100ml of SB ( Carb 50ug/ml and Tet 10ug/ml ) and shaken for 2 hours at 37°C. Kanamycin was added to a final concentration of 70ug/ml and the cultures were shaken overnight at 37°C. The following morning phage were precipitated as previously described. For the first round of panning each libraries were reamplified separately, and for the second round eluates from the first selection were combined.

## 4. Coating of wells for panning.

Biotinylated peptide was purchased from Chiron, mimotopes peptide systems ( San Diego, California, USA ). Avidin was purchased from Sigma ( St-Louis, Maryland, USA ).

100ul of avidin ( 5ug/ml $H_2O$ ) was first added to wells of a microtiter plate ( F96 Maxisorp Nunc-Immuno plate ) and dried at 37°C until water evaporated. The wells were washed 3x with 0.05% Tween 20 in PBS ( PBST), and then blocked with 3% BSA in PBS, then rinsed with PBS. After blocking overnight at 4°C or 2 hours at 37°C, 100ul of biotinylated peptide was added. The sequence of the peptide used is B-SGSGTAEKLK. This peptide was resuspend in water, 1mg/ml, and diluted in PBS, 0.1% BSA, 0.1% $NaN_3$ to a final concentration of 5ug/ml. Biotinylated peptide coating was done for 1 hour at RT. Coated wells were then washed 4x with PBST 0.05%. For the first round of panning 2 wells per library were coated and, for the subsequent pannings 2 wells were coated per round.

## 5. Panning.

Panning selection was done using the method outlined in the manual prepared for the 1994 Cold Spring Harbor Laboratory course on: monoclonal antibodies from combinatorial libraries ( Barbas CF III, Burton DR, 1994 ).

300ul of freshly reamplified phage library was incubated with 100ul of 5ug/ml avidin for 30 minutes at RT, then added to 2 wells ( 200ul per well ) and incubated for 2 hours at 37°C. Unbound phage were removed and wells were washed with TBST 0.05% by pipetting vigorously up and down and incubated for 5 minutes before removing washing solution. For the first round of selection wells were washed once, second round 5x, third and subsequent 10x. After the washing step, bound phage were eluted twice with 50ul of 0.1M HCl pH2.2 with BSA 1mg/ml per well. Eluate was neutralized with 3ul of 2M Tris.base per 50ul of elution buffer. Fresh XL-1 Blue cells ( $OD_{600}$ = 1 ) were also added to the panning wells for 1 hour at 37°C to allow infection by the remaning phage.

## 6. Titration of phage isolates.

Titration of the amplified phage before the panning step ( input ) and of the eluted phage ( output ) was done by infecting fresh XL-1 Blue cells ( $OD_{600}$ = 1.0 ) with phage then doing appropriate 10x serial dilutions and plating them on LB plates ( Carb 50ug/ml ). The number of colonies were counted the next morning and the number of colony forming units/ml ( cfu/ml ) was calculated.

## IV. Characterization of isolates.

### 1. Microtiter plate rescue of phage-antibody clones.

100ul of SB medium ( Carb 50ug/ml, Tet 10ug/ml ) was added to each well of a 96-well microtiter V bottom plate. Individual colonies were transferred to separate wells with sterile toothpicks and transferred also to another SB plate ( Carb 50ug/ml ) kept as an original stock. The microtiter plate was incubated overnight at 37°C on a shaker ( 150 rpm ). The next morning, 200ul of SB containing Carb 50ug/ml, Tet 10ug/ml and VCSM13 helper phage ( about $5x10^9$ pfu/ml of SB ) was added to each well of a second V bottom plate. Then 20ul of saturated cultures were transferred to the second plate and incubated for 2 to 4 hours ( until the cultures appeared turbid ). Kanamycin 70ug/ml was added and the plate was shaken overnight at 37°C ( 150 rpm ). The next day, the cells were spun down for 10 minutes at 480g ( IEC Centra-7 centrifuge, International Equipment Company, Needham Heights, Mass., USA ). The supernatants were transferred to a blocked plate and assayed for binding by ELISA.

### 2. ELISA for antigen binding phage.

The detection module; recombinant phage antibody system was purchased from Pharmacia ( Uppsala, Sweden ).

$\beta_1$ integrin was purified by Dr. John Wilkins from human placenta on JB1 affinity column ( 2 ).

Avidin was coated into wells of a microtiter plate ( F96 Maxisorp Nunc-Immuno plate ) as specified above. Peptides were either coated as specified before or by drying the wells at 37°C. $\beta_1$ integrin was coated by diluting it in coating buffer ( 0.795g $NaCO_3$, 1.465g $NaHCO_3$ per 500ml $H_2O$ pH 9.6 ) and was incubated overnight at 4°C. Wells were then blocked with 3% BSA in PBS, 2% skim milk,or 1% BSA/2% skim milk/0.5% gelatin for 2 hours at 37°C or overnight at 4°C. In a separate preblocked plate or tubes, phage-antibodies were mixed with an equal volume of blocking solution and incubated at RT for 15 to 30 minutes. 200ul of the diluted phage-antibodies were added to the antigen-coated wells. 200ul of M13K07 or VCSM13 helper phage diluted 1:1000 in PBS was also added as a control. The plate was incubated for 2 hours at 37°C. At the same time 50ul of phage-antibodies were added to non-coated wells to see the amount of production and incubated for 1 to 2 hours then blocked with blocking solution for 1 to 2 hours at 37°C. The plates were washed 5x with PBST 0.05% using a squeeze bottle or an ELISA plate washer. Plates were blotted dry on paper to remove any remaining wash buffer between each washing step. HRP/anti-M13 conjugate was diluted 1:5000 3% BSA in PBS and 200ul was added to each wells. Plates were incubated for 1 hour at 37°C. The plates were then washed 6 times as described above. Then the OPD substrate was prepared by adding 1ml of OPD 20x to 20ml citric buffer pH 5.4 and 20ul of 30% $H_2O_2$. 200ul of substrate was added per well and the plates were incubated at RT until a suitable color reaction occured. The reaction was stopped with 100ul of 10N $H_2SO_4$ per well. Optical Density was read in a microtiter plate reader set at 490nm substracting 690nm as background.

## V. Characterization of soluble Fab clones.

### 1. Conversion of pComb3H from phage display form to soluble Fab producing form.

Restriction enzymes SpeI, NheI and T4 DNA ligase were purchased from Gibco BRL, Life Technologies ( Gaitherburg, MD, USA ).

Generation of soluble Fab producing form was performed based on the method provided in the manual prepared for the 1994 Cold Spring Harbor Laboratory course on: monoclonal antibodies from combinatorial libraries ( Barbas CF III, Burton DR, 1994 ).

Single phage clones showing a positive signal on ELISA after selection were chosen for production of soluble Fab. Double stranded DNA from single clones was isolated then digested with SpeI and NheI restriction enzymes. The digested DNA was run on a 0.6% agarose gel electrophoresis. The band corresponding to the construct without the pIII gene was isolated by cutting a well in the gel in front of the band. The band was allowed to migrate into the well and DNA countaining TAE electrophoresis buffer was collected and EtOH precipitated. The purified band was self-ligated using T4 DNA ligase for 16 hours at 16°C then the enzyme was heat inactivated at 70°C for 10 minutes. The ligated DNA was then EtOH precipitated with 2ul glycogen and resuspended in 10ul of $H_2O$. The ligated product was transformed into 40ul of electrocompetent XL-1 Blue using an EC100 electroporator ( E-C Apparatus Corporation, St-Petersburg, Florida, USA ). The ligated product was mixed with 40ul of electrocompetent host cells and transferred into an

ice chilled cuvette and pulse in a 0.1cm electrode gap cuvette set at 1800V, 25mF and 220ohms. The cuvette was immediately flushed with 1ml of SOC medium and shaken ( 250 rpm ) for 1 hour at 37°C. Then the cells were plated on LB agar plates ( Carb 100ug/ml ) and incubated overnight at 37°C. The next day single colonies were ready to be picked for induction of soluble Fab production. Single colonies were innoculated into 10ml of SB ( Carb 50ug/ml, 20mM $MgCl_2$ ) and incubated on a shaker for 6 hours at 37°C. IPTG was then added at a final concentration of 1mM and the cultures were incubated overnight at 30°C. The next morning, cells were centrifuged at 1,320g for 20 minutes ( IEC Centra-7 centrifuge, International Equipment Company, Needham Heights, Mass., USA ). Cell pellets were resuspended in 1ml PBS. Cells were then lysed by freezing at -80°C for 10 minutes followed by thawing in a 37°C water bath. This process was repeated 4 or 5 times. Cell debris were pelleted by centrifugation at 16,000g in a microcentrifuge and supernatants were transferred to new tubes.

## 2. Plasmid DNA preparation ( miniprep ).

Bacterial cultures ( up to 10-20ml ) grown overnight were pelleted for 10 minutes at 850g ( IEC Centra-7 centrifuge, International Equipment Company, Needham Heights, Mass., USA ). Supernatant was discarded and any residual liquid removed by inverting the tubes on a paper. Cells were resuspended in 1ml TE buffer and transferred to Ependorf tubes, then pelleted again at 16,000g for 1 minutes in a microcentrifuge. The supernatant was discarded and pellets were resuspended in 200ul TE buffer and tubes were placed on ice. 200ul of lysis buffer ( 835ul $H_2O$, 125ul 10% SDS, 40ul 5N NaOH ) was added to each tube and mixed gently by inverting

the tubes several times and tubes were put back on ice. After 7 or 8 tubes , 150ul of 3M Na acetate ( pH 5.2 ) was added and mixed. Tubes were stored on ice for 30 minutes then centrifuged at 16,000g for 20 minutes in a microcentrifuge. Pellets were removed with sterile toothpicks. 1ml 100% EtOH was added and tubes were stored at -20°C for at least 1 hour. DNA was then pelleted at 16,000g for 25 minutes and supernatant was discarded. DNA pellets were rinsed with 70% EtOH and air dried. The dried pellets were resuspended in 200ul of TE buffer and digested for 1 hour at 37°C with 5ul of RNAse A ( 10mg/ml ). A phenol/chloroform extraction was performed by adding 100ul each of phenol and chloroform, vortexing, and centrifuging for 5 minutes at 16,000g. The supernatant ( upper phase ) was transferred to another tube, and 200ul of chloroform, 4% isoamyl alcool was added and the tube vortexed, then centrifuged again 5 minutes. A 1/10 volume of Na acetate ( pH 5.2 ) and 2.5 volumes of 100% EtOH were added. The tubes were stored at -20°C for at least 1 hour. Then the DNA was pelleted, and pellets were rinsed with 70% EtOH and air dried. Pellets were resuspended in TE buffer and samples were analysed on a 0.6% agarose gel.

## 3. Adsorption of $\beta_1$ integrin.

This procedure was done due to the fact that the integrin preparation is contaminated with Ig which cause a strong background on ELISA.

Goat anti-human $F(ab')_2$ was purchased from Pierce ( Rockford, Illinois, USA ). Gammabind Plus Sepharose beads were purchased from Pharmacia ( Uppsala, Sweden ).

20ul of $\beta_1$ integrin was mixed with 110ul of coating buffer and 1ul of anti-human F(ab')$_2$ and incubated 2 hours at 37°C. This was then added to 20ul of Gammabind Plus Sepharose beads and rotated for 1 hour at RT. The beads were then pelleted by centrifugation at 3000 rpm in a microcentrifuge and supernatant was transferred to a fresh tube and diluted to 1ml in $\beta_1$ coating buffer. This cleared $\beta_1$ was used for ELISA using goat alkaline phosphatase-labeled anti-human F(ab')$_2$ to characterized the binding of soluble Fab clones.

## 4. Soluble Fab ELISA.

Goat anti-human F(ab')$_2$-alkaline phosphatase conjugate was purchased from Pierce ( Rockford, Illinois, USA ). Alkaline phosphatase tablets were purchased from Sigma ( St-Louis, Maryland, USA ).

Antigens were coated as described previously. Plates were also blocked as above. 50ul of freshly prepared Fabs were added to appropriate wells and incubated for 2 hours at 37°C. Plates were then washed 5 to 10 times with TBST 0.05% as described above. 50ul of anti-human F(ab')$_2$ diluted 1:1000 in 3% BSA in PBS was added to each well and the plates were incubated for 1 hour at 37°C. Plates were then washed 6 to 10 times with TBST 0.05%. 1 tablet of phosphatase substrate was dissolved in 5ml of alkaline phosphatase developing buffer and 50ul was added to each well. The plates were developed at RT and read in a microtiter plate reader set at 405 nm after 15, 30, and 60 minutes of reaction.

## VI. Immunoblotting.

### 1. SDS-PAGE electrophoresis.

Rainbow protein molecular weight marker ( 14,300-200,000 Daltons ) was purchased from Amersham Life Science ( Oakville, Ontario, Canada ).

SDS-polyacrylamide gel electrophoresis of proteins was done as described in Molecular Cloning, a laboratory manual, second edition ( Sambrook, Fritsch, Maniatis ). 10ul of soluble Fabs and 1ug of human IgG were run under reduced and non-reduced conditions on a 12% acrylamide gel using a Mighty Small II SE 250 gel apparatus ( Hoefer Scientific Instruments, San Francisco, California, USA ). The gel was run at 200 Volts until the dye reached the bottom.

### 2. Western blotting.

Semi-dry electrophoretic transfer of protein from SDS-PAGE to nitrocellulose membrane was done as described in Molecular Cloning, a Laboratory Manual, second edition ( Sambrook, Fritsch, Maniatis ). Proteins were transferred for 1 hour or more at 60mAmperes using a LKB 2117 Multiphor II Electrophoresis Unit ( Sweden ).

**3. Detection with alkaline-phosphatase-labeled antibodies.**

Goat anti-human γ chain, goat anti-human λ chain, and goat anti-human κ chain were purchased from Chemicon ( Temecula, California, USA ). Anti-goat IgG-alkaline phosphatase conjugate was purchased from Sigma ( St-Louis, Maryland, USA ). Goat anti-human $F(ab')_2$-alkaline phosphatase conjugate was purchased from Pierce ( Rockford, Illinois, USA ). Nitro blue tetrazolium ( NBT ) and 5-bromo-4-chloro-3-indolyl phosphate ( BCIP ) were purchased from Sigma ( St-Louis, Maryland, USA ).

Detection was done following the protocol suggested in Antibodies a Laboratory Manual ( Harlow and Lane ).

The nitrocellulose membranes were blocked with 3% BSA in PBS overnight at 4°C. The next day, the membranes were washed twice for 5 minutes each in PBS. Anti-human $F(ab')_2$-AP conjugate ( diluted 1:1000 with 3% BSA in PBS ), anti-human γ chain ( 1:1000 ),or anti-human λ and κ chains ( 1:1000 ) were added to the membrane and incubated with agitation for 1 hour ( anti-human $F(ab')_2$-AP conjugate ) or 2 hours or more for the anti-human Ig chain antibodies at RT. The blots were washed 4 times in PBS for 5 minutes each, and the anti-goat IgG-AP conjugate ( diluted 1:1000 with 3% BSA in PBS ) was added and incubated for 1 hour with agitation at RT. The membranes were washed 4 times in PBS for 5 minutes each cycle. 70ul of NBT was added to 10ml of alkaline phosphatase buffer, mixed well, then 35ul of BCIP was added. The blots were developed at RT with agitation until bands were visible. The reaction was stopped with PBS containing 20mM EDTA.

# RESULTS

## I. Characterization of semi-synthetic human antibody libraries.

The construction of semi-synthetic libraries by the laboratory of Dr. Carlos F. Barbas III (Department of Molecular Biology, The Scripps Research Institute, La Jolla, California) led to libraries with a diversity greater than $1x10^8$ different members per library (V13/V1G, V13/V3G, V13/8H, V13/Si#1, V7/V3G, V7/8H, V7/Si#1).

To assess the proportion of clones that had successfully combined both cDNAs from the Lc and the Hc, the analysis of 70 randomly picked clones of the semi-synthetic libraries was performed. This analysis was important to assess the quality of the libraries, which is the percentage of the libraries that encodes both Hc and Lc fragments. 10 clones from each of the 7 libraries were isolated and plasmid DNA was prepared from them (Figure 1). Figure 2 shows the restriction analysis of 42 of those 70 clones which were picked for their various size (ones which were thought not to have both inserts or ones that were thought to contain the inserts for each libraries). For the library V13/V1G, 5 clones (50% of insertion) did not contain the Hc and Lc fragments. For libraries V13/V3G, V13/Si#1, and V7/V3G, 2 clones (80% of insertion) did not contain the inserts. For libraries V7/8H and V7/Si#1 only 1 clone (10%) did not contain both chains. All 10 clones (100% of insertion) for library V13/8H contained the Hc and Lc. Overall 81% of the clones contained both Hc and Lc cDNA sequences. Some of the clones contained inserts that varied from the predicted size of approximately 680bp. In Figure 2, clones at lane 11 and 22 did not contain the Hc fragment but still contained the Hc stuffer fragment which is approximately 300bp. The clone at lane 35

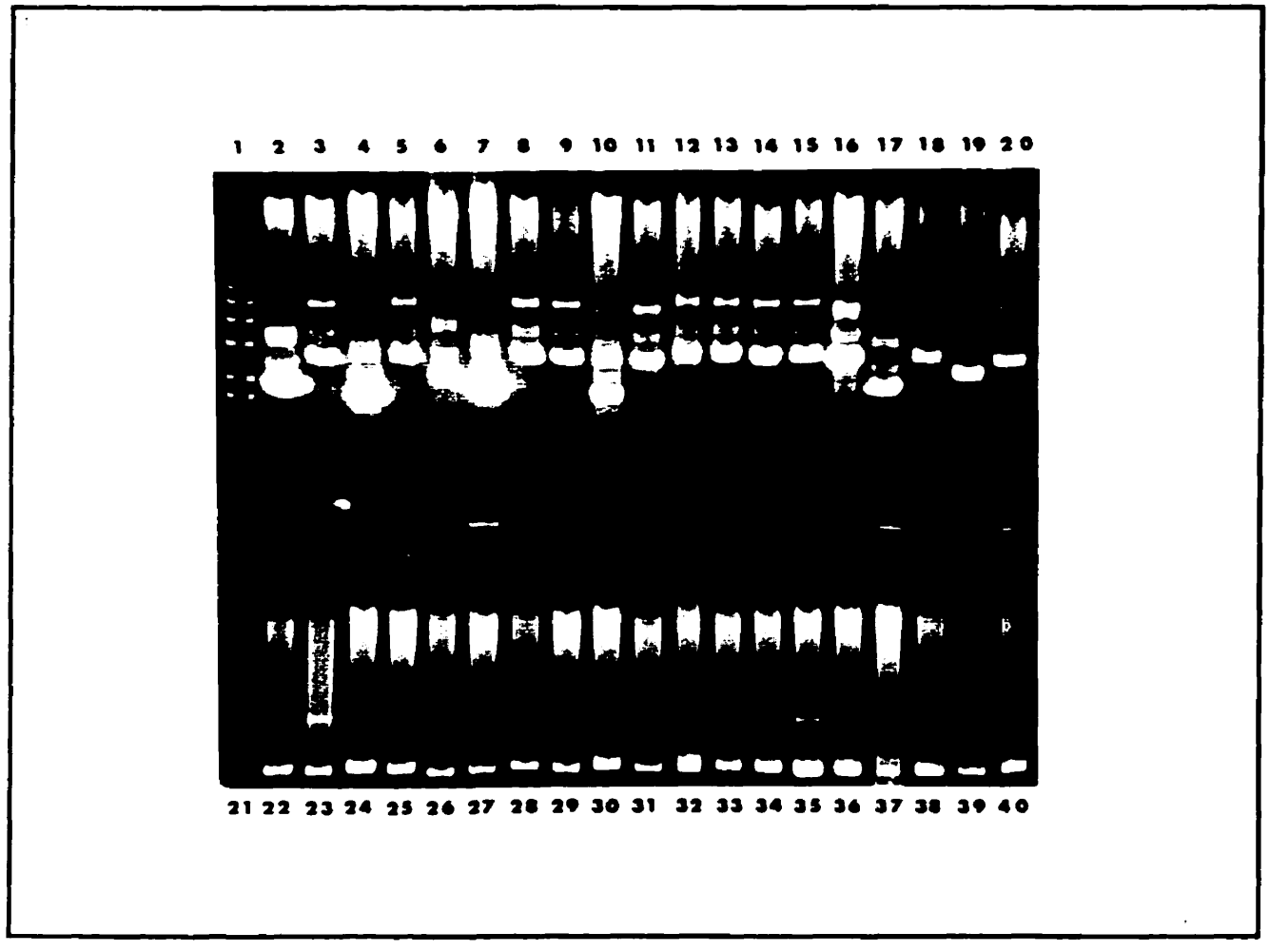
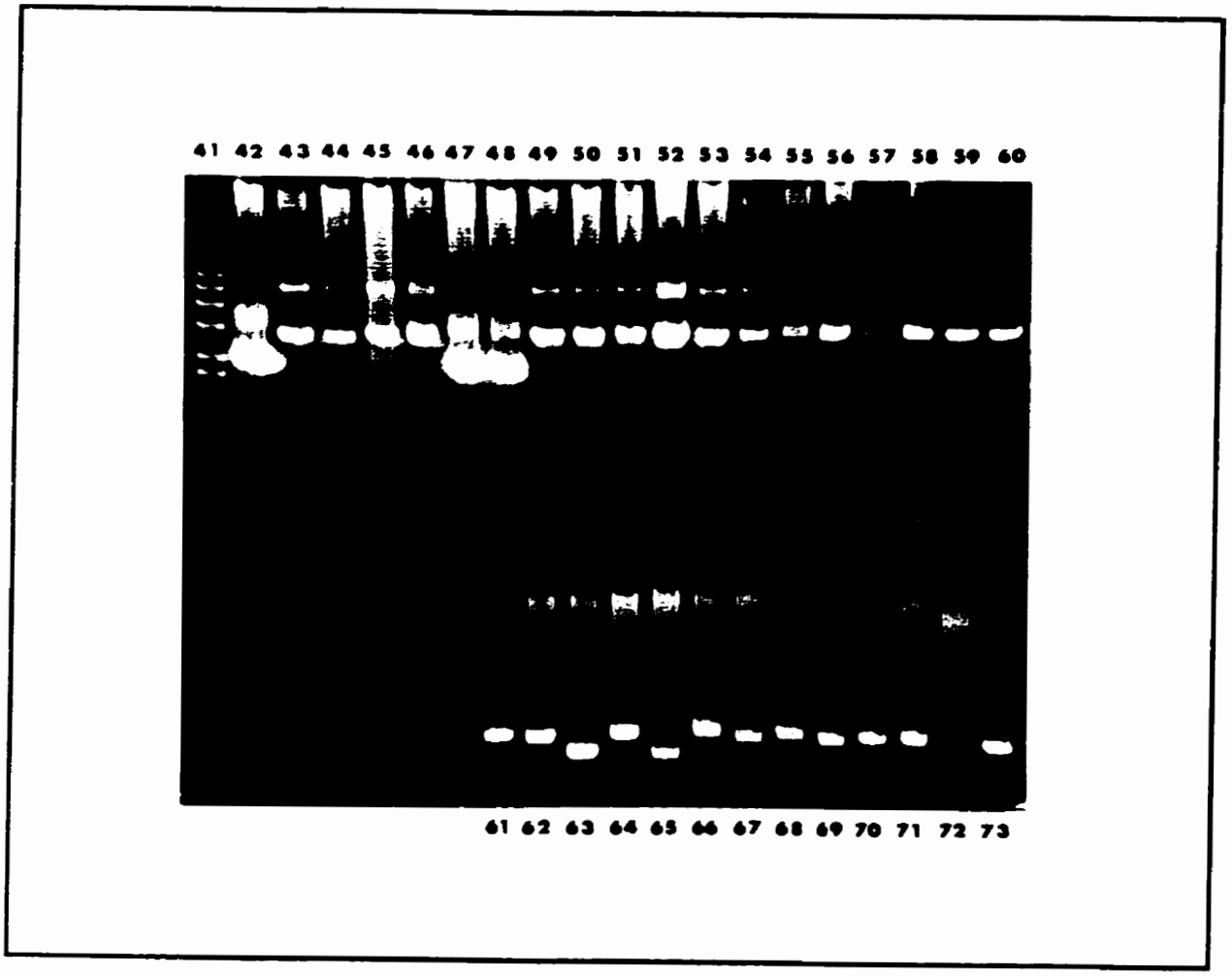



**Figure 1.** Plasmid preparation of clones from unselected semi-synthetic human antibody libraries to show the variability (heterogeneity) of the libraries. 10 clones from each of the 7 libraries were randomly picked and plasmid DNA was isolated and run on a 0.6% agarose gel. V13/V1G, lane 2 to 11; V13/V3G, lane 12 to 22; V13/8H, lane 23 to 32; V13/Si#1, lane 33 to 43; V7/V3G, lane 44 to 53; V7/8H, lane 54 to 63; V7/Si#1, lane 64 to 73; 250ng 1kb ladder molecular weight marker, lane 1, 21, and 41. Lane 8 represent the predicted size of a clone containing both Lc and Hc inserts.


41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
61 62 63 64 65 66 67 68 69 70 71 72 73

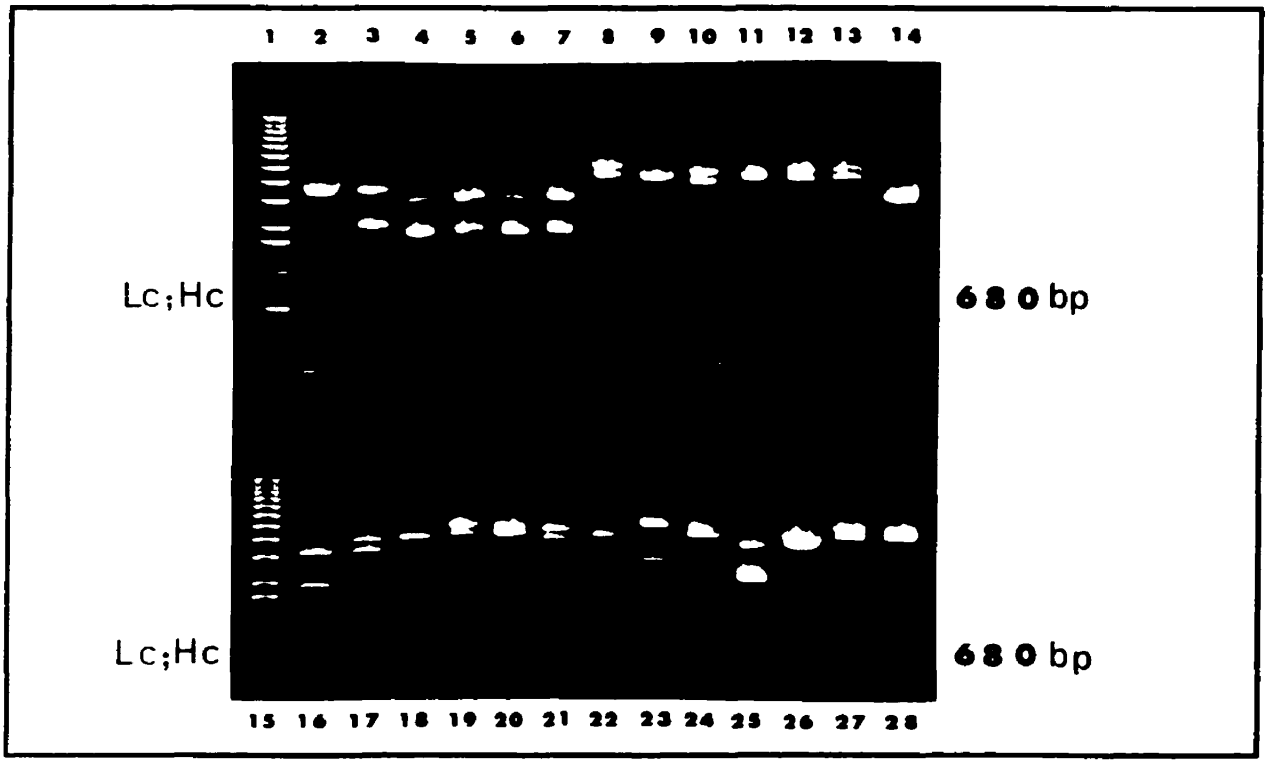
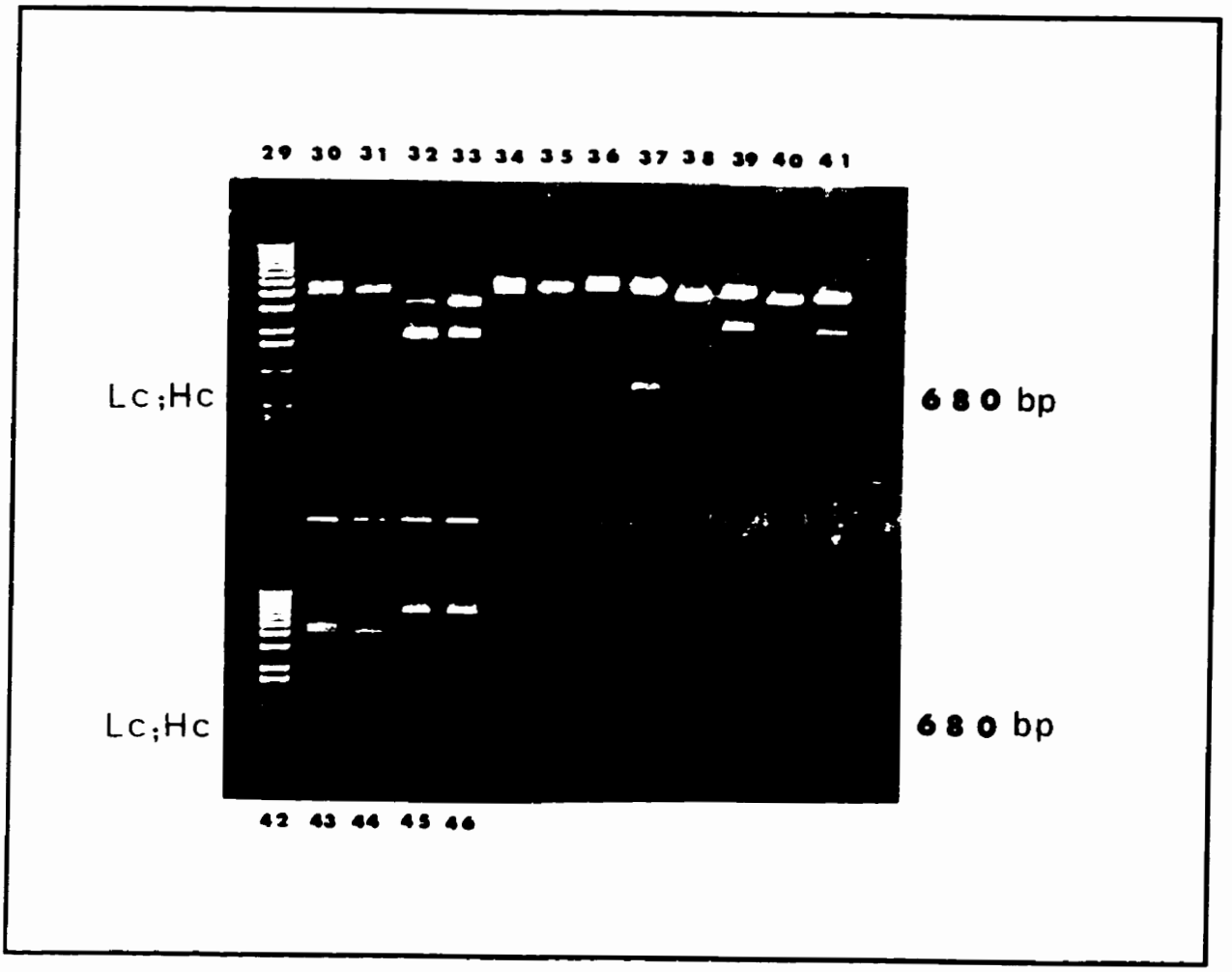



**Figure 2.** Frequency of the presence of the Lc and Hc fragments into clones from unselected semi-synthetic human antibody libraries. 42 clones from the 70 randomly picked clones in Figure 1 were analysed on a 0.6% agarose gel for the presence of Lc and Hc fragments by restriction analysis. Plasmid DNA from the clones was cut with SacI and XbaI restriction enzymes to release the Lc fragment and with XhoI and SpeI to release the Hc fragment. Lc and Hc fragments migrated at approximately 680bp. Lane 2 and 3 correspond to clone lane 2 in Figure 1; lane 4, 5 -> 4; lane 6, 7 -> 7; lane 8, 9 -> 8; lane 10, 11 -> 11; lane 12, 13 -> 15; lane 14, 16 -> 17; lane 17, 18 -> 19; lane 19, 20 -> 24; lane 21, 22 -> 26; lane 23, 24 -> 35; lane 25, 26 -> 42; lane 27, 28 -> 43; lane 30, 31 -> 45; lane 32, 33 -> 48; lane 34, 35 -> 50; lane 36, 37 -> 59; lane 38, 39 -> 63; lane 40, 41 -> 65; lane 43, 44 -> 70; clone 46, lane 45 and 46; 250ng 1kb ladder molecular weight marker, lane 1, 15, 29, and 42. First lane of each clone shows Lc digestion and second lane shows Hc digestion.


29 30 31 32 33 34 35 36 37 38 39 40 41
Lc;Hc
680 bp
Lc;Hc
680 bp
42 43 44 45 46

contained both Lc, Hc, and Hc stuffer fragment. The clone in lane 23 contained the Hc but did not contain the Lc fragment. Some of the clones contained a fragment of 2-2.5kb that could not be accounted for.

## II. Determination of the concentration of purified $\beta_1$ to use for panning selection using microtiter plate, and for ELISA.

The $\beta_1$ integrin used for the panning selection and also for the characterization of clones on ELISA was affinity purified from human placenta. An ELISA was performed with different concentrations of that $\beta_1$ preparation to assess the proper concentration used to coat wells of microtiter plates for panning selection and ELISA. Serial doubling dilutions of $\beta_1$ integrin were coated and ELISA was performed as described. Dilution 1:10 was used for panning selection which correspound to the saturation of coating to have the maximum concentration of antigen coated in an attempt to optimize the conditions of selection. Dilution 1:50 was used for ELISA purposes which correspound to the beginning of the saturation curve plateau. Dilution 1:50 was enough to obtain a good signal on ELISA(Figure 3).

## III. Design of a selection strategy for anti-$\beta_1$ integrin Fab selection.

The rationale for selecting phage-antibody clones that interact with human $\beta_1$ integrin is based on the fact that integrin molecules are highly conserved between species which make it impossible to produce antibodies

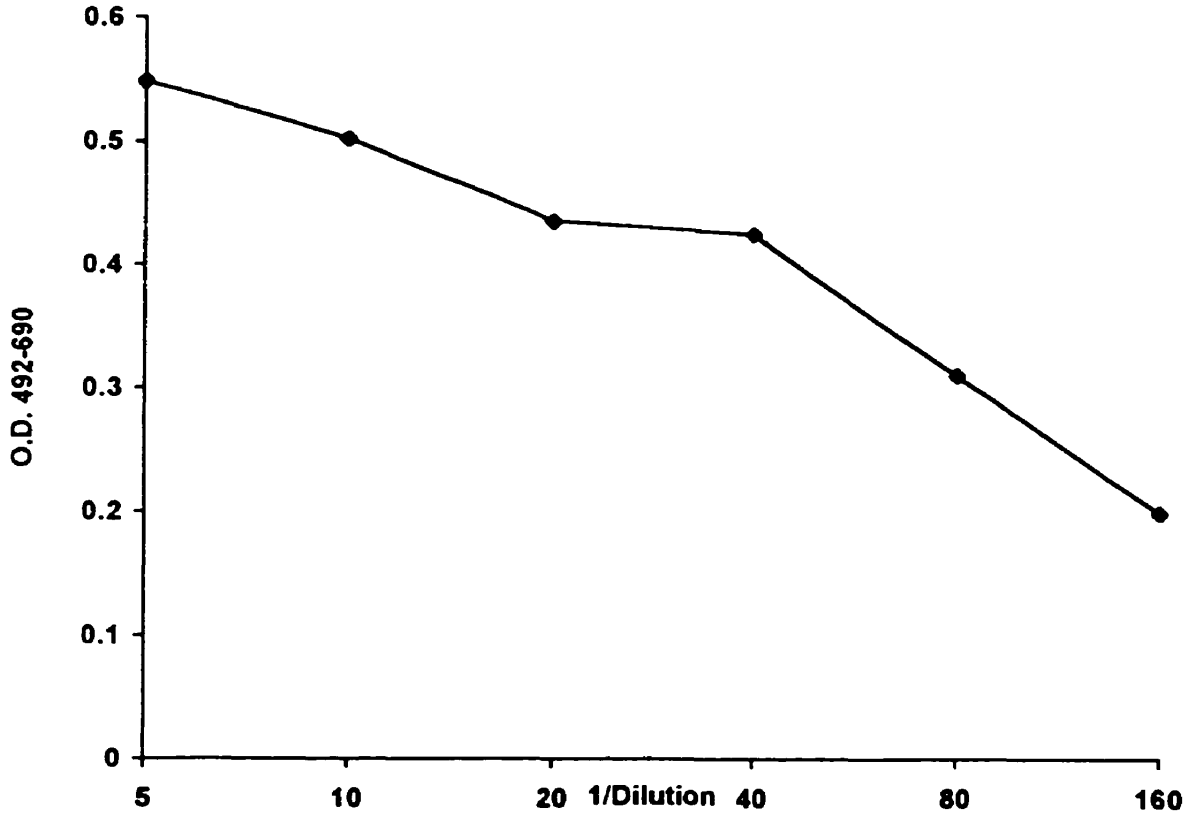



**Figure 3.** Determination of the concentration of purified $\beta_1$ integrin to use for panning and ELISA in microtiter plates. $\beta_1$ integrin purified from human placenta on a JB1 affinity column was diluted at different concentrations from eluted fraction 4 and coated on ELISA plate. Detection was done with JB1A antibody and anti-mouse IgG-HRP conjugate.

against such a conserved molecule. Based on that consideration, we employed 7 semi-synthetic human antibody libraries cloned into pComb3H to identify possible clones that could specifically recognize regions of the human $\beta_1$ integrin.

In a first attempt, panning of the semi-synthetic libraries was performed using a biotinylated $\beta_1$ peptide (B-SGSGTAEKLK). A panning stategy was used in which avidin was first coated into wells of a microtiter plate. Then, the biotinylated JB1A peptide was added and captured by the biotin binding sites of the avidin molecules. Freshly amplified libraries were incubated with avidin in solution then added to the antigen-coated wells in an attempt to remove possible phage that could interact with avidin. 7 rounds of panning selection were performed with increasing stringency of the washing steps.

In a second attempt, panning of the semi-synthetic libraries was done using purified $\beta_1$ integrin from human placenta. Freshly amplified phage were directly applied to the antigen-coated wells of a coated microtiter plate. 7 rounds of panning selection were performed with increasing number of washing steps.

Between panning round 1 to 3, the number of washes increased from 1 to 5 to 10, it is then expected to see the titer of recovered phage dropping. The panning selection on captured $\beta_1$ biotinylated peptide revealed a modest enrichment of 17 fold ($7 \times 10^6$ cfu / $4 \times 10^5$ cfu = 17 x) between round 3 and 7 (Table 1A and C). Panning on captured $\beta_1$ integrin did not result in significant phage enrichment (Table 1B and C).

A)

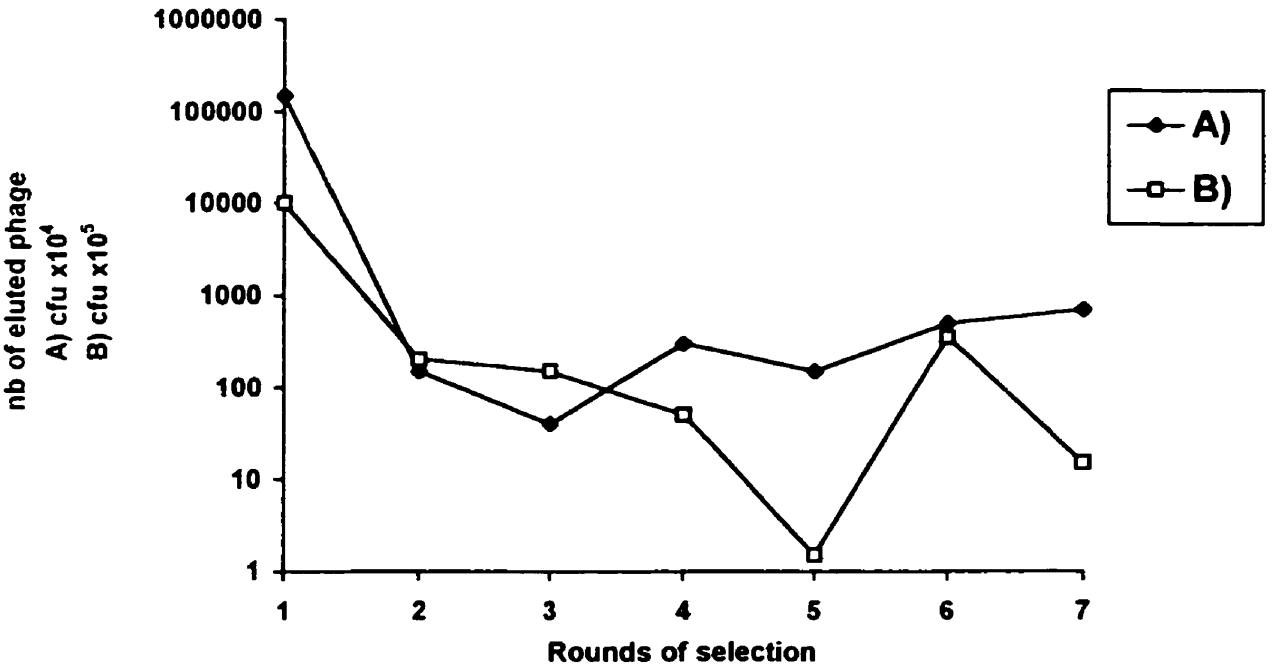
| round of panning | input ( cfu ) | output ( cfu ) | number of washes |
|---|---|---|---|
| 1 | $2.5x10^{12}$ * | $1.5x10^{9}$ * | 1 |
| 2 | $1x10^{10}$ | $1.5x10^{6}$ | 5 |
| 3 | $1.5x10^{10}$ | $4x10^{5}$ | 10 |
| 4 | $4x10^{9}$ | $3x10^{6}$ | 10 |
| 5 | $2x10^{10}$ | $1.5x10^{6}$ | 10 |
| 6 | $5.5x10^{11}$ | $5x10^{6}$ | 10 |
| 7 | $2.5x10^{11}$ | $7x10^{6}$ | 10 |

B)

| round of panning | input ( cfu ) | output ( cfu ) | number of washes |
|---|---|---|---|
| 1 | $6x10^{12}$ * | $1x10^{9}$ * | 1 |
| 2 | $6x10^{11}$ | $2x10^{7}$ | 5 |
| 3 | $4x10^{11}$ | $1.5x10^{7}$ | 10 |
| 4 | $5x10^{11}$ | $5x10^{6}$ | 10 |
| 5 | $1.5x10^{11}$ | $1.5x10^{5}$ | 10 |
| 6 | $4.5x10^{11}$ | $3.5x10^{7}$ | 10 |
| 7 | $2.5x10^{11}$ | $1.5x10^{6}$ | 10 |

**Table 1.** Panning of phage display Fabs from 7 semi-synthetic human antibody libraries. The semi-synthetic libraries were panned on biotinylated JB1A peptide attached to avidin coated microtiter wells (A), or purified $\beta_1$ integrin (B). * The sum of all libraries. Enrichment of the recovered phage titer over 7 rounds of selection (C).

C)


1000000
100000
10000
1000
100
10
1
nb of eluted phage
A) cfu x10^4
B) cfu x10^5
1
2
3
4
5
6
7
Rounds of selection
A)
B)

## IV. Characterization of phage-antibodies binding specificity.

For the selection with biotinylated JB1A peptide and the selection with purified $\beta_1$ integrin, clones were randomly picked and were amplified after 3, 5, and 7 rounds of panning and phage particles were produced. Screening was performed by ELISA. For the selections with purified $\beta_1$ integrin, no positive clones were obtained (data not shown), either after 3, 5, or 7 rounds of selection. For the selection with biotinylated JB1A peptide, no clones were obtained after 3 rounds of panning, 1 clone was isolated after 5 rounds (named clone 19), and 3 clones were obtained after 7 panning (respectively named clones 37, 42, and 46). Clone V13/8H 11 was randomly picked from an unselected library (V13/8H) to use as a negative control on ELISA to assess nonspecific background.

Figure 4 shows that clone 19 appears to bind to avidin and purified $\beta_1$ integrin but did not bind to JB1A peptide which was surprising since the selection of this phage was performed on the peptide. Whereas clones 37, 42, and 46 bound to avidin and also bound to both peptide and $\beta_1$ integrin. None of the clones bound to BSA. The negative control V13/8H 11 had a low background on ELISA, suggesting low nonspecific background of phage particle. The last column for each clones in Figure 4 show the relative amount of phage produced in microtiter wells. 50ul of phage were coated directly into wells and phage were detected with anti-M13 HRP conjugate. As shown, clone 19 is represented in a lower number of phage concentration limiting the level of binding compared to the other clones. At this point, we felt that these clones were specific for the desired epitope.

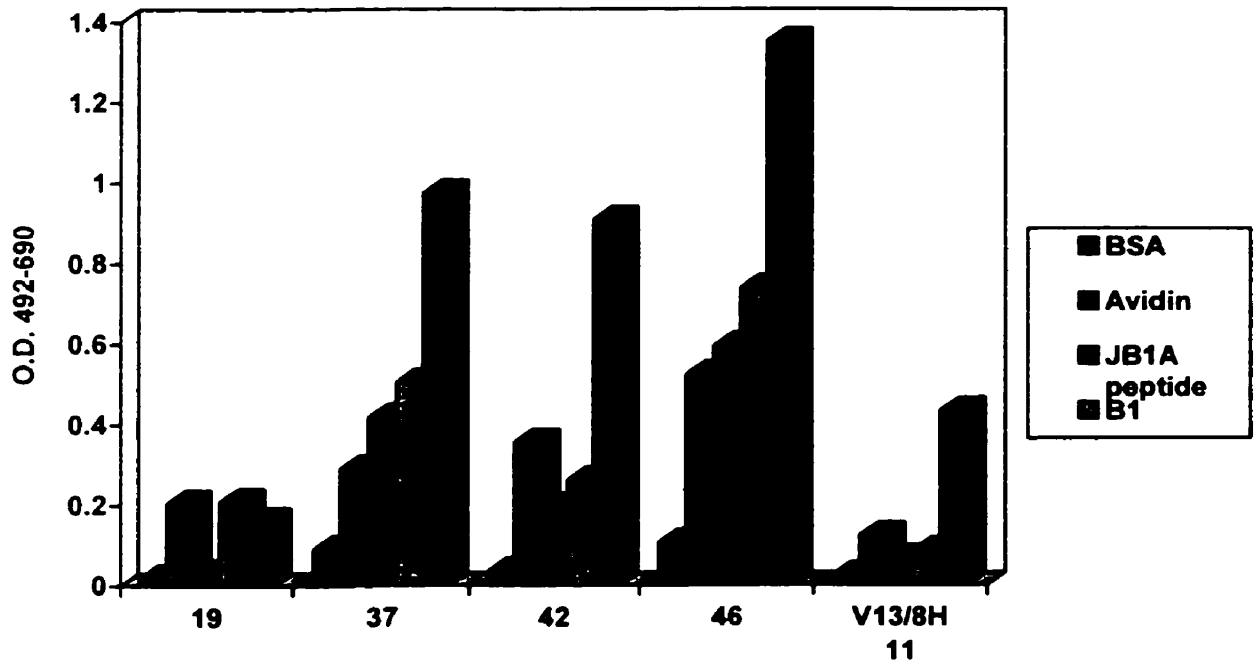



**Figure 4.** Specificity of phage-antibody clones selected from semi-synthetic human antibody libraries determined by ELISA. 3% BSA/PBS was used as a blocking solution. Phage-antibodies were first incubated with an equal volume of blocking solution prior to application to the antigen coated wells. The first columns show the background of non-specific binding to BSA. The last columns show the relative amount of phage that were applied to the wells.

To further characterize the binding of clone 19 to antigen, we determined the influence of the concentration of VCSM13 helper phage used for microtiter plate rescue of phage-antibody clone 19 on the binding to avidin to show that binding is maximized. Serial 10X dilutions of VCSM13 helper phage was used from $5x10^{10}$ to $5x10^{7}$ pfu/ml of SB. These different concentration of helper phage were used for the microtiter plate rescue of clone 19. When using $5x10^{10}$ pfu/ml of VCSM13 helper phage, more phage were produced but the binding to avidin was completely inhibited probably because only native pIII is expressed at the surface of the phage particle. Fab fragments were competed out by native pIII for a space on the surface of the phage. Reducing helper phage to $5x10^{7}$ did not affect the binding capacity of clone 19 to avidin (Figure 5) and reduced only by a little fraction the relative number of phage particles produced. Therefore the signal could not be improved by changing the titer of VCSM13 helper phage used.

Figure 6 shows that the binding of clone 19 was directly proportional to the amount of avidin coated on a microtiter plate which might suggest specific binding for avidin.

## V. Presence of the Hc, Lc, and pIII gene inserts in phage-antibody clones.

The following study was to determine if phage-antibody clones 19, 37, 42, and 46 previously selected with biotinylated $\beta_1$ peptide contained Hc and Lc inserts as well as the pIII gene fragment to demonstrate that it is really encoded in the phagemid vector. As shown in figure 7 these selected clones were of unexpected sizes. This control analysis was done before any further characterization of the soluble forms. Restriction analysis shows in Figure 7

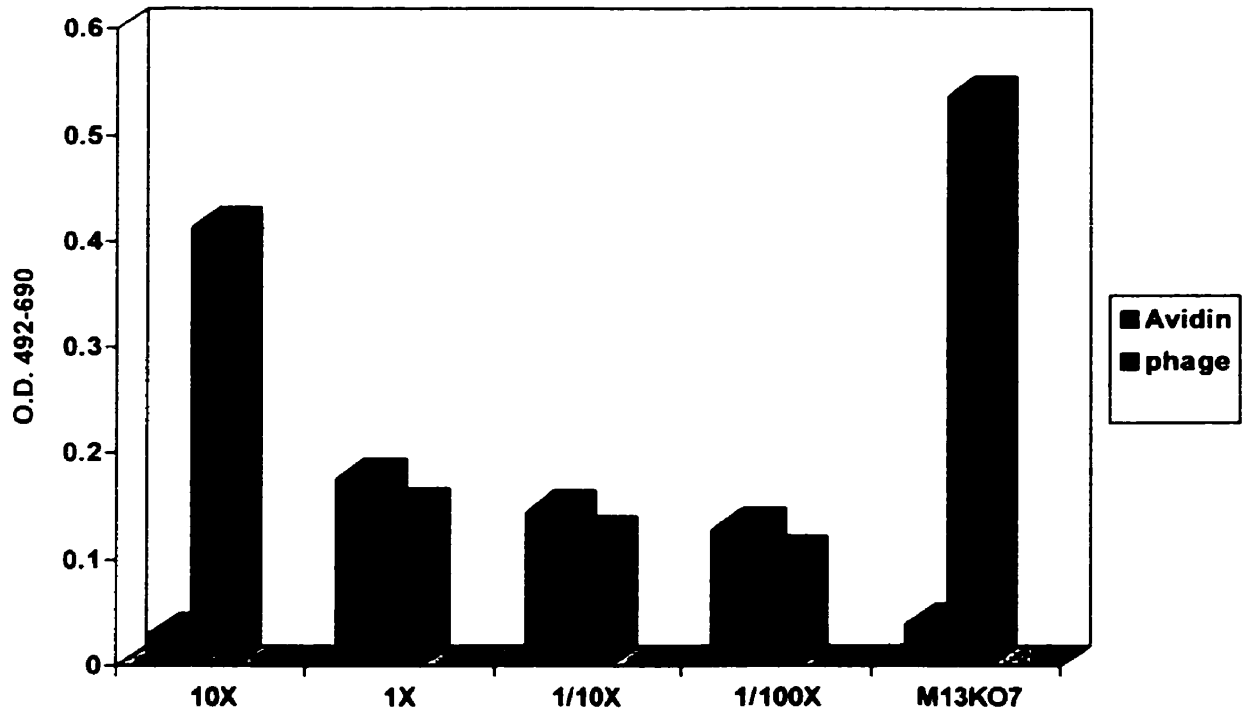



**Figure 5.** Influence of the amount of VCSM13 helper phage on phage-antibody clone 19 binding to avidin. The second columns show the relative amount of phage that were applied to the wells.

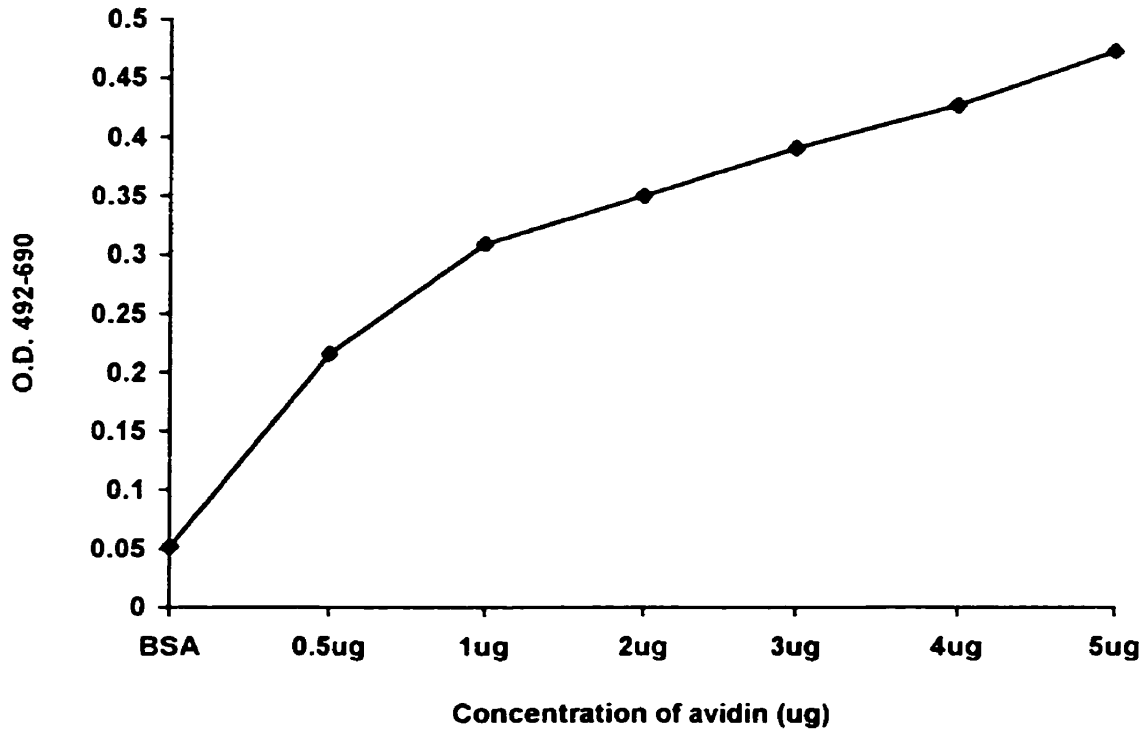



**Figure 6.** Binding activity of phage-antibody clone 19 in function of the concentration of avidin. Same titer of clone 19 was applied to wells coated with different concentrations of avidin.

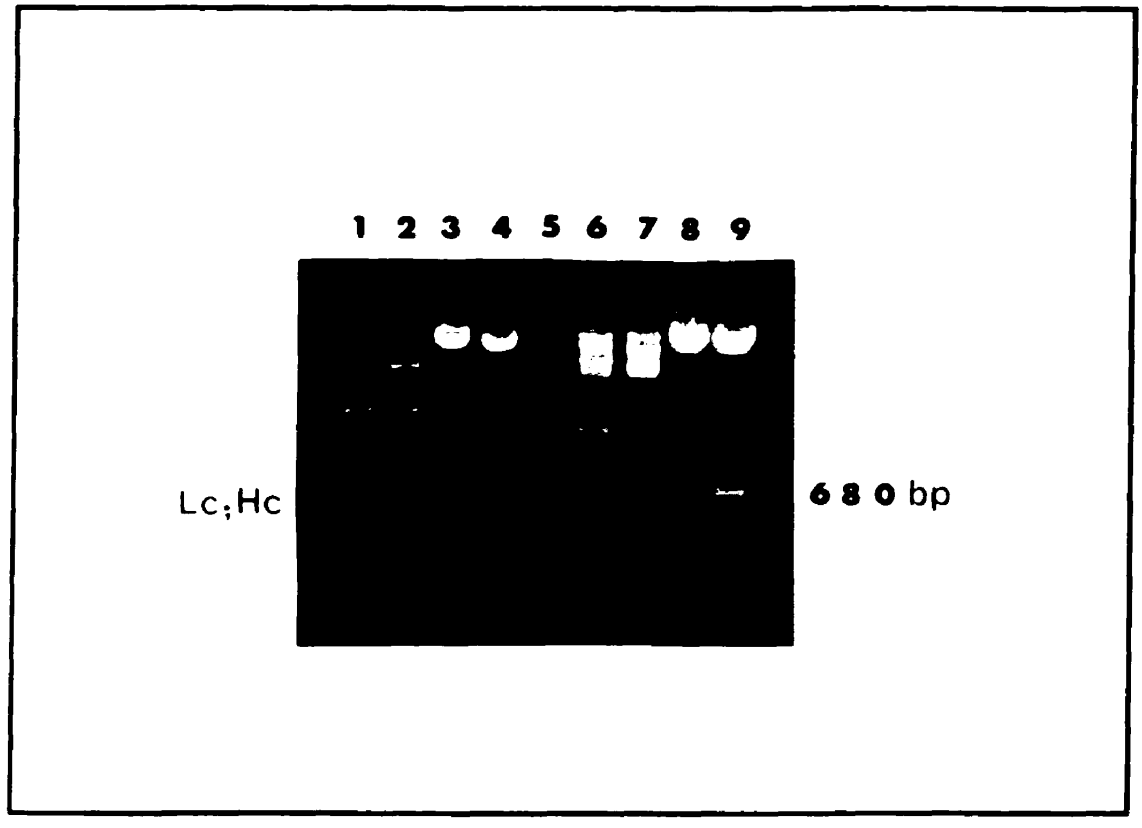



**Figure 7.** 0.6% agarose gel showing the insertion of Lc and Hc fragments into clones selected from semi-synthetic human antibody libraries. Clone 19, lane 1 and 2; clone 37, lane 3 and 4; clone 42, lane 6 and 7; clone 46, lane 8 and 9; 500ng 1kb ladder molecular weight marker, lane 5. First lane of each clone shows the Lc and second lane shows the Hc. Plasmid DNA from the clones was cut with SacI and XbaI restriction enzymes to release the Lc fragment, and with XhoI and SpeI to release the Hc fragment. Lc and Hc fragments migrated at approximately 680 bp.

that clones 37, 42, and 46 contained the Hc and Lc inserts of the proper size (approximatively 680bp), whereas clone 19 did not seem to contain any of the two chains by restriction digestion (could not be cut out by restriction digestion). Agarose gel electrophoresis of restriction digested phage-antibody clones shows in Figure 8 that clones 37, 42, and 46 contained the pIII gene fragment of 555bp whereas clone 19 did not seem to contain the pIII gene fragment. Clone V13/8H 11 which is used as a negative control in ELISA contained both Hc, Lc, and pIII gene inserts (data not shown) of the appropriate sizes.

## VI. Characterization of soluble Fab clones.

The next step was to produce the soluble Fab form by removing the pIII gene by restriction digestion, religation, and transformation into the host cell XL1-blue. Production of soluble Fab was induced with 1mM IPTG, overnight. Supernatant of lysed cells was directly used to further characterize the clones by ELISA. Due to the fact that the pIII gene fragment of clone 19 could not be removed by restriction digestion, this clone could not be converted to the soluble Fab producing form.

A problem that was encountered is that $\beta_1$ integrin preparation from human placenta is contaminated with human IgG, which caused a strong background on ELISA using goat anti-human $F(ab')_2$-alkaline phosphatase conjugate used to detect soluble Fab on $\beta_1$ integrin. To resolve this problem the $\beta_1$ integrin preparation was absorbed with goat anti-human $F(ab')_2$ and then Gammabind Plus Sepharose (protein G coupled beads) was added. The beads-goat anti-human $F(ab')_2$ complexes were then removed by centrifugation. As shown in Figure 9, when the $\beta_1$ integrin preparation is

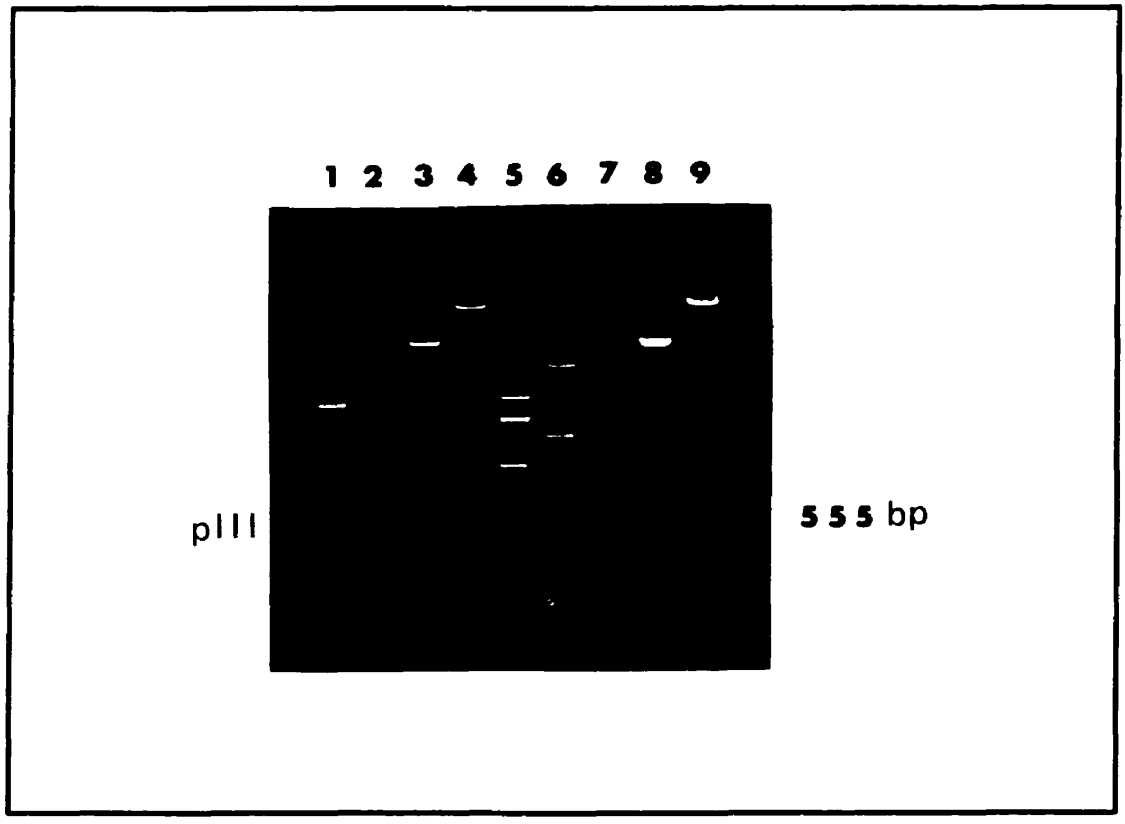



**Figure 8.** Restriction analysis of phage-antibody clones. 0.6% agarose gel showing the presence of pIII gene fragment into phage-antibody clones selected from semi-synthetic human antibody libraries. Clone 19, lane 1 and 2; clone 37, lane 3 and 4; clone 42, lane 6 and 7; clone 46, lane 8 and 9; 500ng 1kb ladder molecular weight marker, lane 5. First lane of each clone shows undigested plasmid and second lane shows digestion with SpeI and NheI. Plasmid DNA from the clones was cut with SpeI and NheI restriction enzymes to release the pIII fragment to produce soluble Fab, which migrated at 555bp.

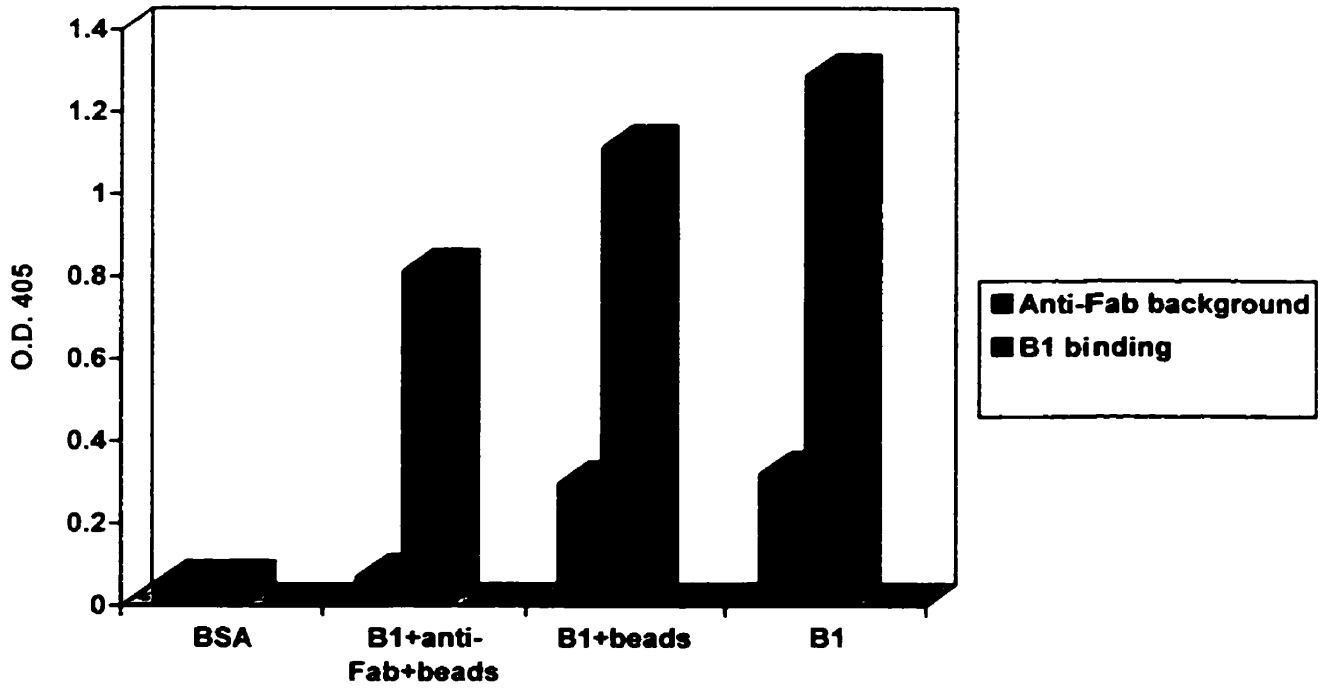



**Figure 9.** Adsorption of human IgG from $\beta_1$ integrin preparation from human placenta. $\beta_1$ preparation was treated with goat-anti human F(ab')2 and Gammabind Plus Sepharose or with Gammabind Plus Sepharose alone. First columns show background produced when goat-anti human F(ab')2-AP conjugate was used in ELISA. Second columns show the relative amount of $\beta_1$ integrin in the preparation after adsorption.

absorbed with goat anti-human $F(ab')_2$ and Gammabind Plus Sepharose beads, the background can be completely removed to a basal level. The background could not be removed when only the beads were used. This removal of the background could be achieved with only a small lost of the $\beta_1$ integrin in the preparation.

A control experiment was done to make sure that the host cell XL1-blue itself did not contribute to any of the background on ELISA. XL1-blue cells that were not transformed with semi-synthetic human antibody libraries were lysed as done when Fab were prepared from periplasm and the supernatant was used directly on ELISA. XL1-blue host cell did not contribute to the background on BSA, avidin, biotinylated JB1A peptide, or $\beta_1$ integrin as shown in (data not shown).

Analysis of the soluble Fab clones by ELISA revealed that all soluble Fab clones were binding to avidin, biotinylated JB1a peptide, and $\beta_1$ integrin. Clone V13/8H 11-25 (picked from an unselected library) that was negative as a phage display form bound as well in a nonspecific way (Figure 10). Another clone randomly picked from an unselected library, V13/V1G 7-9, was also binding to the antigens. None of the clones were binding to BSA. As mentioned previously, clone 19 could not be tested as a soluble Fab form.

## VII. Influence of the concentration of soluble Fab on the binding specificity.

One of the questions that was asked at this point was if the concentration of soluble Fab could influence the binding to antigens and discriminate between clones. To assess that question, an ELISA was

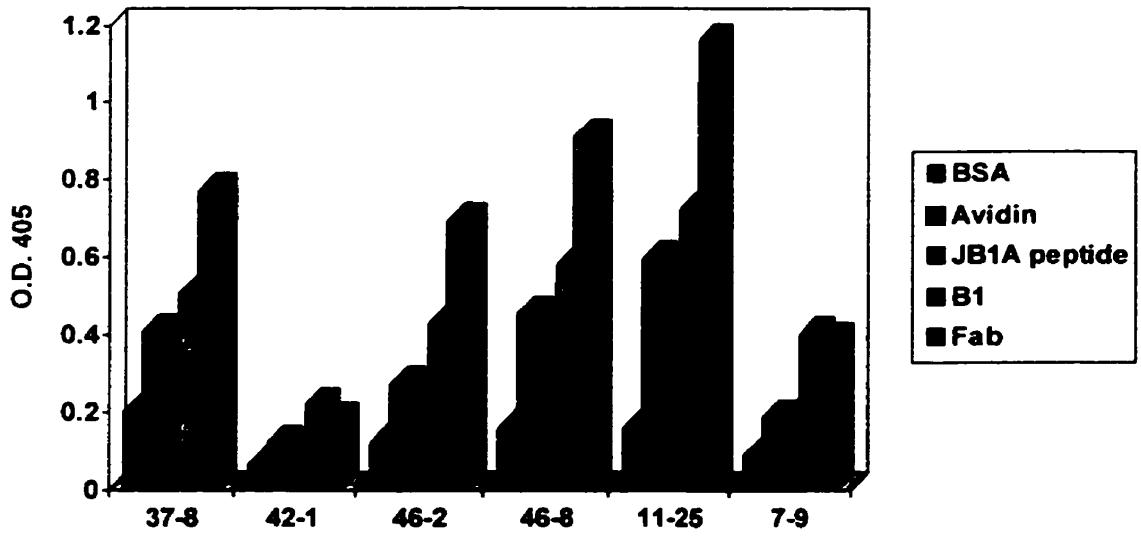



**Figure 10.** Specificity of monoclonal Fab from semi-synthetic human phage-Ab libraries determined by ELISA. Clones V13/8H 11-25 and V13/V1G 7-9 were randomly picked from a library without any selection, to use as a negative control. 3% BSA in PBS was used as a blocking agent. Last columns indicate the level of Fab in the sample. First columns show the level of non-specific background on BSA. Fab clones 46-8, V13/8H 11-25,

performed in which soluble and V13/V1G 7-9 were applied as serial 2X dilutions to different antigens. No differences were observed between the 3 clones on either BSA (Figure 11A), avidin (Figure 11B), or biotinylated JB1A peptide (Figure 11C). The binding was decreasing proportionaly with the dilutions for each of the clones. No discrimination in binding was observed between the different antigens.

Curiously, when dilutions of soluble Fab clones 46-8, V13/8H 11-25, and V13/V1G 7-9 were coated directly to wells of a microtiter plate to assess the soluble Fab production. a different pattern was observed between the clones (Figure 11D). The optical density of clones 46-8 and V13/8H 11-25 was decreased in a fashion while the optical density of clone V13/V1G 7-9 was stable up to dilution 1:8, then increased up to dilution 1:32 and finally decreased progressively. The reason of this pattern is not known.

## VIII. SDS-Page and Western blot analysis.

At this point it was questioned whether the problem might be that the two antibody chains did not assemble properly. To assess this concern, equal volumes of periplasmic preparations containing expressed soluble Fab clones were run on a 12% SDS-Page gel under nonreducing conditions and analysed by Western blotting using goat anti-human Lc and Hc (Figure 12). 1ug of human IgG was used as control. The Hc tends to be sticky if not paired with a Lc. It was revealed that in the control V13/8H 11-25, some Hc-Lc dimers (50kD) were formed but most of the Hc (25kD) was not paired with the Lc (25kD). More dimers were formed in proportion to monomeres for the other

A)

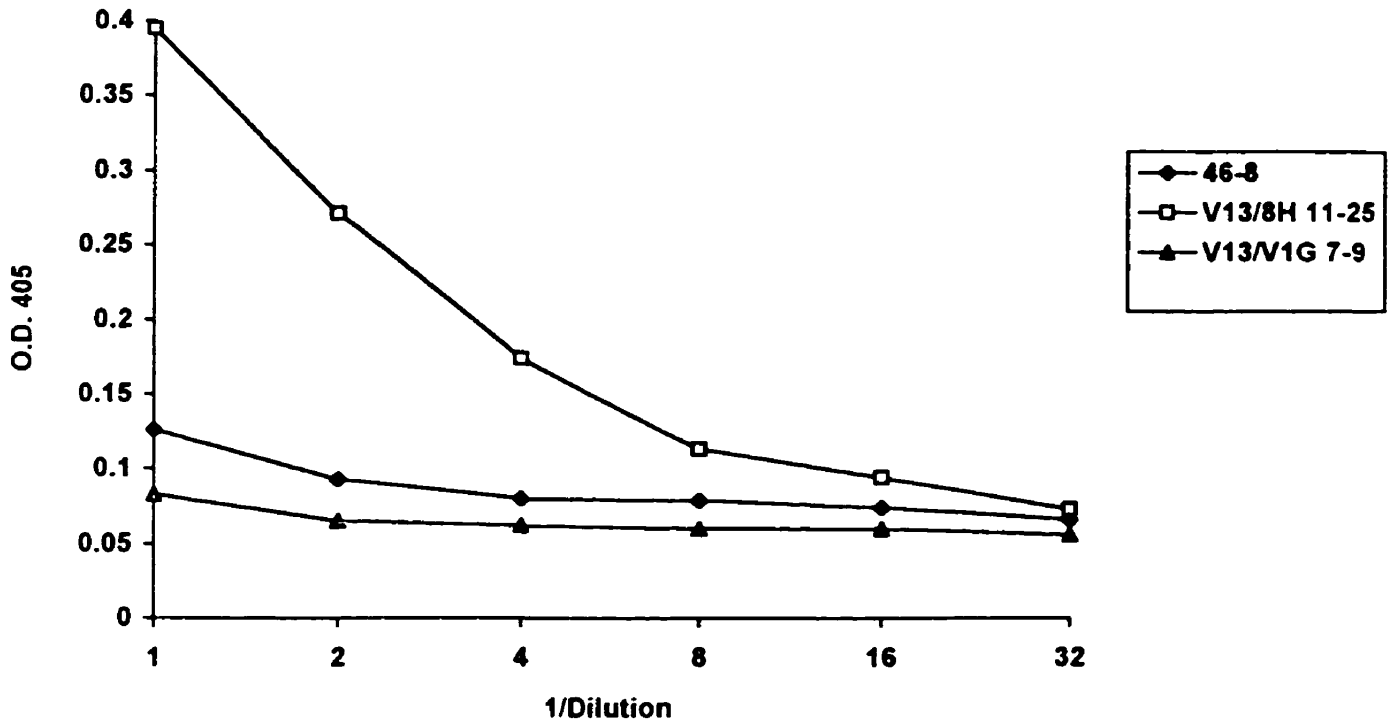
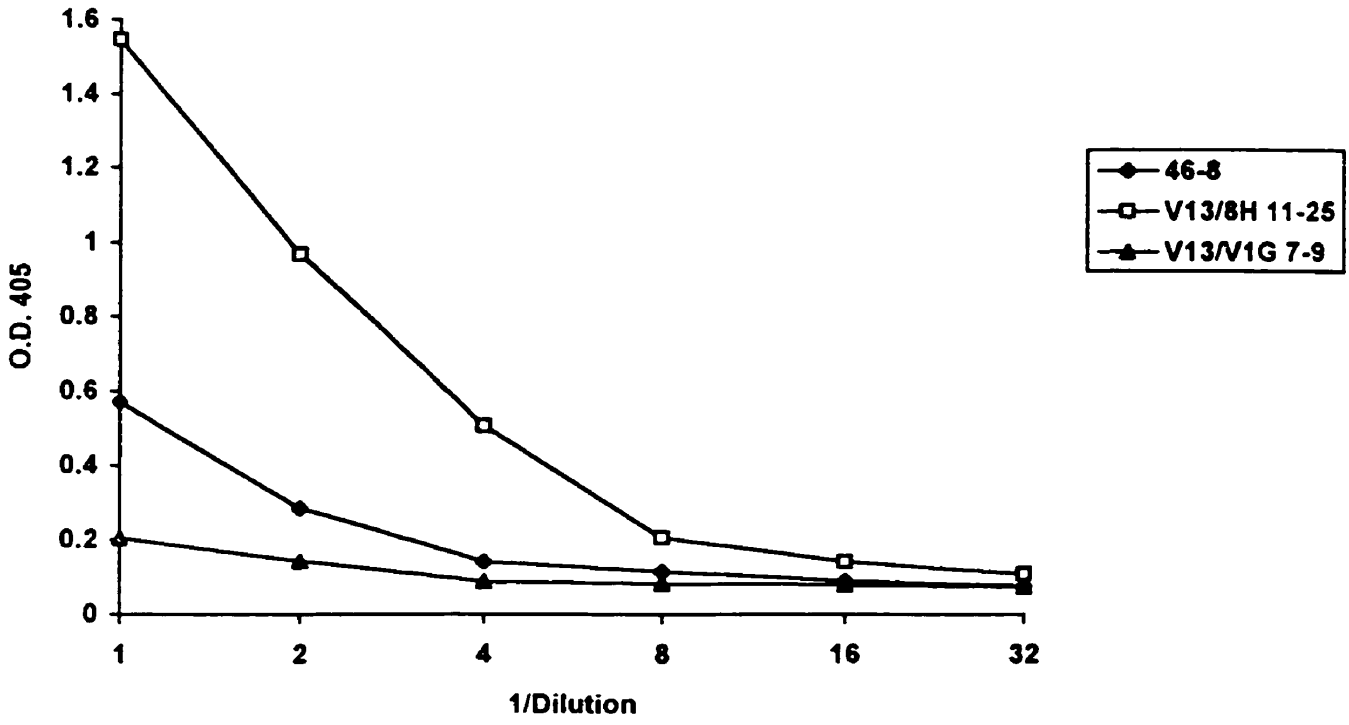



**Figure 11.** Influence of the concentration of soluble Fab on the binding to antigen coated wells. Clone 46-8, V13/8H 11-25, and V13/V1G 7-9 were applied as 2 fold dilutions to BSA (A), avidin (B), and JB1A peptide (C). The relative amount of soluble Fab produced was determined by coating these at different concentrations (D).

B)


1.6
1.4
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1
0.8
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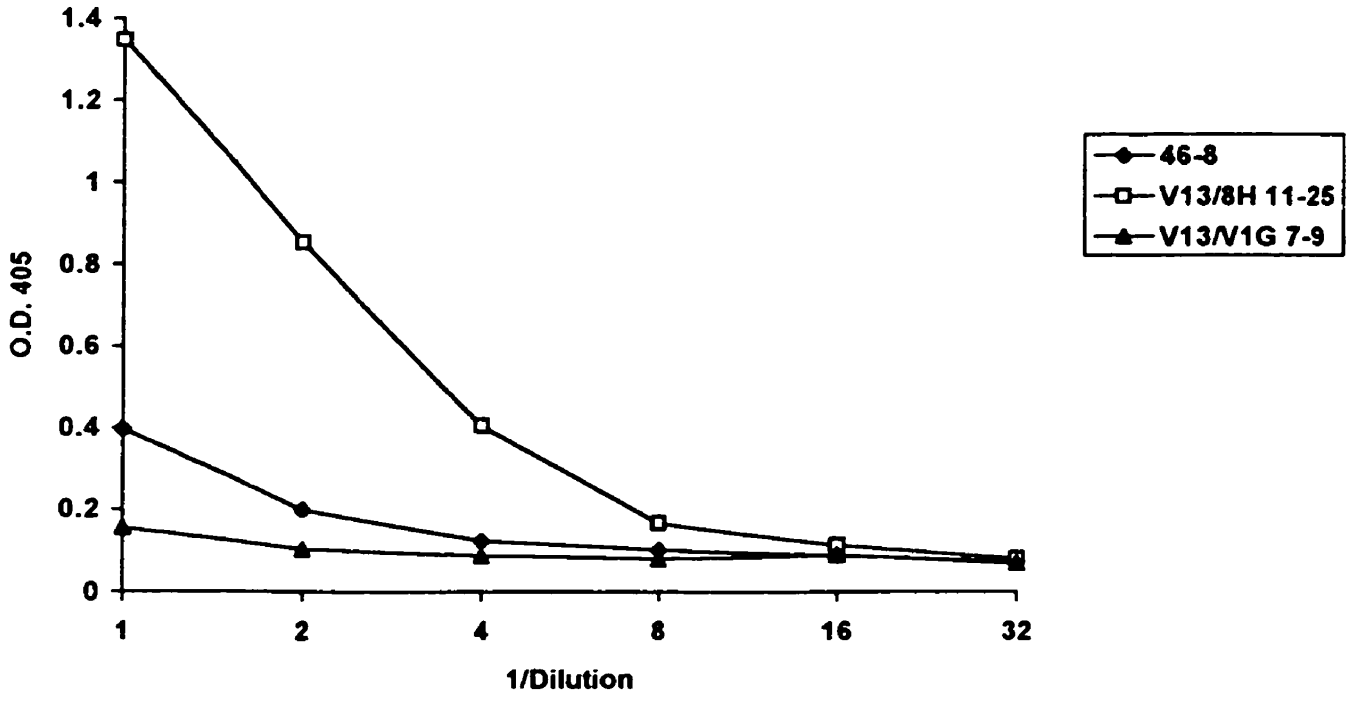
16
32
1/Dilution
46-8
V13/8H 11-25
V13/V1G 7-9

C)


1.4
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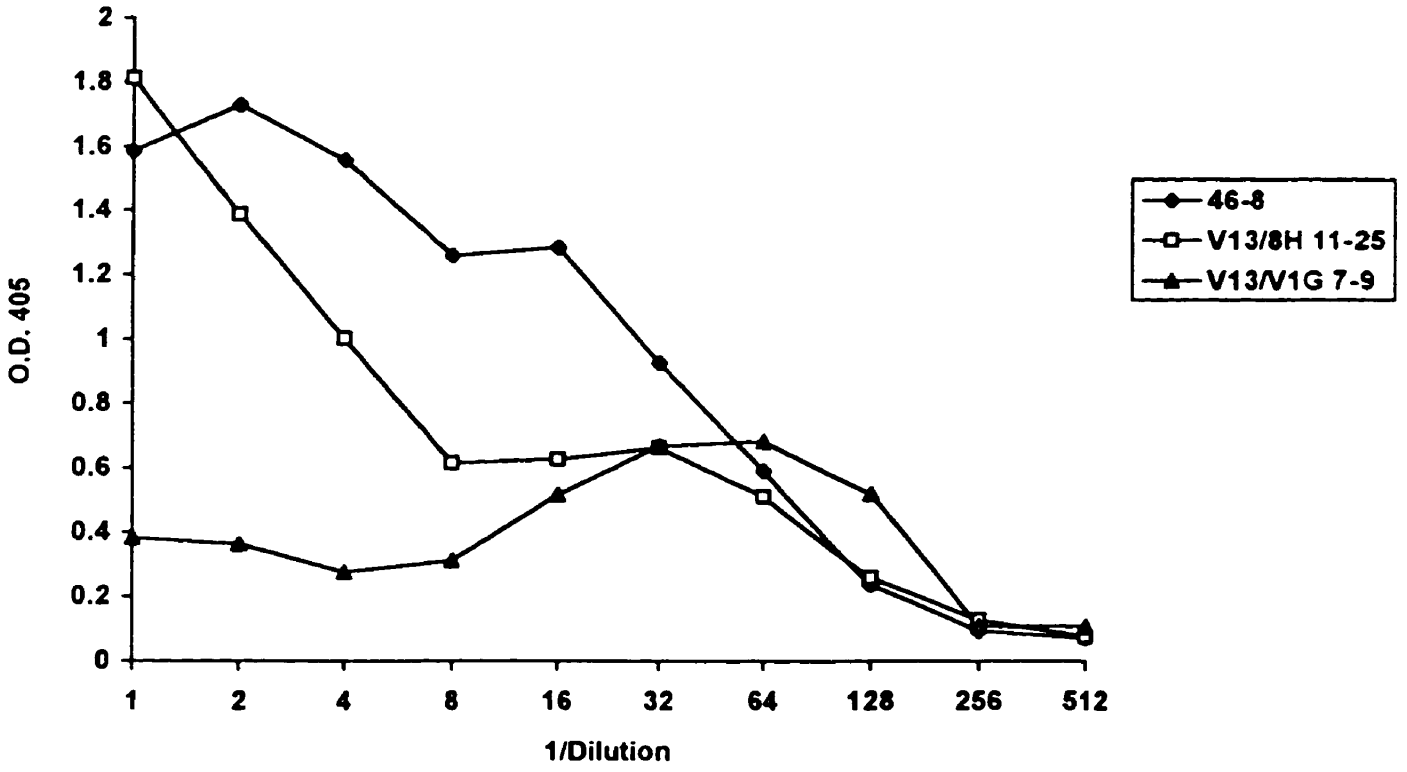
32
1/Dilution
46-8
V13/8H 11-25
V13/V1G 7-9

D)


O.D. 405
2
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1.6
1.4
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1
0.8
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46-8
V13/8H 11-25
V13/V1G 7-9

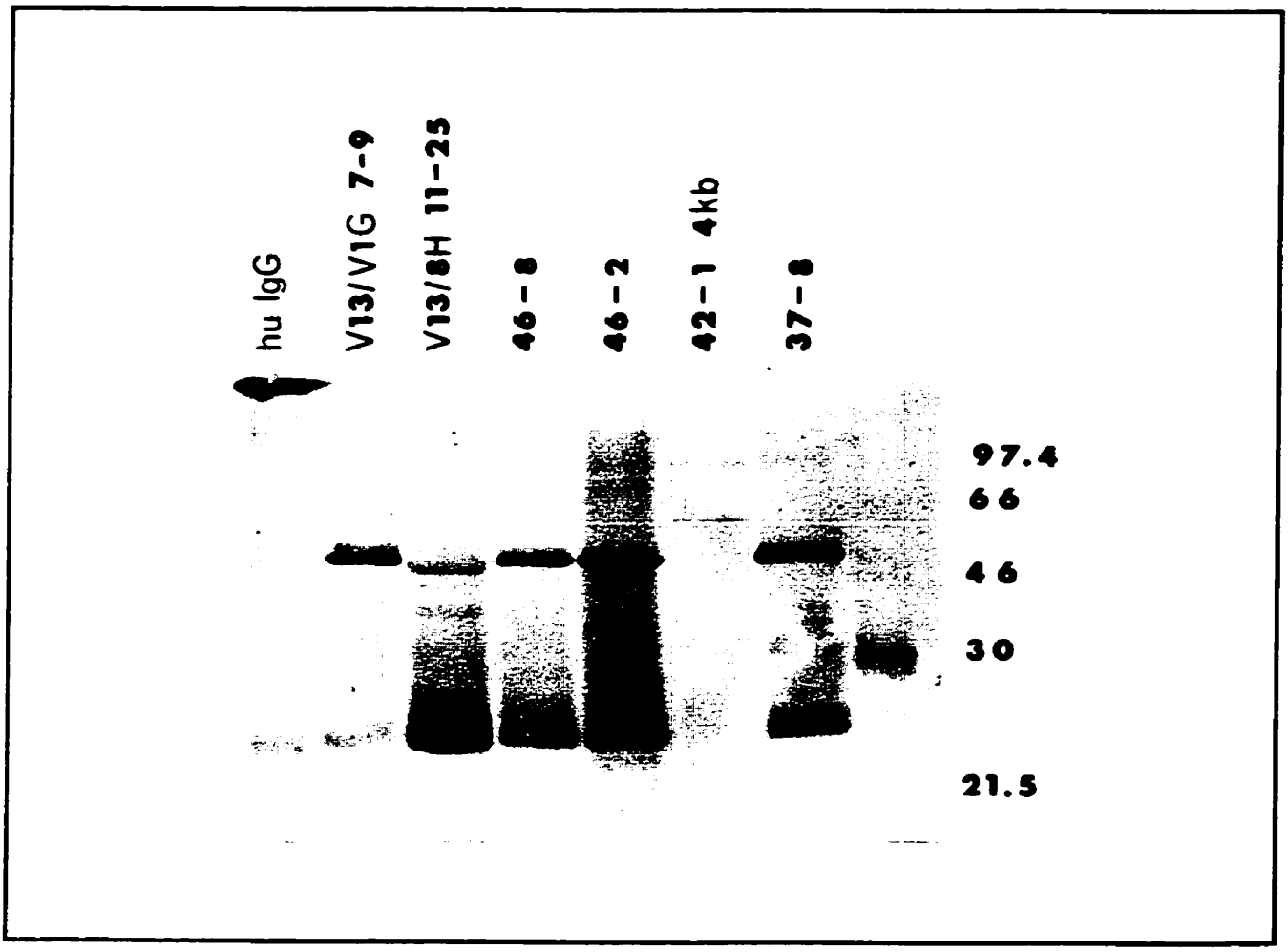
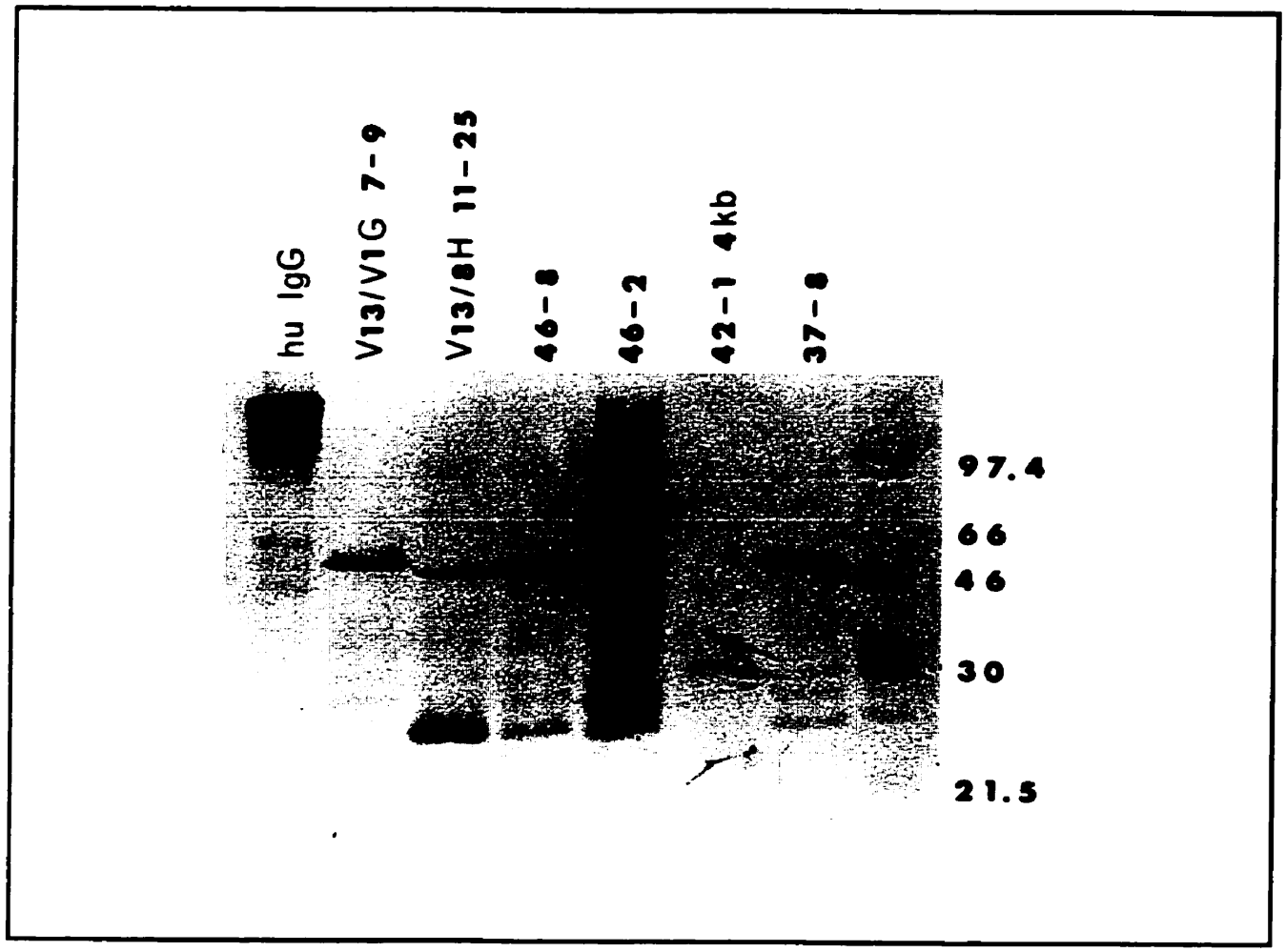



**Figure 12.** Western blot analysis of soluble Fab clones from semi-synthetic human antibody libraries. Soluble Fab clones were run on a 12% SDS-Page gel under nonreducing conditions and analysed by Western blot using goat anti-human Lc 1:1000 **(A)**, and goat anti-human Hc 1:1000 **(B)**. Detection was done with anti-goat IgG-alkaline phosphatase conjugate.


hu IgG
V13/V1G 7-9
V13/8H 11-25
46-8
46-2
42-1 4kb
37-8
97.4
66
46
30
21.5

clones. Moreover, a larger amount of Lc seems to be expressed in comparaison to the Hc.

## IX. Nonspecificity of phage-antibody clones.

In another attempt to understand, reanalysis of the phage-antibodies by ELISA using different peptides available in the laboratory determined that the same clones bound to these additional peptides except clone #19, thus indicating that adhesion was apparently nonspecific (data not shown). Alteration of the conditions of blocking and washing were tested for their ability to block nonspecific adherence. Blocking the plate with a solution of 1% BSA/2% skim milk/0.5% gelatine, and increasing to 0.1% Tween 20/PBS instead of 0.05% were tested. All the phage-antibody clones lost their binding to the different antigens coated, independent of the Tween 20 concentration used, except clone #19 (Figure 13). Unfortunately we were not able to cut either the Lc or the Hc out of clone #19 as shown in figure 7.

JB1A peptide: B-SGSGTAEKLK
B3B11 peptide: B-SGSGTPAKLR
1-14 peptide: QTDENRCLKANAKS

## X. Determination of the expression of Fab fragment at the surface of phage-antibody clone 19.

It was thought that it might be possible to capture clone #19 on ELISA plate coated with anti-Fab, anti-Hc, or anti-Lc antibodies because Fab

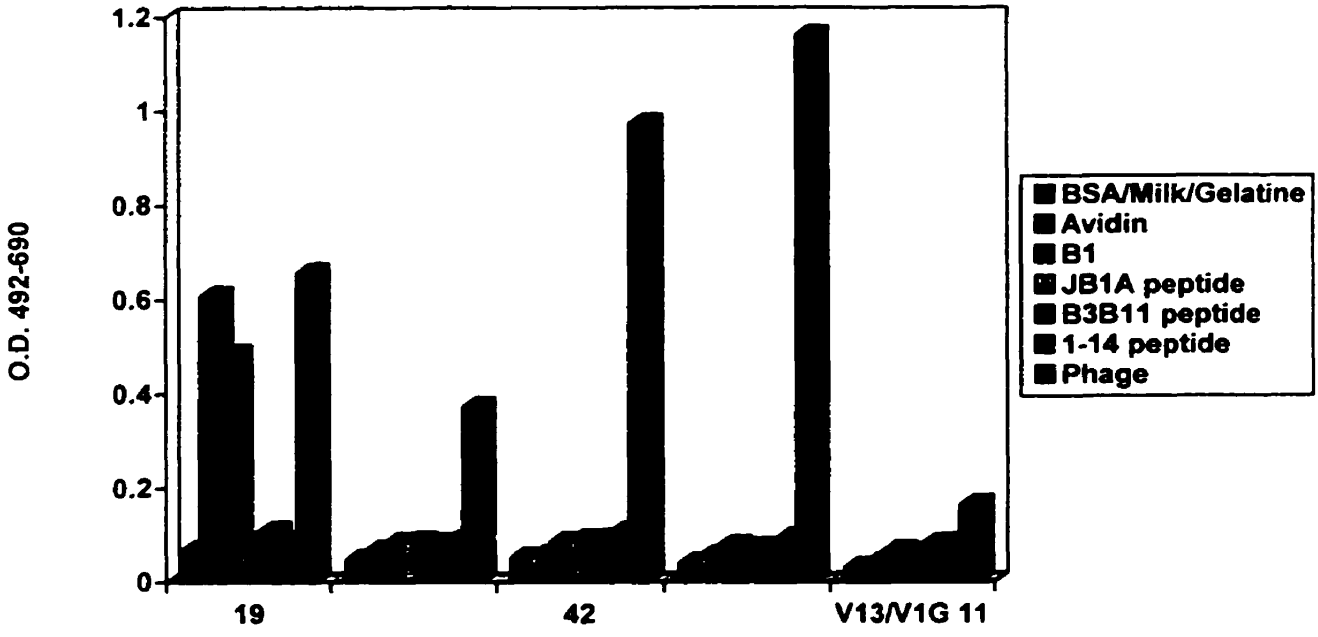



**Figure 13.** Specificity of monoclonal phage-Ab from semi-synthetic human librairies determined by ELISA. 1% BSA/ 2% skim milk 0.1% gelatine in PBS was used as a blocking agent. Phage-Ab clones were first incubated with an equal volume of blocking solution prior to be applied to the Ag coated wells. 0.1% PBST was used as washing solution.

fragment could not be detected by restriction digestion or Western blot analysis. It seems that this clone has a kappa Lc, but the data are not convincing due to the fact that this clone does not produce a large amount of phage-antibody particles so binding is barely above background level (Figure 14). If kappa Lc is expressed on the phage surface, Hc should be present also. Hc is linked to pIII which is inserted into the membrane of the phage whereas Lc is not attached, therefore the Lc has to be linked to the Hc to be present at the surface of the phage particle.

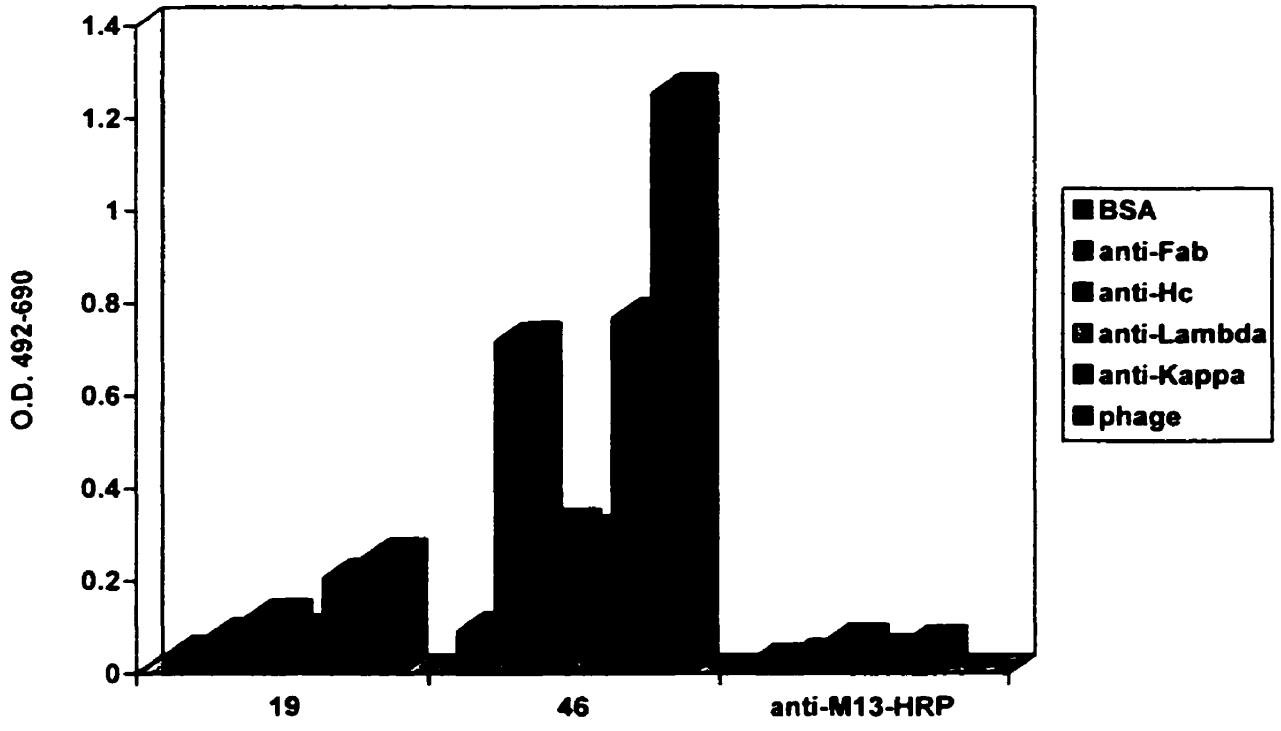



**Figure 14.** Determination of the expression of Fab fragment at the surface of phage-antibody clones. 3% BSA/PBS was used as blocking solution. Clones were first incubated with an equal volume of blocking solution prior to be applied to the antibody coated wells. Anti-M13 HRP conjugate was applied on the antibody coated wells without any previous incubation with phage-antibody to assess the level of background. #46 was used as a positive control.

# DISCUSSION

It is felt that the semisynthetic repertoires may be the method of choice to produce human monoclonal antibodies targeting highly conserved proteins.

In Dr. John Wilkins laboratory, the regulation og human $\beta_1$ integrin is being examined. Attempts to raise monoclonal antibodies against the most highly conserved regions of the $\beta_1$ integrin have failed. These conserved regions are known to be important for the function of integrins (Lewing-Hagesteijn, C.Y., 1994).

Successful use of the semisynthetic approach could allow for the production of antibody fragments against conserved regions of the $\beta_1$ integrin. Such probes could further improve the understanding of the role and function of $\beta_1$ integrin in the inflammatory diseases such as rheumatoid arthritis. Selection on a peptide (JB1A peptide) known to be the epitope of an inhibitory antibody called JB1A was attempted as well as selection on purified human $\beta_1$ integrin.

## I. Summary of the results.

The diversity and quality (evaluation of the library stability) of a library is of critical importance to increase the chance of obtaining high affinity monoclonal antibody fragments. To assess the success of combination of both Lc and Hc fragment cDNA into the phagemid vector, clones were randomly picked from each libraries and analysed by restriction

digestion. Overall of 81% of the clones contained both cDNA sequences. Within the 19% that had not inserted both successfully, some had lost all or part of the inserts. Others contained inserts that varied from the predicted size of approximately 680 bp. This is probably due to the instability of some libraries using *E. coli* expression systems. As mentioned by Rapoport, B., et al. (1995), there is a general consensus in the research community that there are serious problems such as instability with the present generation of phage display vectors. The host cells tend to cope with the stress caused by the expression of the protein fusion by eliminating all or part of the antibody fragment or the entire plasmid. Deletants expressed at the phage surface are often sticky and cause increases in the nonspecific background (Method in enzymology, vol 267, 1996). These deletants also often have a growth advantage over the correct fusion clones, consequently outgrowth by these deletants will occur and overtake the phage population because the amplification steps are done in solution (rescue by helper phage cannot be performed on solid media). The frequency of antibody inserts in the vector should be monitored at each panning step because a high proportion of deletants may indicate that the selection efficiency is low (Method in enzymology, vol 267, 1996). It is reported that a higher frequency of deletants occurs with Fab libraries compaired to scFv libraries. To reduce the frequency of deletion, it is possible to shorten the time of growth of a library (the yield would be smaller consequently less copies of each clones would be produced). Stickyness caused by instability is problematic when trying to isolate a rare high affinity clone which frequency is lower than the nonspecific background binding to the selection surface (Portolano, S., et al., 1993; Marks, J.D., et al., 1993).

For the selection on biotinylated JB1A peptide, a modest enrichment of 17 fold was observed over 7 rounds of selection. No enrichment was seen for the selection on purified human $\beta_1$ integrin. The enrichments were

monitored by ELISA after the fifth and seventh panning to check for positive clones. As reported by Yu, J., and Smith, G.P. (1996), the number of eluted phage are low in the early rounds for a typical selection (about $10^5$-$10^6$ cfu), increasing in the later rounds to about $10^9$ cfu. But if the numbers do not increase, this does not necessarily indicate a failure to select positive clones. Selection on proteins tend to be more difficult (Hoogenboom, H.R., and Winter, G., 1992; Marks,J.D. et al.,1991; Garrard, L.J., and Henner, D.J., 1993) than on smaller molecules such as haptens. For the same area of selection, the concentration of antigen is lower for proteins than for haptens. Perhaps the structural diversity of these synthetic repertoires were not enough large to obtain $\beta_1$ integrin binding phage.

In the selection with biotinylated JB1A peptide, one clone (clone 19) was isolated after 5 rounds of panning, and three more clones were isolated after seven rounds (clones 37, 42, and 46). Clone 19 appears, by ELISA, to have specificity for avidin and purified $\beta_1$ integrin, but not to JB1A peptide and BSA. This was contradictory to the expected results as the selection was performed with peptide. In most cases antibodies raised against peptides cross-react with the folded protein (Fieser, T.M., et al., 1987; Niman, H.L., et al., 1983) even if it seems paradoxal because anti-peptide antibodies are very different from those raised against intact proteins (Dyson, H.J., et al., 1988). Antipeptide antibodies raised against peptides derived from the surface regions of the protein would be more likely to recognize the protein (Dyson, H.J., et al., 1988; Enea, V., et al., 1984). Avidin has been reported to cause nonspecific binding (Pierce, Rockford, Illinois, USA). The presence of carbohydrate on avidin, and its high isoelectric point (10-10.5) contribute to the nonspecific background. $\beta_1$ integrin is also a glycosylated protein. Possibly, clone 19 might recognize carbohydrates at the surface of both

molecules. The interaction between clone 19 and avidin or $\beta_1$ integrin might also be an electrostatic interaction based on charges.

Clones 37, 42, and 46 appeared, by ELISA, to have specificity for avidin, $\beta_1$ integrin, and JB1A peptide, but not to BSA. Clone 19 could not be further analysed due to the inability to generate the soluble form. Two negative control clones V13/8H 11-25 and V13/V1G 7-9, were picked from two unselected libraries. When testing these negative controls as well as clones 37, 42, and 46 as soluble Fab, on ELISA, they all bound to $\beta_1$ integrin, avidin, biotinylated JB1A peptide, but not to BSA. Clone V13/8H 11-25 as a phage did not bind to any of these antigens (V13/V1G 7-9 was only tested as a soluble form). It would be of some interest to know what kind of interactions were occuring, and why in this case Fab behaved differently than the phage-antibody form. Are these interactions possibly be based on similarities (common sequences or charges) between the set of antigens chosen, or a common feature of soluble Fabs based on a longer off-rate shared by the clones of these particular libraries? The only certainty is that the binding is not due to the blocking agent or to the panning matrix (plastic) itself as no clones bound to the BSA coated wells.

In an attempt to discriminate between clones, the influence of the concentration of soluble Fab on the binding was tested by ELISA. The binding decreased proportionaly with the concentration of soluble Fab used for each clones as well as the two control clones. No discrimination in binding could be observed. A surprising observation was made in Figure 11D. The relative amount of soluble Fab produced was determined by coating these at different concentrations and detected by ELISA. The signal for clones 46-8 and V13/8H 11-25 was decreased linearly. However for clone V13/V1G 7-9, the signal was low at high concentration of Fab, then increasing at lower concentration, and finally reaching background level when diluted more.

The reason for this pattern is not known but it was also observed in the laboratory of Greg Silverman (personnal communication). It could possibly be that some components in the supernatant compete with the Fab fragments when the supernatant is at high concentrations. At lower concentrations the blocking agent cannot compete soluble Fab due to a lower affinity or shorter off-rate.

It was observed that Fab fragments are not as efficiently expressed as scFv fragments because of more folding problems (Hawkins, R.E., et al.,1993). Huse et al. (1989) observed that the absence of the $V_L$ domain leaves a large hydrophobic patch on one face of the $V_H$ fragment, which increase nonspecific binding. Hc alone tend to be sticky. To assess whether assembly problems of the two chains could be the cause of nonspecific binding in our system Western blot analysis was performed using specific antibodies for human Hc and Lc under nondenaturing conditions. For the control V13/8H 11-25, the majority of Hc fragments were not paired to the Lc, but for the control V13/V1G 7-9 good pairing was observed with a low proportion of monomer expression. For the selected clones more dimers were formed in proportion to monomers. Even if a low proportion of monomers were formed this could cause nonspecific background at saturation of binding. If this is the case, patterns in figure 11 should have shown differential binding of the clone compared to the controls assuming that these clones have some specificity for the antigens, since background binding due to the monomers would have been probably diluted out. A larger amount of Lc seems also to be expressed in comparaison to the Hc, possibly because the expression of each chain is regulated by a different promoter.

Additional peptides available in the laboratory where used in an attempt to better define the binding specificity of the selected clones in phage form. Clones 37, 42, and 46 as well as the negative control V13/V1G 11 also

bound to these additional peptides except for clone 19.

Different blocking conditions where then tested in an attempt to eliminate the nonspecific binding. Using 1% BSA/ 2% skim milk/ 0.5% gelatin as a blocking mixture, only clone 19 still bound to the antigens. Binding of other clones was inhibited to a background level. How the binding of these clones is inhibited as a result of blocking with 1% BSA/ 2% skim milk/ 0.5% gelatin is not understood. We can therefore assume that the selection of these clones was based on nonspecific interactions, except maybe for clone 19.

Clone 19 being the only clone that seems to have specificity to some extent, we wanted to determine if there was effective expression of Fab fragments at the surface of clone 19, by ELISA using anti-Fab, anti-Hc, anti-lambda, and anti-kappa antibody coated microtiter plate. Clone 19 seems to have a kappa Lc. The results are not convincing due to the fact that clone 19 does not produce a large amount of particles, so binding is barely above backgroung level. Therefore it is reasonable to think that if a kappa Lc is expressed on the phage surface, Hc should also be present. Hc is fused to pIII which is anchored into the membrane of the phage, whereas Lc is not attached. Therefore the Lc has to be linked to the Hc to be present at the surface of the phage particle. Detection of the phage was done with anti-M13-HRP conjugated antibody, so Lc or soluble Fab not expressed as surface molecules would not be detected.

In parallel, selection in solution using peptide coated magnetic beads was performed by Dr Wang Di. Only clones with broad binding to several unrelated antigens were obtained as in the results presented here. Improvement and careful tuning of the system currently used should be done

in any future attempts to select antibodies against $\beta_1$ integrin or any other molecules of interest in this laboratory.

Eventual sequencing of these clones could bring some light on the understanding of the interaction between these selected phage-antibodies and the target molecules.

When using the synthetic repertoire approach instead of libraries from an immunized source, one must accept the risk that the desired sequence might not be represented in the repertoire. If this is the case, even using a perfectly optimized selection procedure will be vain.

Several aspects concerning the improvement and choice of the best method of selection and the system to use will be discussed in the following sections.

## II. Choice of a system and library.

The pComb 3H system seems to be less stable than the scFv based systems such as the pCantab system due to inherent characteristics. The risk of wrong pairing or folding problems might be increased as Lc is not attached to the Hc with a linker. This might also increase the chance of having phage expressing only a Hc fragment, these being selectable without Lc. This is reflected by a number of publications being higher for scFv based systems than for the Fab systens. The chance of obtaining antibody fragments specific for large proteins might be increased by the use of more diverse libraries (in number of different clones as well as diversity of shapes).

## III. Bias during selection process.

In a library there can be clonal variations in display level which results in differences in stability of the fusion and also in growth rates that will cause biais in the selection process. These clonal variations occur even among closely related sequences (McCafferty, J., 1996), mainly caused by differences in translation efficiency, transport to the periplasm, folding of the antibody fragment, or stability of the fusion (McCafferty, J., 1996).

In a library there will probably be a bias for the selection of antibody fragment sequences that are favorable for bacterial expression or well expressed, these genes will be preferred and contribute in a larger extend to the selection process (Begent, R.H.J.,et al., 1996; Method in enzymology, vol 267, 1996). As discussed by Makowski, L. (1993), some sequences might be fatal by disrupting the stability of the phage particle structure or by interferring with the assembly and infection process. As pointed out, a synthetic repertoire could never be totally random. Even by maximizing the proportion of functional antibody fragments, individuality of antibodies and protein folding problems will continue to limit the phage-antibody display approach.

## IV. Crucial role of the first round of panning.

The first round of panning is of critical importance, in particular when target phage are present at low frequency in large repertoire, or have a reduced affinity like for nonmature or synthetic repertoires. The first round

of selection has to be optimized to increase the chance of not losing the rare binders.

In the first round of panning, the input consists theoritically of all clones in the initial library. Within large repertoires, each clone is represented by few particles (respectively, in theory, approximatively 3500 copies of each for the selection against biotinylated JB1A peptide, and approximatively 8500 copies for the selection against $\beta_1$ integrin). These numbers are in fact much lower as for particles derived by rescue of phagemid clones, the majority of pIII molecules present are of wild-type pIII size and not fusion. This is even lower than the 20-30% of intact fusion present from phage-display vectors (McCafferty, J., 1996) where intact fusion is barely detectable by Western. In fact, a large proportion of phage are bald, not expressing any functional Fab fragments. Therefore, if a binding clone has a low frequency or yield in the first round of panning, that clone has a good chance of being lost, and can never be enriched. Consequently, stringency has to be low in the first round, then in later rounds it can be increased in order to select the best binders. But stringency must not be set too high otherwise specific phage binding will be eliminated (Method in enzymology, vol 267, 1996).

To use a higher phage input is not necessarily a good way to increase the chance of capturing rare binders. As dicussed by McCafferty, J. (1996), the proportion of binding phage is reduced as the input is increased. It has been observed that nonspecific interaction between phage particles themselves occurs (Day, L.A., et al., 1988). Therefore it is suggested by McCafferty, J. (1996) that phage could possibly form aggregates resulting in steric inhibition of incoming phage by aggregates already bound. This would result in the impossibility for a significant proportion of phage to bind and also reducing the capacity of the selection surface.

## V. Choice of a method of selection.

As the first round of panning is crucial especially when selecting rare binders from a nonmatured repertoire, the choice of selection method is critical. It is important to capture all possible binders in the first round of panning as these would be lost and could not be recovered. Consequently, the highest targeted antigen concentration should be used since with limiting antigen rare binders could be competed out (Kretzschmar, T., et al., 1995). Lower coating density of antigen might be used in later rounds to promote discrimination between high affinity phage from low affinity binders (Winter, G., et al., 1994). The use of lower coating density mimics the *in vivo* situation where as the immune response develops in an organism, more antibodies are produced and the concentration of available antigens decreases. Thus, B cells that bind the antigen with higher affinity are preferentially stimulated.

Increasing the surface selection increase the chance to capture all possible binders.Kretzschmar, T., et al. (1995) compared selection in microtiter well, immunotube, and batch or column chromatography. Selection in microtiter plate was the least efficient as less phage were captured after the first round of panning. Batch or column chromatography were the recommended methods of selection. Batch chromatography will more closely ressemble the behavior of antibody-antigen interaction as the selection is performed in solution using soluble biotinylated antigen. Selection with soluble antigen also provides a means to discriminate between

clones with closely related affinities by controling the concentration of the soluble biotinylated antigen. Preabsorption or deselection (to remove unwanted specificities) can also be carried in solution by adding an excess of the "deselecting antigen" in solution with the phage (McCafferty, J., 1996) instead of doing preabsorption on immobilized deselecting antigen. Batch chromatography using biotinylated peptide coated on magnetic beads was carried out by Dr Wang Di with limited success.

## VI. Elution.

pH elution is usually performed, but elution with a large excess of antigen has been shown to give more consistant enrichments by presumably providing a more selective enrichment for the target antigen over nonspecific binding. pH elution does not discriminate between specific and nonspecific binders. If unlimited quantity of antigen is available, elution with the antigen is recommanded (Levitan, B., 1998)

## VII. Second generation libraries.

Once a phage-antibody clone with the desired properties has been isolated, it is possible to improve or modify the binding properties (Adey, N.B., et al., 1996). Second generation library displays variants of the original phage-antibody clone selected from the primary library. Additional mutations are generated in the second generation library which is screened to yield optimal binding. Clones with improved affinities (Barbas, C.F., et al., 1994) or altered specificity (Widersten, M., and Mannervik, B., 1995) can

be selected from these libraries. Site directed mutagenesis, cassette mutagenesis, random mutation in the HCDR3 introduced via PCR (Fujii, I., and al., 1998), error-prone PCR, DNA shuffling (Crameri, A.,et al., 1996), or CDR walking mutagenesis (Yang, W.-P., et al., 1995) can be used to create second generation libraries. The production and selection of second generation libraries mimics the affinity maturations of antibodies occuring after repeated antigen stimulations of a population of B cells *in vivo*.

In conclusion, phage display technology is an extremely attractive and valuable approach in that it mimics *in vitro* the essence of the *in vivo* immune response by linking the fundamental processes of recognition and replication. The phage display approach is still at an immature stage, but hopefully it will evolve into an established technology.

## APPENDIX A: solutions, medium and buffers.

### I. Bacterial growth medium.

1. SB: 30g bacto tryptone
20g bacto yeast extract
10g MOPS
Complete to 1 liter with distilled water and adjust to pH 7.0.

2. LB: 10g bacto tryptone
5g bacto yeast extract
5g NaCl
Adjust to 1 liter with distilled water.

3. LB plates: LB broth + 15g bacto agar per liter.

4. 2XYT: 17g bacto tryptone
10g bacto yeast extract
5g NaCl
Adjust to 1 liter with distilled water.

5. B: 10g bacto tryptone
8g NaCl
Adjust to 1 liter with distilled water.
After autoclaving and cooling add 10ml of sterile 20% glucose.
20% glucose is sterilized through a 0.45μm filter.

6. B plates: B broth + 15g bacto agar per liter.

7. B top agar: B broth + 6g bacto agar per liter.

8. SOC: To 950ml distilled water,add
20g bacto tryptone
5g bacto yeast extract
0.5g NaCl
Dissolve. Add 10ml of a 250mM KCl. Adjust to pH 7.0.
Adjust to 1 liter with distilled water and autoclave.
Just before use, add 5ml of 2M $MgCl_2$ and 20ml of 1M glucose.
2M $MgCl_2$ and 1M glucose is sterilized through a 0.45μm filter.

## II. Panning buffers.

1. PBS: 8g NaCl
0.2g KCl
1.44g $Na_2HPO_4$
0.24g $Kh_2PO_4$
Adjust to 1 liter with distilled water and adjust to pH 7.4.

2. 3% BSA/PBS: Sterilized through a 0.22μm filter.

3. PBST: PBS containing 0.05% Tween 20.

**4. TBS:** 50mM Tris base
150mMNaCl
Adjust to pH 7.5.

**5. TBST:** TBS containing 0.05% Tween 20 and 1% BSA.

**6. Elution buffer:** 0.1M HCl
1mg/ml BSA
Adjust to pH 2.2 with glycine.

## III. ELISA buffers.

**1. 0.05M citric buffer:** 10.507g of citrate monohydrate ( MW 210.14 ) in 1 liter of distilled water.
Adjust to pH 5.4.

**2. Alkaline phosphatase buffer:** 10% diethanolamine
0.01% $MgCl_2$ ( 1.05M )
3mM $NaN_3$
Adjust to pH 9.8 with HCl.

**3. 20X OPD:** 240mg diluted in 30ml citric buffer.

**4. $\beta_1$ coating buffer:** pH 9.6
0.795g Na2CO3
1.465g Na.HCO3
Adjust to 500ml with distilled water.

## IV. Immunoblotting solutions.

**1. SDS-page:** 1 gel ( 12% ):

3.3ml $H_2O$

4.0ml 30% Acrylamide mix

2.5ml 1.5M Tris ( pH 8.8 )

0.1ml 10% SDS

0.1ml 10% ammonium persulfate

0.004ml TEMED.

Stacking gel:

3.4ml $H_2O$

0.83ml 30% acrylamide mix

0.63ml 1M Tris ( pH 6.8 )

0.05ml 10% SDS

0.05ml 10% ammonium persulfate

0.005ml TEMED.

**2. Tris-glycine SDS-page electrophoresis buffer:**

25mM Tris

250mM glycine pH 8.3

0.1% SDS

Adjust to 1 liter with distilled water.

**3. SDS-page loading buffer:** reducing conditions: 2X buffer:
0.125M Tris-HCl pH 6.8
4% SDS
20% glycerol
10% 2-mercaptoethanol
non-reducing conditions:
no 2-mercaptoethanol added.

**4. Transfer buffer pH 8.3:** 39mM glycine
48mM Tris base
0.037% SDS
20% methanol
Adjust to 1 liter with distilled water.

**5. Alkaline phosphatase buffer:** 100mM NaCl
5mM $MgCl_2$
100mM Tris pH 9.5.

**V. DNA buffers:**

**1. TE:** 10mM Tris-HCl pH 8.0
1mM EDTA pH 8.0.

**2. TAE:** 50X stock solution:

242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA ( pH 8.0 )

Adjust to 1 liter with distilled water.

**3. Gel-loading buffer:** 0.25% bromophenol blue

0.2% xylene cyanol FF

30% glycerol in water.

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